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Developmental acquisition of enhancer function requires a unique coactivator activity

Sadhan Majumder[1], Zhaoyang Zhao, Kotaro Kaneko[2] and Melvin L.DePamphilis[2]

University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Box 100, Houston, TX 77030, USA and [2]National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-2753, USA

[1]Corresponding author

Enhancers are believed to stimulate promoters by relieving chromatin-mediated repression. However, injection of plasmid-encoded genes into mouse oocytes and embryos revealed that enhancers failed to stimulate promoters prior to formation of a two-cell embryo, even though the promoter was repressed in the maternal nucleus of both oocytes and one-cell embryos. The absence of enhancer function was not due to the absence of a required sequence-specific enhancer activation protein, because enhancer function was not elicited even when these proteins either were provided by an expression vector (GAL4:VP16) or were present as an endogenous transcription factor (TEF-1) and shown to be active in stimulating promoters. Instead, enhancer function *in vivo* required a unique coactivator activity in addition to enhancer-specific DNA binding proteins and promoter repression. This coactivator activity first appeared during mouse development in two- to four-cell embryos, concurrent with the major onset of zygotic gene expression. Competition between various enhancers was observed in these embryos, but not competition between enhancers and promoters, and competition between enhancers was absent in one-cell embryos. Moreover, enhancer function in oocytes could be partially restored by pre-injecting mRNA from cells in which enhancers were active, the same mRNA did not affect enhancer function in two- to four-cell embryos.

Keywords: coactivators/enhancers/mouse development/transcription/zygotic gene activation

Introduction

Regulation of transcription involves at least two primary *cis*-acting DNA sequence components: promoters (short distance) and enhancers (long distance). Promoters determine where transcription begins; they function upstream and proximal to the initiation site and consist of a binding site for the basal level transcription complex and often one or more sequence-specific transcription factor binding sites. Enhancers stimulate weak promoters in a tissue-specific manner; they consist of sequence-specific transcription factor binding sites that function distal to the initiation site from either an upstream or downstream position (Felsenfeld, 1992; Workman and Buchman, 1993; Paranjape *et al.*, 1994). Enhancers also can function as components of origins for DNA replication (DePamphilis, 1993). The ability of an enhancer to stimulate either a promoter or replication origin has been observed only under conditions where the DNA template has been assembled into a chromatin structure that represses promoter or origin activity (Prives *et al.*, 1987; Paranjape *et al.*, 1994; Majumder and DePamphilis, 1995; Majumder, 1997). When this repression is relieved *in vivo* by inhibition of histone deacetylase, then the activity of promoters is restored and they are no longer stimulated by enhancers (Majumder *et al.*, 1993; Wiekowski *et al.*, 1993). Therefore, it appears that the primary role of enhancers is to alleviate repression of weak promoters or replication origins that is mediated through chromatin structure (Felsenfeld, 1992; Majumder and DePamphilis, 1994b, 1995; Paranjape *et al.*, 1994; Studitsky *et al.*, 1995; Struhl, 1996; Wolffe and Pruss, 1996). However, while chromatin-mediated repression is necessary to elicit enhancer function, here we show that it is not sufficient.

One approach to understanding regulation of DNA replication and gene expression at the beginning of mammalian development has been to inject plasmid DNA into the nuclei of mouse oocytes and preimplantation embryos (Majumder, 1997). The injected DNA can replicate or express an encoded reporter gene only when specific *cis*-acting regulatory sequences are present and provided with their cognate transacting proteins. Moreover, replication and transcription of DNA or translation of nascent mRNA occur only when the cellular genome executes the same function during its normal developmental program (Majumder and DePamphilis, 1995; Nothias *et al.*, 1995). Thus, these studies indicate that the response of injected plasmids is not an artifact of the experimental protocol, but reflects physiological controls that govern expression of cellular genes, and reveals the embryo's capacity for DNA replication and gene expression, and its requirements for specific regulatory elements. Using this approach, we have discovered that the developmental acquisition of enhancer function in two-cell mouse embryos involves the appearance of an enhancer-specific coactivator activity.

Mouse oocytes are terminally differentiated cells that can express genes, but not replicate them (Schultz, 1993; Majumder and DePamphilis, 1994b, 1995). Transcription stops when oocytes undergo meiotic maturation to form unfertilized eggs. 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. Although a transcriptionally permissive state occurs at the late one-

cell stage in mouse development (Latham *et al.*, 1992), zygotic gene expression (ZGE) is regulated by a time-dependent mechanism ('zygotic clock') that delays transcription of zygotic genes and translation of nascent mRNA until a specified time (~40 h) after fertilization, which corresponds to the two-cell stage of normally developing embryos (Conover *et al.*, 1991; Schultz, 1993; Majumder and DePamphilis, 1995; Nothias *et al.*, 1995, 1996; Majumder, 1997). However, when one-cell embryos are arrested in S-phase, ZGE still occurs ~40 h after fertilization, even though morphological development has ceased. This phenomenon has facilitated dissection of the pathway regulating ZGE at the beginning of mammalian development.

Injection of plasmid-encoded genes into the nuclei of mouse oocytes, one-cell and two-cell embryos has revealed that transcription always requires a functional eukaryotic promoter, but that stimulation of promoters or origins by enhancers does not appear until formation of a two-cell embryo (Martínez-Salas *et al.*, 1989; Majumder *et al.*, 1993; Wiekowski *et al.*, 1993; Majumder and DePamphilis, 1995; Nothias *et al.*, 1996; Majumder, 1997). This developmental acquisition of enhancer function is not due to differences in the amount or composition of transcription factors required for promoter activity in two-cell embryos (Majumder *et al.*, 1993), nor is it due to the formation of a zygotic nucleus (Wiekowski *et al.*, 1993; Henery *et al.*, 1995). It does, however, require the presence of chromatin-mediated repression. This repression can be relieved by addition of butyrate to the cell culture medium (Majumder *et al.*, 1993; Wiekowski *et al.*, 1993). Butyrate inhibits histone deacetylase, causing destabilization in the chromatin of mammalian cells (Grunstein, 1990; Turner, 1991). Thus, based on the relative levels of promoter activity and the ability of butyrate to stimulate promoter activity, chromatin-mediated repression is found to be present in the maternal nucleus of oocytes and one-cell embryos, and the zygotic nuclei and cytoplasm of two- to eight-cell embryos, but repression is absent from the paternal pronucleus of one-cell embryos (Majumder *et al.*, 1993; Wiekowski *et al.*, 1993; Henery *et al.*, 1995). Furthermore, the ability to repress transcription after formation of a two-cell embryo correlates with changes in histone synthesis and modification (Wiekowski *et al.*, 1997). In addition, plasmid DNA injected into oocytes and two- to four-cell embryos, and not into paternal pronuclei of one-cell embryos, is rapidly assembled into chromatin (Martínez-Salas *et al.*, 1989). Butyrate strongly stimulates promoter activity in cleavage-stage embryos, but has little effect on promoters that are already stimulated by an enhancer (Majumder *et al.*, 1993; Wiekowski *et al.*, 1993). Inhibition of histone deacetylase with either butyrate, trichostatin A or trapoxin increases the amount of nuclear hyperacetylated histone H4 in two- to eight-cell embryos (Thompson *et al.*, 1995; Worrad *et al.*, 1995; Wiekowski *et al.*, 1997), consistent with the effect of these inhibitors on promoter activity either in injected plasmids (Majumder *et al.*, 1993; Wiekowski *et al.*, 1993) or in transgenes (Thompson *et al.*, 1995).

Although the promoter repression observed in the maternal pronucleus of S-phase-arrested one-cell embryos, like that observed in cleavage-stage embryos (two or more cells), could be relieved by butyrate, this repression could not be relieved by enhancers such as the polyomavirus F101 or SV40 enhancers that function effectively in cleavage-stage embryos (Chalifour *et al.*, 1986; Martínez-Salas *et al.*, 1989; Majumder *et al.*, 1993; Wiekowski *et al.*, 1993). These enhancers could alleviate repression only after formation of a two-cell embryo, regardless of whether the resulting cleavage-stage embryos continue morphological development or are arrested in S-phase (Majumder *et al.*, 1993; Wiekowski *et al.*, 1993). Thus, while stimulation by enhancers requires the presence of chromatin-mediated repression, this repression alone is not sufficient to elicit enhancer function. Experiments described in this paper demonstrate that enhancer function requires the presence of a coactivator activity in addition to the presence of promoter repression, and enhancer activation proteins. Furthermore, the absence of this coactivator activity prior to formation of a two-cell mouse embryo, would help to prevent premature transcription of zygotic genes, while its presence after zygotic gene activation would help to regulate the activity of groups of genes that depend on enhancers. Thus, it may serve as a critical regulator in ZGE.

Results

Enhancer stimulation of promoters first appears in two-cell embryos

Previously we observed that the activity of weak promoters is very high in the paternal pronucleus of one-cell embryos, and is repressed in the zygotic nuclei of two-cell embryos. The promoter repression in two-cell embryos could be relieved by the presence of an enhancer, or a transactivator, and that enhancers or transactivators had little effect in the paternal pronucleus of one-cell embryos. This led us to propose that the role of enhancers *in vivo* is to relieve promoter repression (Martínez-Salas *et al.*, 1989; Wiekowski *et al.*, 1991; Majumder *et al.*, 1993; Majumder and DePamphilis, 1994a). Here, we extended these studies to mouse oocytes. In order to determine the relative promoter and enhancer activities in oocytes and two-cell embryos, plasmid DNA (ptkluc or pF101tkluc) containing the firefly luciferase (luc) reporter gene placed under the control of herpes simplex virus (HSV) thymidine kinase (tk) promoter, linked or unlinked to the polyomavirus F101 enhancer (F101) was injected into the germinal vesicle of oocytes and one of the zygotic nuclei of two-cell embryos. In order to determine the effect of a transactivator, ptkluc was also co-injected with an expression vector (pMEX4) encoding the HSV transactivator, ICP4. The tk promoter and F101 enhancer were selected because they use cellular transcription factors exclusively and function in a wide variety of mouse cell types, including undifferentiated embryonic stem cells and cleavage-stage embryos (McKnight and Kingsbury, 1982; Martínez-Salas *et al.*, 1989; Majumder *et al.*, 1993; Majumder and DePamphilis, 1994a). HSV tk promoter has been found to respond to stimulation by various enhancers and transactivators, including the F101 enhancer and the HSV-ICP4 transactivator (Stow and Stow, 1986; Boni and Coen, 1989; Majumder and DePamphilis, 1994a). The F101 enhancer is the strongest enhancer found so far for stimulating promoter activity in two- to eight-cell mouse embryos (Mélin *et al.*, 1993). Enhancer elements

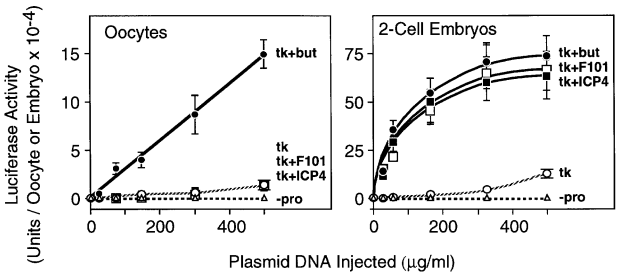

Fig. 1. Butyrate can stimulate promoters in mouse oocytes and in two- and four-cell embryos, while enhancers or transactivators can stimulate promoters in two- and four-cell embryos, but not in oocytes. CD-1 mouse oocytes were cultured in dibutyryl-cAMP to prevent meiotic maturation, and two-cell embryos were cultured in aphidicolin to arrest development at the beginning of S-phase in four-cell embryos. Plasmid DNA (~2 pl) was injected into the germinal vesicles of oocytes (left) or one of the two zygotic nuclei of two-cell embryos (right) at the concentrations indicated. Plasmids carried the firefly luciferase gene (luc) under control of the HSV-tk promoter (ptkluc, ○), and HSV-tk promoter linked to the F101 enhancer (pF101tkluc, ■). A promoterless control (pluc, △) was also tested. ptkluc was also co-injected with 15 μg/ml pMEX4 (□), an expression vector for the transactivator HSV-ICP4 (Resnick *et al.*, 1989). Some of the oocytes and embryos injected with ptkluc were cultured in the presence of 2.5 mM butyrate (●). Butyrate did not significantly increase luciferase gene expression with pluc. Luciferase activity was measured quantitatively in individual embryos or oocytes and expressed as light units (Miranda *et al.*, 1993). Each data point indicates the mean value ± SEM for 40–60 successfully injected oocytes or embryos.

were placed 600 bp upstream of the promoter. The plasmid pluc, containing the reporter gene without any promoter element, was used as a negative control in these experiments to determine the background level of luciferase expression. Promoter/enhancer activity was quantitatively evaluated by their ability to express the luciferase gene.

Mouse oocytes were isolated and cultured in the presence of dibutyryl-cAMP to prevent meiotic maturation and the inhibition of transcription that accompanies it. Two-cell embryos were isolated and cultured in the presence of aphidicolin in order to arrest development as they entered S-phase. When different amounts of plasmid ptkluc DNA were injected into these oocytes and embryos, the amount of luciferase activity observed was dependent on the amount of DNA injected (Figure 1), although the tk promoter activity in general was found to be ~5- to 10-fold lower in oocytes than in two-cell embryos. When pF101tkluc was introduced into oocytes and two-cell embryos, the promoter activity could be stimulated ~15-fold in two-cell embryos, but not in oocytes. Similar results were also observed in oocytes and two-cell embryos when ptkluc was co-injected in the presence of pMEX4. These experiments suggest that enhancers or transactivators can stimulate promoters in two-cell embryos, but not in oocytes.

Previously, we found that incubating two-cell embryos with butyrate, a potent inhibitor of histone deacetylase, strongly stimulated the activity of plasmid-encoded promoters (Majumder *et al.*, 1993; Wiekowski *et al.*, 1993). Moreover, this stimulation was specific for promoters; butyrate did not increase the activity of promoters that were already stimulated by an enhancer. These and other experiments (see Introduction) suggested that the repression of promoter activity observed in two-cell embryos was mediated by chromatin structure, and that either enhancers or butyrate could relieve this repression. Therefore, to determine whether or not the promoter activity observed in oocytes resulted from chromatin-mediated repression, oocytes and two-cell embryos were isolated and cultured in the presence of butyrate and then injected with ptkluc. The results (Figure 1) showed that, at the same DNA concentration, tk promoter activity in oocytes was ~5-fold lower than in two-cell embryos (Figure 1), but that tk promoter activity could be stimulated ~15-fold by butyrate in both oocytes and two-cell embryos. These experiments suggest that F101 enhancer or ICP4 transactivator can relieve chromatin-mediated repression in two-cell embryos, but not in oocytes.

Transcription factor Gal4:VP16 can activate a promoter but not an enhancer prior to formation of a two-cell embryo

Absence of enhancer function in oocytes and one-cell embryos could result either from insufficient amounts of activation proteins that bind to specific DNA sequences within the enhancer, or from the absence of an enhancer-specific coactivator, a protein that might mediate interaction between the enhancer-bound activation protein and the promoter-bound transcription complex. To distinguish between these two possibilities, a tandem series of yeast GAL4 DNA binding sites was used either as an enhancer (GAL4 enhancer), placed 600 bp upstream of the tk promoter, driving the luciferase gene ($pGAL_9$tkluc; Majumder *et al.*, 1993), or as a promoter (GAL4 promoter), 10 bp upstream of a TATA box, driving the bacterial chloramphenicol acetyltransferase (CAT) gene ($pGAL_5$-TCAT; Majumder *et al.*, 1993). GAL4:VP16, a strong transcriptional activator that functions through GAL4 DNA binding sites, was provided by co-injection of pSGVP, an expression vector that encodes the GAL4:VP16 gene driven by the Sp1-dependent SV40 T-antigen promoter stimulated by the SV40 enhancer. The SV40 promoter is very active in mouse oocytes and early embryos (Chalifour *et al.*, 1986; Martínez-Salas *et al.*, 1989; Majumder *et al.*, 1993).

The GAL4 promoter ($pGAL_5$TCAT) alone was inactive when injected into oocytes, paternal (P) and maternal (M) pronuclei of S-phase-arrested one-cell embryos, or the zygotic nuclei of two-cell embryos (Z). Co-injecting $pGAL_5$TCAT with increasing amounts of pSGVP identified the amount of pSGVP required to provide saturating levels of functional GAL4:VP16 (Figure 2A; +GAL4:VP16). GAL4 promoter activity was readily detected in all three cell types, but the maximum levels of activity could vary up to 10-fold (Figure 2A). The ability of the GAL4 enhancer to stimulate the tk promoter was then examined by co-injecting the enhancer construct, $pGAL_9$-tkluc, with sufficient pSGVP to provide saturating amounts of functional Gal4:VP16 protein. In the absence of GAL4:VP16, $pGAL_9$tkluc and ptkluc ($pGAL_9$tkluc without the enhancer), produced the same level of luciferase. In the presence of saturating levels of GAL4:VP16, however, the GAL4-dependent enhancer was active only after injection of two-cell embryos. This enhancer stimulated tk promoter activity ~30-fold in two- and four-cell embryos, but only 1- to 1.2-fold in oocytes, 1- to 2-fold in the maternal pronuclei and 1- to 1.2-fold in the paternal pronuclei of one-cell embryos (Figure 2B). Thus, even in

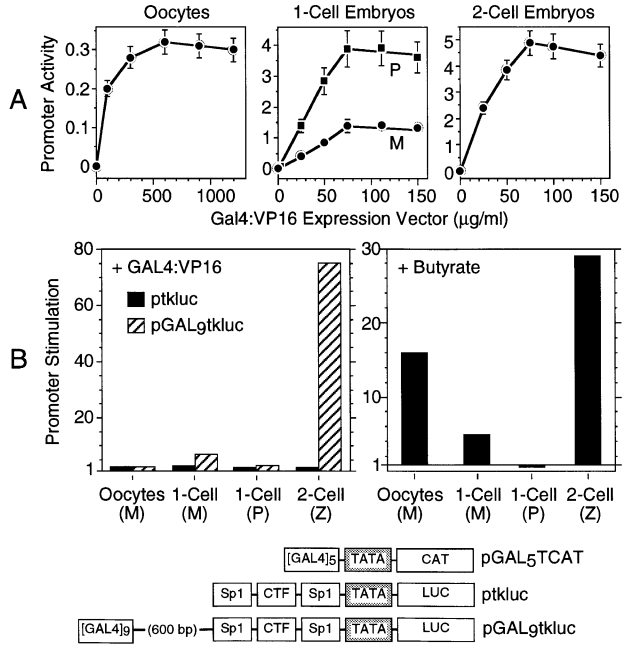

Fig. 2. Gal4-DNA binding sites in the presence of Gal4:VP16 protein can function as a promoter in oocytes and in one- and two-cell embryos, but cannot function as an enhancer until formation of a two-cell embryo. (**A**) GAL4:VP16 protein can stimulate a promoter in oocytes, one- and two-cell embryos. A Gal4-dependent promoter linked to the *E.coli* CAT gene ($pGAL_5TCAT$) was injected into oocytes, paternal (P) and maternal (M) pronucleus of aphidicolin-arrested one-cell embryos and one of the zygotic nuclei (Z) of aphidicolin-arrested two-cell embryos together with increasing amounts of an expression vector for the transcription factor GAL4:VP16 (pSGVP). Oocytes, and one- and two-cell embryos were injected with a plasmid DNA solution containing 100 μg/ml $pGAL_5TCAT$ plus various amounts of pSGVP. For oocytes, the total plasmid DNA concentration was maintained at 1350 μg/ml by including an appropriate amount of pBR322 DNA, the vector used to construct both $pGAL_5TCAT$ and pSGVP. For one- and two-cell embryos, the concentration of plasmid DNA was maintained at 250 μg/ml. After injection, oocytes were cultured for 20 h and embryos were cultured for 42 h before the extracts were prepared and assayed for CAT activity. (**B**) Left panel '+GAL4:VP16'; GAL4:VP16 protein can stimulate an enhancer in two-cell embryos, but not in oocytes or one-cell embryos. ptkluc or $pGAL_9tkluc$, the same plasmid containing a Gal4-dependent enhancer 600 bp upstream of the tk promoter, was injected into the germinal vesicle of oocytes, either the paternal (P) or maternal (M) pronucleus in one-cell embryos and one of the two zygotic (Z) nuclei in two-cell embryos. These luciferase expression vectors were injected either alone or together with sufficient pSGVP to produce saturating amounts of GAL4:VP16 transcription factor activity based on the data in (A). Oocytes received 100 μg/ml of either ptkluc or $pGAL_9tkluc$ plus 600 μg/ml pSGVP or 600 μg/ml pML-1. One-cell and two-cell embryos received 25 μg/ml of either ptkluc or $pGAL_9tkluc$ plus 75 μg/ml pSGVP or 75 μg/ml pBR322. Injected ova were cultured for time intervals as described above, and then assayed for luciferase activity. Stimulation by GAL4:VP16 is the ratio of +GAL4:VP16/–GAL4:VP16. A ratio of 1 indicated no stimulation. Right panel '+Butyrate'; some injected embryos were cultured in the presence of 2.5 mM butyrate. Stimulation was measured as the ratio of ptkluc + butyrate/ptkluc.

the presence of saturating amounts of a functional sequence-specific enhancer activation protein, enhancer function was not evident until formation of a two-cell embryo.

This failure of enhancers to stimulate promoter activity prior to formation of a two-cell embryo was not due to limited transcriptional capacity of oocytes and S-phase-arrested one-cell embryos, because addition of butyrate to either oocytes or two-cell embryos stimulated tk promoter activity ~15- to 20-fold by relieving chromatin-mediated repression (Figures 1 and 2B). Similarly, butyrate stimulated promoter activity in maternal pronuclei of one-cell embryos ~4- to 5-fold (Figure 2B). When the promoter was already stimulated by an enhancer in two-cell embryos, then butyrate had little effect (see Figure 5; Majumder *et al.*, 1993; Wiekowski *et al.*, 1993). [Note that the actual levels of expression from genes injected into oocytes and preimplantation embryos depend directly on the endogenous levels of transcription and translation of the injected cells. In fertilized eggs and cleavage-stage embryos, optimal levels are achieved by arresting these cells as they enter S-phase. In oocytes, optimal levels are achieved by injecting growing oocytes from young females (see Materials and methods).] Thus, if chromatin-mediated repression was all that was required to elicit enhancer function, then at a minimum, the GAL4 enhancer should have stimulated promoters ~15-fold in oocytes and ~5-fold in the maternal pronucleus of one-cell embryos. Therefore, a coactivator activity must be required for enhancer function that is absent in oocytes or one-cell embryos, and first appears with formation of a two-cell embryo.

Transcription factor TEF-1 can activate a promoter but not an enhancer prior to formation of a two-cell embryo

The lack of enhancer function in oocytes and one-cell embryos is also valid for enhancers driven by endogenous transcription factors. Previous studies (Mélin *et al.*, 1993) have shown that two copies of the 30 bp GTIIc sequence encoding the TEF-1 DNA binding site are responsible for the ability of the F101 enhancer to stimulate promoter activity, from a site 600 bp upstream of the promoter, in either developing or S-phase-arrested two- and four-cell mouse embryos. In fact, five tandem copies of the GTIIc sequence (GT enhancer), similarly placed at 600 bp upstream of the promoter (pGT_5Pyluc) could completely replace the F101 enhancer in two- to four-cell embryos (pF101Pyluc; Figure 3B). However, neither the F101 enhancer nor the GT enhancer stimulated promoter activity when injected into oocytes or S-phase-arrested one-cell embryos (Figure 3B). To determine whether or not TEF-1 transcription factor activity was present in oocytes and one-cell embryos, four tandem copies of GTIIc sequence were placed 10 bp upstream of a TATA-box, serving as a promoter (GT promoter), to drive luciferase reporter gene expression (pGT_4Tluc). When expressed at the optimum DNA concentration (Majumder *et al.*, 1993), GT promoter activity was undetectable in oocytes, but was clearly present in S-phase-arrested one-, two- and four-cell embryos (Figure 3A). For comparison, the activity of the Sp1-dependent HSV tk promoter is also shown. This promoter was active in oocytes as well as in one- to eight-cell embryos. The 3- to 4-fold greater activity observed in S-phase-arrested one-cell embryos than in S-phase-arrested two-cell embryos reflects the absence of repression in paternal pronuclei of one-cell embryos. This difference would have been 10-fold had these two-cell embryos been injected with the same DNA concentration used for one-cell embryos. Similar results were obtained using six tandem Sp1 sites as a promoter (Majumder *et al.*, 1993).

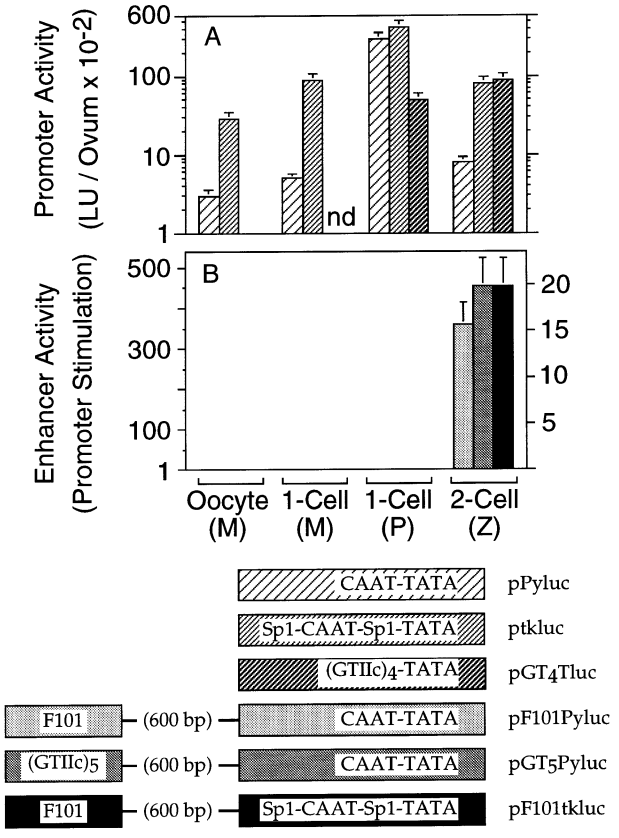

Fig. 3. DNA binding sites for the TEF-1 family of transcription factors can function as a promoter in S-phase-arrested one- and two-cell embryos, but not as an enhancer until the two-cell stage of mouse embryonic development. Injection and assay of expression vectors were carried out as described in Figure 2. Panel (**A**) shows optimum promoter activities in terms of luciferase activity (RLU) per ovum produced by pPyluc (coarse hatched) ptkluc (medium hatched) and pGT_4Tluc (fine hatched) following injection of ~2 pl of 550 μg/ml into oocytes, 150 μg/ml into maternal (M) or paternal (P) pronucleus of one-cell embryos, or 300 μg/ml into one of the zygotic nuclei (Z) of two-cell embryos. ptkluc encodes the luciferase gene driven by the HSV tk promoter as described above. pGT_4Tluc contains four tandem copies of the polyomavirus TEF-1 DNA binding site placed 10 bp upstream of the adenovirus late gene promoter TATA box driving the luciferase gene, and thus serving as a promoter (GT promoter). The luciferase gene alone (pluc) produced 30 light units in oocytes, 700 in one-cell embryos, and 150 in two-cell embryos. nd, not determined. Panel (**B**) shows the optimum enhancer activity in terms of ratio of luciferase activities produced by pF101Pyluc/pPyluc (fine stipple), pGT_5Pyluc/pPyluc (coarse stipple), and pF101tkluc/ptkluc (solid). The values for stimulation of the tk promoter by the F101 enhancer are shown on the right-hand *x*-axis. pPyluc represents the same as ptkluc, except that the tk promoter is replaced by polyomavirus (Py) promoter. pF101Pyluc (F101 enhancer), pF101tkluc (F101 enhancer) and pGT_5Pyluc (GT enhancer) contain either the F101 enhancer or the GT enhancer (five tandem copies of the TEF-1 DNA binding site), placed 600 bp upstream of the corresponding promoter.

Thus, sufficient TEF-1 activity was present in S-phase-arrested one-cell embryos to drive a GT promoter, but not a GT enhancer.

Enhancers compete with one another in two-cell embryos, but not in one-cell embryos

The presence of an enhancer coactivator activity in mouse cleavage-stage embryos was further demonstrated by competition experiments between enhancers whose activities did not depend upon binding the same sequence-specific transcription factors (Figure 4). In these experiments, the total plasmid DNA concentration for each competition was constant, so that neither the amount of DNA, nor the expected level of luciferase expression arising from such an amount of injected DNA, nor the amount of luciferase produced, exceeded their saturating levels as previously determined (see Figure 2 in Majumder *et al.*, 1993). The test molecule used was pGT_5Pyluc (GT enhancer), a plasmid encoding the luciferase gene driven by the polyomavirus T-antigen promoter stimulated by an enhancer consisting of five tandem copies of the TEF-1 DNA binding site placed 600 bp upstream of the promoter. When co-injected with an 8-fold excess of the enhancer construct, pF101tk (pF101tkluc with an inactivating internal deletion of the luciferase gene), into two-cell embryos, luciferase activity was reduced 12-fold. This resulted from competition for one or more *trans*-acting factors that limited luciferase gene expression. This limiting factor was specific for enhancer function, because competition with a different GAL4 enhancer construct, $pGAL_9tk$ (luciferase gene deleted) alone, did not interfere with luciferase gene expression unless saturating amounts of GAL4:VP16 were produced by co-injecting pSGVP, and thus activating the GAL4 enhancer. Under these conditions, luciferase gene activity was reduced 11-fold. Furthermore, if the tandem GAL4 DNA binding sites were used as a GAL4 promoter ($pGAL_5T$) instead of a GAL4 enhancer, then in the presence of GAL4:VP16, GAL4 promoter inhibited the GT enhancer only 2.5-fold, and about half of this inhibition resulted from competition between the SV40 enhancer/promoter present in pSGVP and pGT_5Pyluc. The SV40 enhancer/promoter in pSGVP was only weakly competitive, because it was present in only 3-fold excess over pGT_5Pyluc (as opposed to 8-fold excess for other enhancers). The specificity of these experiments for enhancer-specific factors present in two- to four-cell embryos was further confirmed by repeating the competition experiment in paternal pronuclei of S-phase-arrested one-cell embryos where enhancers have little to no effect on promoter activity. Under these conditions, competition between pF101tk and pGT_5Pyluc was not observed (Figure 4).

Enhancer coactivator activity can be restored in mouse oocytes

The results described above reveal that enhancer function requires a specific coactivator activity that is not present until the two-cell stage in mouse development. To determine whether or not oocytes can be provided with the missing coactivator, mRNA was isolated from mouse embryonic stem (ES) cells and preinjected into the cytoplasm of mouse oocytes (Figure 5). ES cells, like cleavage-stage embryos, utilize the F101 enhancer efficiently (Mélin *et al.*, 1993), and therefore provided a convenient source of mRNA encoding both the TEF-1 family of sequence-specific transcription factors and the putative enhancer coactivator protein. Expression of coactivator activity in mouse oocytes was then assayed by co-injecting $pGAL_9tkluc$ and pSGVP. As shown above, the tk promoter (ptkluc) was active in oocytes (Figures 1–3) where it could be stimulated at least 15-fold by butyrate (Figure 2B). However, it could not be stimulated by the GAL4-dependent enhancer ($pGAL_9tkluc$) in the presence of

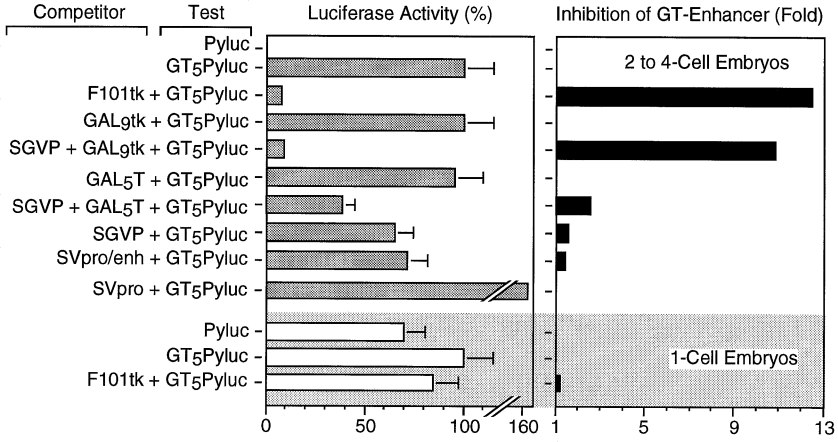

Fig. 4. Enhancers can be inactivated in mouse two-cell embryos by competition with other heterologous enhancers. Mouse early one-cell embryos and late two-cell embryos were cultured in aphidicolin to arrest development when they entered S-phase. The test construct containing GT enhancer, pGT_5Pyluc (25 μg/ml), produced ~2.35×10^5 RLU in the zygotic nuclei of S-phase-arrested two- and four-cell embryos. This was ~500-fold more than pPyluc produced in these embryos. Each competitor plasmid was 200 μg/ml except for pSGVP (75 μg/ml) which was used only to provide GAL4:VP16 at saturating levels. pSVpro contained the SV40 promoter consisting of six tandem Sp1 DNA binding sites linked to a TATA box. pSVpro/enh contained the complete SV40 promoter and enhancer region. The total concentration of DNA in the microinjected solution was kept constant at 300 μg/ml in two-cell embryos by addition of the appropriate amount of pBR322. Each set of plasmid DNA was diluted into half before injecting them into the paternal pronucleus of one-cell embryos, to keep the total DNA concentration at 150 μg/ml. pGT_5Pyluc produced ~0.5×10^5 RLU of luciferase activity in these embryos. This was ~1.5-fold more than that produced by pPyluc under similar conditions.

GAL4:VP16 (pSGVP). These controls were reproduced and compared with oocytes that had been preinjected with ES cell mRNA. The results revealed that ES cell mRNA expressed a factor that allowed the GAL4 enhancer to stimulate the tk promoter.

Pre-injection of oocytes with ES cell mRNA, and not *Escherichia coli* tRNA, stimulated $pGAL_9tkluc$ 3.5-fold in the presence of pSGVP, or ~25% of the maximum amount of enhancer stimulation one might expect based on the maximum ability of butyrate to stimulate tk promoter activity in oocytes (Figure 5, Oocytes). ES cell mRNA did not stimulate the tk promoter in the absence of an enhancer. Injection delivered ~0.5 pg of mRNA per cell, which is ~2.5-fold more than the poly(A)$^+$ RNA content of a two-cell embryo (Zimmerman and Schultz, 1994). Since oocytes contain 2- to 3-fold more poly(A)$^+$ RNA, the amount of ES cell mRNA delivered to oocytes was equivalent to the amount of endogenous mRNA already present. Higher concentrations of ES cell mRNA could not be tested, because they were toxic to the cells, but a 1:1 mixture of ES cell mRNA and *E.coli* tRNA gave ~50% stimulation. Since *E.coli* tRNA alone had no effect, enhancer-specific stimulation was in proportion to the amount of ES cell mRNA provided. Therefore, these mRNA preparations provided one or more factors required for enhancer activity that were absent in oocytes.

The specificity of the enhancer-specific stimulation observed by preinjecting ES cell mRNA into oocytes was confirmed by repeating similar experiments in two-cell embryos (Figure 5, 2-Cell Embryos). As described before, the activity of the tk promoter was repressed in cleavage-stage embryos, and this repression could be relieved either by addition of butyrate to the culture medium (Figures 1–3) or by linking the promoter to an active enhancer, such as the F101 enhancer or the GAL4-dependent enhancer in the presence of GAL4:VP16. When these controls were reproduced and compared with two-cell embryos that had been preinjected with ES cell mRNA, the results revealed that ES cell mRNA could not further stimulate either the

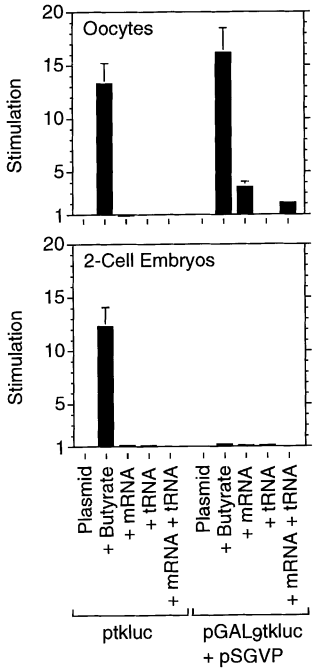

Fig. 5. Enhancers can be activated in mouse oocytes by preinjection of mRNA from mouse embryonic stem (ES) cells. Oocytes or two-cell embryos were injected with either ptkluc or $pGAL_9tkluc$ plus sufficient amount of pSGVP as described in Figure 2. Some injected ova were cultured in the presence of 2.5 mM butyrate. In some experiments, ~2 pl of 500 μg/ml of ES cell mRNA, *E.coli* tRNA (Sigma), or a mixture of ES cell mRNA and tRNA (1:1) was preinjected into the cytoplasm of oocytes or two-cell embryos (both blastomeres) 1 h before either ptkluc or $pGAL_9tkluc$ + pSGVP was injected into one of the nuclei of these cells. Stimulation by Gal4-dependent enhancer in oocytes was marginal when mRNA and luciferase expression vectors were co-injected into the nuclei. ES cells were generated from mouse blastocysts and propagated on lysed PMEF cells as feeder layer in DME plus 15% heat-inactivated fetal bovine serum and in the presence of 1000 U/ml ESGRO murine leukemia inhibitory factor to prevent them from differentiating. mRNA from ES cells was isolated using an RNA isolation kit from Stratagene.

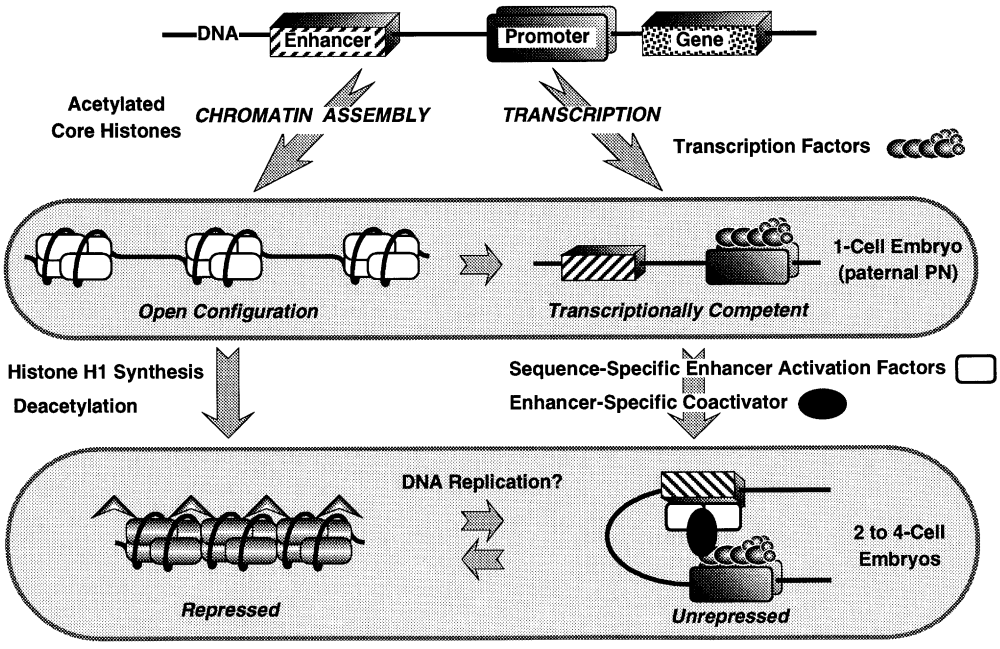

Fig. 6. The role of enhancers in relieving chromatin-mediated repression of promoters. Enhancer activity requires both sequence-specific transcription factors that bind to the enhancer and a unique coactivator that mediates interaction of the enhancer with one or more of the proteins that activate a promoter. Chromatin-mediated repression is absent from the paternal pronucleus of one-cell embryos, so that enhancers are dispensable under these conditions. Repression does occur in the maternal pronucleus of oocytes and one-cell embryos, and in the zygotic nuclei of cleavage-stage embryos as well as in later stages of development, but the enhancer-specific coactivator first appears in two-cell embryos. Thus, it is not until the major ZGE begins that enhancers can be utilized to relieve chromatin-mediated repression.

tk promoter alone (ptkluc), or the tk promoter in the presence of a functional GAL4 enhancer ($pGAL_9$tkluc). *E.coli* tRNA also had no effect. Since two-cell embryos already contained both promoter- and enhancer-specific factors, the additional factors provided by ES cell mRNA were apparently not needed.

Discussion

As a fertilized mouse egg develops into an adult animal, gene expression passes from a state in which all of the zygote's genes are turned off (the one-cell embryo) to one in which zygotic genes can be activated selectively at specific times and in specific cell types. Enhancers provide an important mechanism in this transition. However, our knowledge of how enhancers and other transcriptional elements function has been limited to studies of viral and cellular genes in either cultured cells or cell extracts. Studies of transcription regulation as a function of mammalian development have been difficult due to the limited numbers and sizes of embryos available for biochemical studies. One solution to this problem has been to microinject plasmid-encoded reporter genes in order to identify requirements for specific *cis*-acting sequences and *trans*-acting factors that regulate DNA transcription and replication at the beginning of mammalian development. This approach allows these processes to be characterized within the context of single living embryos as they undergo the transition from dependence on maternally inherited mRNA and proteins to dependence on ZGE. Utilizing this approach, we have confirmed our previous results (Majumder *et al.*, 1993) showing that enhancers provide a special function *in vivo* that is distinct from that of promoters, and have extended them to reveal that enhancer function is developmentally acquired after formation of a two-cell embryo through the appearance of a unique coactivator activity.

A plasmid-encoded reporter gene injected into mammalian nuclei is subject to two competing reactions: assembly into an active transcription complex versus assembly into a repressed chromatin state (Figure 6). Thus, the fraction of active transcription complexes depends on the relative concentrations of the proteins that compose these two pathways. For example, histone H1 synthesis and core histone deacetylation promote formation of repressed chromatin, while basal level transcription factors together with sequence-specific DNA binding proteins that bind to promoters and enhancers facilitate formation of active transcription complexes (Workman and Buchman, 1993; Wolffe and Pruss, 1996). This competition occurs in most cell nuclei, but one exception is the paternal pronucleus of one-cell embryos. Here, the chromatin formed is in an open configuration that allows formation of transcriptionally active initiation complexes without the need for an enhancer. Therefore, it is necessary for a 'zygotic clock' (described below) to delay transcription in these cells in order to prevent premature gene expression. On the other hand, in cleavage-stage embryos (e.g. two- and four-cell embryos) where ZGE begins during mouse development, injected DNA is rapidly converted into a repressed chromatin state, and enhancers are required to prevent formation of this repressed state at promoter sites by interacting directly with one or more of the proteins that bind to promoters. The results presented here suggest strongly that this interaction requires a specific coactivator, in addition to proteins that bind to specific sequences that define the enhancer elements. However, once chromatin has repressed a promoter, DNA replication

may be required to disrupt this chromatin structure and allow enhancer recognition proteins to bind to promoter recognition proteins. DNA replication would then provide a mechanism for reprogramming gene expression.

The enhancer-specific coactivator mediates the interaction of enhancers with promoters presumably by direct interaction between sequence-specific transcription factors that bind to the enhancer and to the transcription complex that forms at the promoter. This interaction has been demonstrated in previous studies on promoter activity during mouse development (Majumder and DePamphilis, 1994a). In differentiated cells, enhancer stimulation of the tk promoter requires a TATA box, a requirement that appears to be a general feature of RNA polymerase II promoters, while prior to cell differentiation, in cleavage-stage embryos, enhancer stimulation of the tk promoter requires an upstream Sp1 binding site. Since the same transcription factors (e.g. TEF-1 and GAL4:VP16) that can function in the capacity of a promoter in oocytes or at the beginning of ZGE in S-phase-arrested one-cell embryos cannot function in the capacity of an enhancer until the two- and four-cell stages in development, separate coactivators specific for either promoter function (short range) or enhancer function (long range) must exist. The fact that some activation domains can function only proximal to the transcription site while others can function in both proximal and distal positions (Seipel *et al.*, 1992, 1994) is consistent with the conclusion that proximal and distal interactions are mediated by different coactivators. Previous studies (Ge and Roeder, 1994; Kretzschmar *et al.*, 1994) have revealed the presence of general coactivators such as PC4 (p15) that are not obligatory for RNA polymerase II basal level transcription, but that facilitate transcription through direct interaction between transcription factors that bind at sites upstream but proximal to the TATA box (e.g. Gal4-based acidic activators) and components of TFIID (e.g. TFIIA). Such coactivators can stimulate promoter activity in the absence of chromatin assembly in *in vitro* reactions. Whether or not the same coactivator can also mediate the distal action of an enhancer remains to be determined. The nature of the enhancer-specific coactivator activity described here and its role, if any, in activating promoter activity from a proximal site remain to be determined. It appears to be required for the activity of many, apparently unrelated, enhancers and as such could serve as a master switch to regulate the activity of several different genes at one time by determining when and under what conditions enhancers could be utilized to relieve chromatin-mediated repression.

The repression observed at the beginning of mouse development appears to be mediated by chromatin structure, because it can be relieved either by treating the cells with butyrate, or, in cleavage-stage embryos (two or more cells), by linking the promoter or replication origin to an embryo-responsive enhancer (Majumder and DePamphilis, 1995; Wiekowski *et al.*, 1997). Butyrate increases the fraction of hyperacetylated core histones by inhibiting histone deacetylation, and thereby stimulates expression of both cellular and plasmid-encoded genes (Turner and O'Neill, 1995). Hyperacetylated core histones increase the accessibility of chromatin to transcription factors and reduce the affinity of oligonucleosomes for histone H1, an important contributor to chromatin condensation and gene repression (Bouvet *et al.*, 1994; Juan *et al.*, 1994). These effects are consistent with the observation that transcriptionally active genes are enriched in hyperacetylated core histones and deficient in histone H1 (Tazi and Bird, 1990; Hebbes *et al.*, 1992). Treatment of mouse oocytes and early embryos with optimum amounts of butyrate stimulates the activity of plasmid-encoded promoters injected into the maternal nuclei of oocytes, activated eggs, and one-cell embryos, or into any nucleus in two-cell embryos, regardless of its nuclear origin or ploidy, but butyrate does not stimulate promoter activity in the paternal pronuclei in one-cell embryos (Majumder *et al.*, 1993; Wiekowski *et al.*, 1993). Thus, butyrate stimulates promoter activity in nuclei that exhibit repression, and does not stimulate promoter activity in nuclei that do not exhibit repression. The fact that butyrate has opposite effects on the maternal and paternal pronuclei in a one-cell embryo strongly suggests that these effects are directed at the structure of chromatin assembled onto the injected plasmid, rather than by changes in transcription factors. Moreover, butyrate and other histone deacetylase inhibitors simply stimulate synthesis of transcription-dependent proteins at the onset of ZGE without changing the pattern of protein synthesis (Wiekowski *et al.*, 1993; Worrad *et al.*, 1995). More recently (Wiekowski *et al.*, 1997), changes have been identified in the synthesis and modification of chromatin-bound histones that are consistent with this hypothesis. The appearance of chromatin-mediated repression of promoters at the beginning of mouse development was consistent with changes in the production of histone H1 and acetylation of core histones: repression was greatest when all five histones were being synthesized and core histones were not hyperacetylated. *In vitro*, enhancers do not stimulate transcription unless the DNA substrate is organized into chromatin and the chromatin is condensed by addition of histone H1 (reviewed in Majumder *et al.*, 1993; Paranjape *et al.*, 1994). However, deletion of the histone H1 gene in *Tetrahymena* reveals that linker histone H1 regulates specific gene expression but not global transcription *in vivo* (Shen and Gorovsky, 1996). Thus, other 'linker' proteins may also play a role in chromatin-mediated repression.

How do 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 ~40 h post-fertilization, a time when a normally developing embryo is at the two-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 two-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 that no spurious transcription occurs during the remodeling period. On the other hand, after the zygotic remodeling, the chromatin-mediated

repression of most promoters in two-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 ZGE provides an additional mechanism for preventing inappropriate transcription of genes destined for expression in specific cell types.

The same mechanisms of transcriptional control that initiate mouse development also seem to occur in other animals. In mammals other than mice, transcription is delayed until the two- to 16-cell stage, presumably by the same zygotic clock mechanism. Thus, the ZGE begins at the two-cell stage in hamsters, the four-cell stage in pigs, the four- to eight-cell stage in humans, and the eight- to 16-cell stage in sheep, rabbits and cows (Telford *et al.*, 1990; Seshagiri *et al.*, 1992; Schultz, 1993). Whether or not repression of promoter activities appears at the two-cell stage in these mammals, or is delayed until the same stage that transcription begins, remains to be seen. The S-phase of a two-cell mouse embryo appears equivalent to the sixth cleavage stage in *Xenopus*, where synthesis of heterogeneous, non-ribosomal mRNA is first detected. The G_2-phase of a two-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 two-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 two-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).

Materials and methods

Mouse embryos and oocytes

Isolation, culture and injection of CD-1 mouse embryos and oocytes were carried out as previously described (Majumder *et al.*, 1993; Miranda and DePamphilis, 1993; Miranda *et al.*, 1993; Majumder, 1996). Growing oocytes were obtained from 13- to 14-day-old females, and were cultured in the presence of 100 μg/ml dibutyryl-cAMP to prevent meiotic maturation. Growing oocytes obtained from 2- to 3-week-old prepubertal mice are more transcriptionally active than mature oocytes obtained from older mice (Worrad *et al.*, 1994). Fertilized eggs (one-cell embryos) were isolated from 8- to 10-week-old pregnant females 17 h after human chorionic gonadotrophin hormone (hCG) was injected and were cultured in the presence of 4 μg/ml aphidicolin (Boehringer Mannheim) to arrest their development at the beginning of S-phase. Two-cell embryos were isolated 40–42 h post-hCG injection, at which time they had completed S-phase. When these embryos are cultured in the presence of aphidicolin, they undergo cleavage into four-cell embryos and are then arrested at the beginning of S-phase. In the absence of aphidicolin, most injected two-cell embryos develop into morula by 44 h.

Plasmids

Various plasmid DNAs used in this study have been described previously (Majumder *et al.*, 1993). Plasmids containing the firefly luciferase gene (pluc) linked to the herpes simplex virus (HSV) thymidine kinase (tk) promoter (ptkluc) or the tk promoter coupled to the polyomavirus (Py) F101 enhancer (pF101tkluc) were previously used for studying enhancer function in mouse oocytes and embryos (Majumder *et al.*, 1993). pSVCAT expresses the bacterial CAT gene driven by the SV40 promoter/enhancer, pSGVP encodes Gal4:VP16 fusion protein, pMEX4 expresses herpes simplex virus ICP4 driven by its natural promoter (Resnick *et al.*, 1989), $pGAL_9$tkluc (Gal4-dependent enhancer construct) contains nine gal4 binding sites placed 600 bp upstream of the tk promoter driving the luciferase reporter gene, and $pGAL_5$TCAT (Gal4-dependent promoter construct) contains five Gal4 binding sites placed 10 bp upstream of a TATA box driving the bacterial CAT reporter gene. The GT promoter construct, pGT_4Tluc, contains four tandem copies of the polyomavirus TEF-1 DNA binding site placed 10 bp upstream of the adenovirus late gene promoter TATA box driving the luciferase gene. pPyluc represents the same as ptkluc, except that the tk promoter is replaced by polyomavirus (Py) promoter. pF101Pyluc (F101 enhancer), pF101tkluc (F101 enhancer) and pGT_5Pyluc (GT enhancer) contain either the F101 enhancer or the GT enhancer (five tandem copies of the TEF-1 DNA binding site), placed 600 bp upstream of the corresponding promoter.

Plasmid DNA was prepared in 10 mM Tris–HCl pH 7.6 and 0.25 mM EDTA (Majumder, 1996) to the desired concentration, and ~2 pl was injected into one-cell embryos 22–28 h post-hCG treatment, and into two-cell embryos 44–48 h post-hCG. Embryos surviving injection were assayed for firefly luciferase or bacterial CAT activities.

Firefly luciferase assay

Firefly luciferase activity was assayed in individual embryos as previously described (Miranda *et al.*, 1993; Majumder, 1996). For each data point the mean value of 40–150 oocytes or embryos was used, and the variation among individual embryos expressed as the standard error of the mean. While the range of luciferase activities among individual embryos could vary as much as 1000-fold (Majumder, 1996), the mean value obtained from several independent experiments was reproducible to within 13–25%. Moreover, the relative activity between different types of embryos and different promoters was always reproducible, even when DNA injection was performed by different people.

CAT assay

About 50 injected embryos were incubated for 44 h, harvested in 250 mM Tris pH 8.0 at a concentration of 0.5 embryo/μl, lysed by freeze–thawing three times in dry ice/ethanol and 37°C baths, centrifuged at 16 000 *g* for 5 min at 4°C, and the supernatant assayed for CAT activity as described by Sambrook *et al.* (1989). The fraction of [^{14}C]acetylchloramphenicol was measured by using a Betascope 603 (Betagen) to collect at least 100 000 emissions. These numbers were then normalized to the average total [^{14}C]chloramphenicol present in the lane of the chromatogram and expressed as c.p.h./embryo.

Assays of promoter activity in transfected cells

Luciferase assays were performed on 50 μl portions of cell extract prepared in CEB (0.1M sodium phosphate pH 7.8, 1 mM dithiothreitol, 0.1% Triton X-100) under the same conditions used for extracts of mouse oocytes and embryos as described previously (Miranda *et al.*, 1993). CAT assays were also carried out on 50 μl portions of cell extract prepared in CEB. Extracts were incubated with 4 mM acetyl coenzyme A, 0.05 μCi [^{14}C]chloramphenicol (Amersham), 0.5 M Tris–HCl pH 8.0 at 37°C for 1 h, and then extracted with 900 μl ethyl acetate and lyophilized. The pellet was dissolved in 25 μl ethyl acetate, chromatographed on silica gel and analyzed by autoradiography (Sambrook *et al.*, 1989).

Embryonic stem cells

Embryonic stem (ES) cells were generated from mouse blastocysts as described by Abbondanzo *et al.* (1993). ES cells were grown on lysed PMEF cells as feeder layer in DME (Specialty Media) plus 15% heat-inactivated fetal bovine serum and in the presence of 1000 U/ml ESGRO murine leukemia inhibitory factor (Gibco-BRL) to prevent them from differentiating. mRNA from ES cells for initial experiments were carried out using a commercially available RNA isolation kit (Stratagene). These experiments were repeated using ES cell mRNA.

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