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Analysis of gene expression in mouse preimplantation embryos demonstrates that the primary role of enhancers is to relieve repression of promoters

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Enhancers are generally viewed simply as extensions of promoters, lacking a function of their own. However, previous studies of mouse preimplantation embryos revealed that 1-cell embryos can utilize enhancer-responsive promoters efficiently without an enhancer, whereas 2-cell embryos require an enhancer to achieve the same levels of expression. This suggested that enhancers relieved a repression in 2-cell embryos that is absent in 1-cell embryos. Results presented here demonstrate first that the ability of 1-cell embryos to dispense with enhancers does not result from the absence of specific activation proteins. Under conditions where GAL4-VP16 activated a GAL4-dependent promoter in both embryos, GAL4-VP16 activated a GAL4-dependent enhancer only in 2-cell embryos. Moreover, the role of an enhancer is not to compensate for either changes in promoter requirements, or for reduced levels of promoter-specific transcription factors. Linker-scanning mutations in a natural promoter revealed that both embryos utilized the same promoter elements, and comparison of different promoters revealed that these embryos have equivalent transcriptional capacities. In addition, titration experiments revealed less Sp1 activity in 1-cell embryos where enhancers are dispensable than in 2-cell embryos where enhancers are required. Therefore, we propose that the primary function of enhancers, first evident with formation of a mouse 2-cell embryo, is to prevent repression of weak promoters, probably by altering chromatin structure. Consistent with this hypothesis is the fact that butyrate, an agent that alters chromatin structure, stimulated promoters in 2-cell embryos, but not in 1-cell embryos.

Key words: chromatin structure/enhancer/mouse preimplantation embryo/promoter/transcription factors

Introduction

Transcription factor binding sites that control gene expression by RNA polymerase II are frequently divided into two groups, promoters, which function proximal to the start site and determine where transcription begins, and enhancers, which function distal to the start site, independently of their orientation or position relative to the gene, and impart tissue specificity by stimulating enhancer-responsive ('weak') promoters (Wasylyk, 1988). Enhancers are also components of some origins of DNA replication (DePamphilis, 1988) where their activity depends on binding specific transcription factors (Guo and DePamphilis, 1992). Since the same transcription factor can act either in the capacity of a promoter or an enhancer, an enhancer is often viewed simply as an extension of the promoter in which transcription factors with the strongest activation domains can act at the greatest distances from the transcription start site (Carey *et al.*, 1990; Schatt *et al.*, 1990). This view does not distinguish the role of promoters from that of enhancers; both transcriptional elements promote assembly of an active initiation complex. Alternatively, enhancers may provide a function *in vivo* that is distinct from that of promoters. For example, promoters could facilitate assembly of a transcription complex while enhancers could prevent repression of promoter activity by a variety of possible mechanisms, the most general of which is chromatin structure (Felsenfeld, 1992). In this paper, we present results from microinjecting plasmid DNA into mouse preimplantation embryos that strongly support the latter model.

DNA injected into the nuclei of mouse oocytes, 1-cell embryos and 2-cell embryos responds to normal cellular signals that regulate DNA replication and gene expression in that it undergoes replication and transcription only in cells competent for that function, and only when unique eukaryotic regulatory sequences are present. For example, mouse oocytes can express some of their genes but, because they are arrested in prophase of their first meiosis, they cannot replicate DNA. Accordingly, plasmid DNA does not replicate when injected into mouse oocytes, even if the injected DNA contains a viral origin and is provided with the appropriate viral proteins (Wirak *et al.*, 1985; Chalifour *et al.*, 1986; De Pamphilis *et al.*, 1988; Martínez-Salas *et al.*, 1988), but injected eukaryotic promoters are active. The same sequence that provides oocyte specific expression of zona pellucida protein-3 (ZP3) when integrated into the chromosomes of transgenic animals (Lira *et al.*, 1990; Schickler *et al.*, 1992) also provides oocyte-specific expressions when present on injected plasmid DNA (Millar *et al.*, 1991). However, while oocytes utilize some of the same promoter elements recognized by somatic cells, promoter activity in oocytes does not appear to require enhancers (Chalifour *et al.*, 1986, 1987). One reason for this may be that oocytes produce unique *trans*-acting factors that mimic certain viral transcription factors (Dooley *et al.*, 1989).

Oocytes mature into eggs which are then fertilized to produce a zygote, but transcription and translation of zygotic genes does not begin until the 2-cell stage of development (Telford *et al.*, 1990). Initiation of zygotic gene expression is governed by a 'clock' that initiates transcription ~20 h post-fertilization, regardless of whether or not the 1-cell embryo (fertilized egg) has completed DNA replication or undergone mitosis (Conover *et al.*, 1991; Manejwala *et al.*, 1991; Wiekowski *et al.*, 1991). Accordingly, promoters injected into the pronuclei of 1-cell embryos remain inac-

tive until this 'zygotic clock' initiates expression of the endogenous genes (Martínez-Salas *et al.*, 1989; Wiekowski *et al.*, 1991), showing that expression of injected genes is governed by the same mechanism that regulates expression of zygotic genes. This delay is observed only in 1-cell embryos whose morphological development is arrested. When injected 1-cell embryos develop to the 2-cell stage or beyond, expression of injected genes is reduced to $<1\%$ of levels observed in arrested 1-cell embryos. Similarly, DNA containing a viral origin replicates in the presence of its cognate recognition protein only in those injected 1-cell embryos that spontaneously remain in appearance as 1-cell embryos; plasmid DNA replication is barely detectable in those embryos that continue morphological development (Wirak *et al.*, 1985). Apparently DNA injected into 1-cell embryos is repressed by changes in its physical state that occur during formation of a 2-cell embryo.

The levels of promoter and origin activity injected into 2-cell embryos are 12- to 500-fold lower than in arrested 1-cell embryos. The exact difference depends on the promoter or replication origin tested, the amount of DNA injected, and whether or not injected 2-cell embryos are arrested at the beginning of S-phase in the 4-cell stage. In order for 2-cell embryos to produce levels of transcription or replication that are equivalent to or slightly greater ($\sim$2-fold) than observed in arrested 1-cell embryos, the DNA injected into 2-cell embryos must carry an embryo responsive enhancer. Enhancers that strongly stimulate promoter or origin activities in 2-cell embryos have no effect in arrested 1-cell embryos or oocytes. Thus, the requirements for replication and expression of genes in mammalian somatic cells appear to be established upon formation of a 2-cell embryo.

The differences in requirements for replication and transcription that are observed prior to this stage reflect changes unique to preimplantation development rather than experimental manipulations. The ability to dispense with enhancers is not a consequence of arresting 1-cell embryos in S-phase, because enhancers are required for full promoter activity in either developing or S-phase arrested 2-cell and 4-cell embryos (Wiekowski *et al.*, 1991). Nor is it determined by the time of injection relative to the cell proliferation cycle, or the developmental history of the embryo. In fact, expression of genes injected into arrested 1-cell embryos is tightly linked to zygotic gene expression. Therefore, the results described above with mouse oocytes and embryos reveal that formation of a 2-cell embryo results in a general reduction of promoter activity, and suggests that the function of enhancers *in vivo* is first required at the 2-cell stage.

The experiments described in this paper demonstrate that the difference between 1-cell and 2-cell embryos in their need for enhancers does not result from differences in the composition or concentration of transcription factor activities. This conclusion is based on four types of experiment. First, a synthetic transcription factor (GAL4-VP16) was expressed at saturating levels and its ability to activate promoters and enhancers was quantitatively evaluated. Second, the effect of linker-scanning mutations in a natural promoter were evaluated in 1-cell embryos, 2-cell embryos and mouse fibroblasts. Third, the activities of different promoters were compared in 1-cell and 2-cell embryos. Fourth, the relative amount of Sp1 activity in 1-cell and 2-cell embryos was measured by competition between the tk promoter and a series of tandem Sp1 DNA binding sites. Furthermore, the repressed promoter activity in 2-cell embryos can be relieved either by a functional enhancer or by butyrate, an agent that alters chromatin structure. Therefore, we propose that the primary function of enhancer, first evident with formation of a mouse 2-cell embryo, is to prevent repression of weak promoters, probably by altering chromatin structure.

Results

Promoter activity is much lower in 2-cell embryos than in 1-cell embryos, and enhancers restore this activity

The relative capacity of 1-cell and 2-cell mouse embryos to utilize common transcriptional elements was determined by injecting their nuclei with plasmid DNA containing the herpes simplex virus (HSV) thymidine kinase (tk) promoter, linked or unlinked to the Py F101 enhancer (F101). 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 (Eisenberg *et al.*, 1985; Martínez-Salas *et al.*, 1989). The F101 enhancer is the strongest enhancer found so far for stimulating promoter activity in 2-cell to 8-cell mouse embryos (F.Melin, unpublished data). Enhancer elements were placed 600 bp upstream of the promoter (Figure 1). The activity of promoters and enhancers was quantitatively evaluated by their ability to express the firefly luciferase gene (luc).

In order to compare the responses of 1-cell and 2-cell embryos under identical experimental conditions, 2-cell embryos were routinely cultured in aphidicolin to arrest their development at the beginning of S-phase. Under these conditions, the relationship between the amount of DNA injected and the cell's ability to utilize an enhancer was examined in order to determine optimal injection conditions. When arrested 1-cell embryos were injected with different amounts of plasmid DNA containing the tk promoter driving the luciferase gene (ptkluc, Figure 1), the amount of luciferase activity observed was dependent on the amount of DNA injected (Figure 2). This level of tk promoter activity was not affected by linking it to the F101 enhancer (pF101tkluc).

In contrast, when mouse 2-cell embryos were isolated and cultured under the same conditions, promoter activity was reduced at least 30-fold relative to 1-cell embryos, and the F101 enhancer was required to restore activity in arrested 2-cell embryos to a level that was 2- to 3-fold greater than observed in arrested 1-cell embryos. As with 1-cell embryos, the level of tk promoter activity depended on the amount of ptkluc injected (Figure 2). However, the amount of ptkluc that produced the maximum level of luciferase activity in 1-cell embryos produced only 3% as much luciferase in 2-cell embryos. This level of tk promoter activity was increased 80-fold by linking it to the F101 enhancer. The extent of stimulation by the F101 enhancer depended upon the amount of DNA injected, because tk promoter activity increased with increasing levels of injected DNA and the cell eventually reached its capacity to utilize these regulatory sequences. Nevertheless, luciferase activity in 2-cell embryos was never >3-fold more than observed in 1-cell embryos. These results were consistent with previously published data in which 2-cell embryos were allowed to develop in the

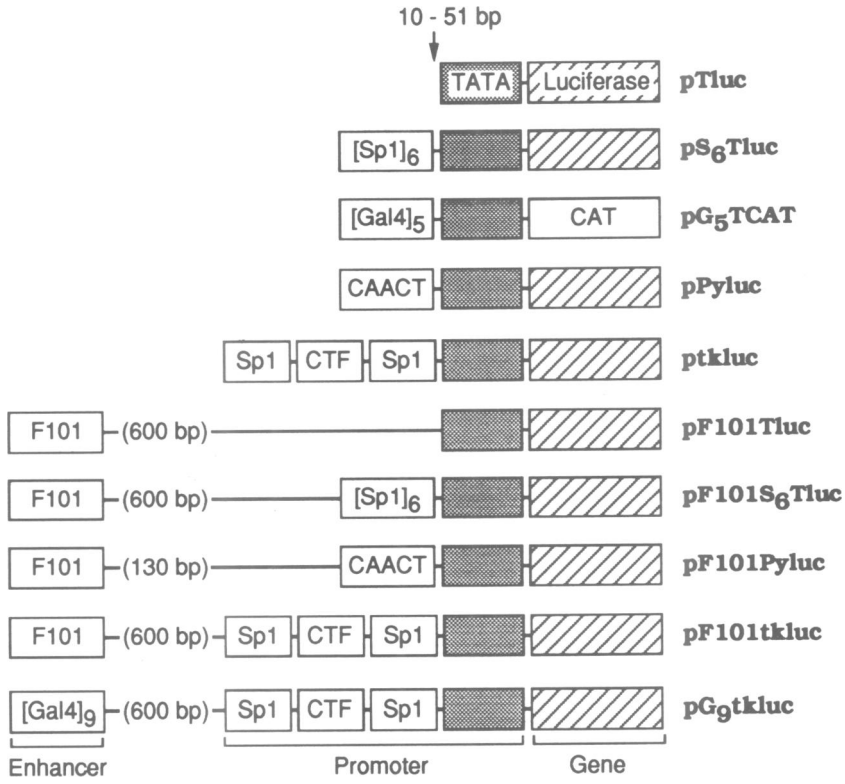

Fig. 1. Transcriptional elements comprising the promoters and enhancers used in the experiments described in this paper.

absence of aphidicolin (Martínez-Salas *et al.*, 1989; Wiekowski *et al.*, 1991) and suggested that the tk promoter is repressed when injected into 2-cell embryos, but not when injected into 1-cell embryos. Furthermore, they suggested that the F101 enhancer alleviates this repression.

The ability of 1-cell embryos to dispense with enhancers does not result from the absence of specific activation proteins

One explanation for the inability of the Py F101 enhancer to stimulate promoters injected into 1-cell embryos might simply be the absence of appropriate transcription factors required to activate this enhancer. If these proteins were present, then activity in 1-cell embryos might increase to even higher levels than previously observed. To test this possibility, a tandem series of yeast GAL4 DNA binding sites were used as a promoter in one plasmid and as an enhancer in another plasmid. This sequence binds the protein GAL4:VP16, a fusion of the DNA binding domain from the yeast transcription factor GAL4, and the acidic activation domain from HSV coat protein 16. GAL4:VP16 can stimulate transcription strongly in differentiated mammalian cells when either the promoter or the enhancer contains tandem copies of its binding site (Sadowski *et al.*, 1988; Gu and DePamphilis, 1992). Since GAL4:VP16 is not present in mammalian cells, Gal4 DNA binding sites are inactive unless the missing transcription factor (GAL4:VP16) is expressed from a coinjected plasmid.

The GAL4-dependent promoter consisted of five tandem copies of yeast Gal4 DNA binding sites proximal to a TATA box (pG_5TCAT, Figure 1). As expected, pG_5TCAT was inactive in 1-cell and 2-cell embryos unless coinjected with the expression vector for GAL4:VP16 (Figure 3). Increasing the amount of the expression vector 25-fold in either 1-cell or 2-cell embryos increased GAL4 promoter activity only 2-fold. This means that the level of GAL4:VP16 produced was saturating the Gal4 DNA sites. Under these conditions, the activity of the GAL4 promoter was essentially the same in 1-cell and 2-cell embryos.

In contrast to the GAL4-dependent promoter, GAL4-dependent enhancer activity was observed only in 2-cell embryos. The GAL4-dependent enhancer consisted of nine Gal4 DNA binding sites 600 bp upstream of the tk promoter (pG_9tkluc, Figure 1). Plasmids ptkluc and pG_9tkluc produced equivalent amounts of luciferase when injected into 1-cell embryos either in the absence (Figure 4, −GAL4:VP16) or presence (Figure 4, +GAL4:VP16) of saturating levels of GAL4:VP16. Although GAL4:VP16 nonspecifically stimulated the tk promoter 2.5-fold, regardless of the presence or absence of GAL4 DNA binding sites, the levels of tk promoter activity observed in 1-cell embryos were obtained in 2-cell embryos only when the tk promoter was linked to a functional enhancer. The F101 enhancer stimulated tk promoter activity 80-fold (Figure 4, −GAL4:VP16), and, in the presence of GAL4:VP16, the GAL4-dependent enhancer stimulated this promoter 75-fold (Figure 4). The GAL4-dependent enhancer did not stimulate tk promoter activity in the absence of GAL4:VP16. Thus, GAL4:VP16 did not activate the GAL4-dependent enhancer in 1-cell embryos but did stimulate promoter activity 75-fold in 2-cell embryos (Figure 4, bottom panel). This was strikingly similar to our previous observations with the F101 enhancer, except that the presence or absence of F101 enhancer activation protein(s) could not be determined. Failure of the GAL4-dependent enhancer to stimulate the tk promoter in 1-cell embryos clearly was not due to a limited

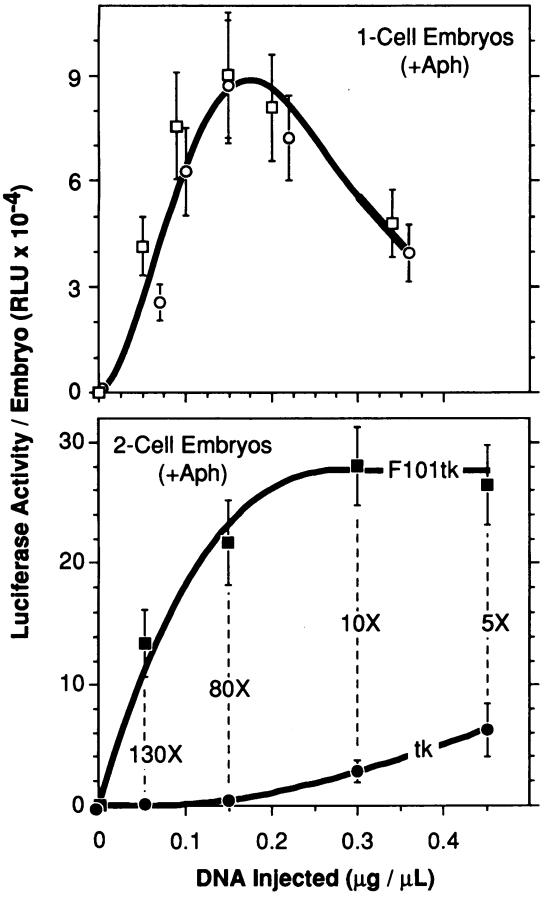

Fig. 2. Expression of luciferase following injection of ptkluc (○, ●) or pF101tkluc (□, ■) into the paternal pronucleus of a 1-cell embryo (top panel), and into one of the zygotic nuclei of a 2-cell embryo (bottom panel). Embryos were cultured in aphidicolin and assayed for luciferase activity. Coenzyme A (CoA) was absent from these assays, but was present in all other assays reported in this paper. Addition of CoA increased luciferase activity ~2.4-fold. Luciferase activity was determined for 40–60 individual embryos per data point. The mean value of these injections ± its standard error were expressed as relative light units (RLU). Similar results were obained when 2-cell embryos were cultured in the absence of aphidicolin, but the levels of activity were 3- to 4-fold less.

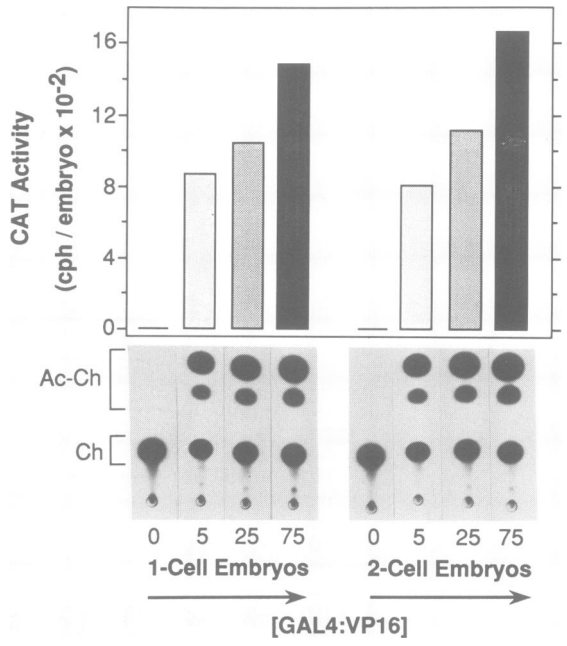

Fig. 3. Activity of a GAL4-dependent promoter in 1-cell and 2-cell embryos. DNA was injected into either 1-cell or 2-cell embryos under the conditions described in Figure 2. Each DNA sample contained a total of 100 μg/ml and consisted of (left to right) pG_5TCAT/pSGVP/pML-1 in the following ratios: 25:0:75 (white bar), 25:5:70 (light grey bar), 25:25:50 (dark grey bar), 25:75:0 (black bar). About 50 injected embryos were incubated for 44 h, harvested in 250 mM Tris (pH 8.0) at a concentration of 0.5 embryo per μl, lysed by freezing and thawing three times using dry ice/ethanol and 37°C baths and centrifuged at 16 000 *g* for 5 min at 4°C, and the supernatant was 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 each lane of the chromatograph and expressed as c.p.h./embryo.

ability of these cells to express luciferase, because the S_6T promoter produced at least 10 times more luciferase than the tk promoter under the same conditions (Figure 6). These results demonstrate that enhancers serve a function in 2-cell embryos but not in 1-cell embryos.

The same promoter elements are utilized in both 1-cell and 2-cell embryos

A second explanation for the ability of 1-cell embryos to dispense with enhancers is that the transcriptional elements in the tk promoter used in 1-cell embryos differ from those used in 2-cell embryos. The tk promoter has been shown to contain four transcription factor binding sites that determine its activity (McKnight and Kingsbury, 1982). Two of these sites bind Sp1, one binds CTF (CAAT box binding protein) and one binds TBP (TATA box binding protein). Linker-scanning mutations were selected that inactivate each of these elements without affecting the distances between various promoter elements (Figure 5), and the mutated promoter was inserted upstream of the luc gene in place of the wild-type tk promoter. The effect of these mutations on tk promoter activity was determined by injecting them into 1-cell and 2-cell embryos, and by electroporating them into mouse 3T3 cells. Each mutation had the same relative effect on tk promoter activity in all three types of mouse cells (Figure 5), demonstrating that the requirements for tk promoter activity were the same from the fertilized mouse egg to differentiated fibroblasts. Therefore, the same promoter elements that are required for transcription under conditions where enhancers stimulate promoter activity (2-cell embryos and 3T3 cells) are also required under conditions where enhancers are dispensable (1-cell embryos). Thus, the need for enhancers that first appears in 2-cell embryos does not reflect changes in the utilization of promoter elements.

The transcriptional capacities of 1-cell and 2-cell embryos are equivalent

A third explanation for the ability of 1-cell embryos to dispense with enhancers is that 1-cell embryos have a greater ability to express promoters than 2-cell embryos (i.e. a greater transcriptional capacity). If the role of an enhancer is to increase promoter strength by providing additional transcriptional elements, then enhancers would have little,

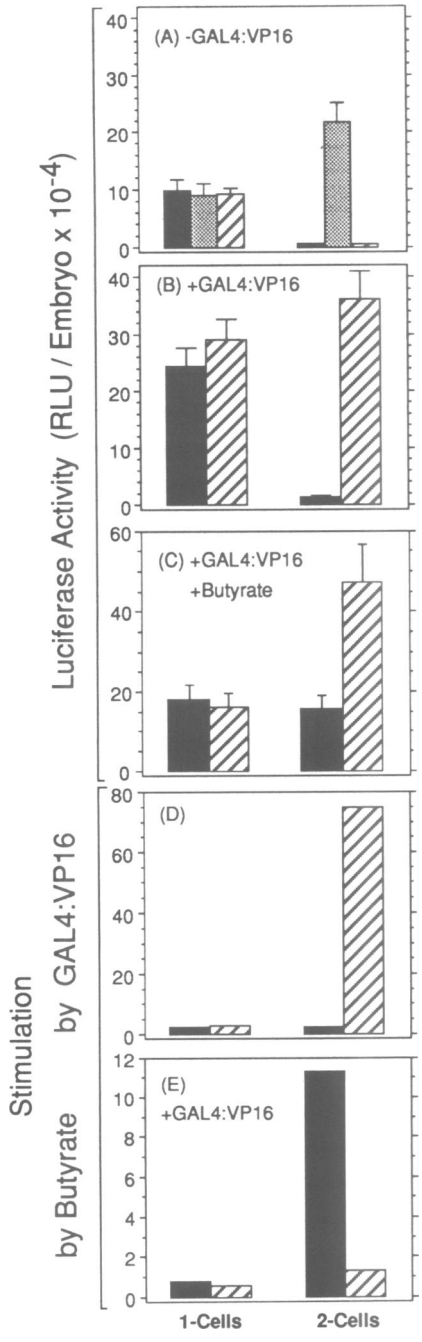

Fig. 4. Activity of a GAL4-dependent enhancer in 1-cell and 2-cell embryos. Conditions were the same as in Figure 2. Panel **A**. ptkluc (black bar), F101tkluc (grey bar) or pG_9tkluc (hatched bar) was combined with pML-1 at a ratio of 25:75 for a total of 100 μg plasmid DNA per ml. Panel **B**. The ratio of ptkluc or pG_9tkluc to pSGVP (GAL4:VP16 expression vector) and pML-1 was 25:25:50 for a total of 100 μg plasmid DNA per ml. Panel **C**. Same as in panel B except that embryos were cultured in the presence of 2.5 mM butyrate as previously described (Wiekowski,M. *et al.*, 1993) Panel D. Stimulation of ptkluc and pG_9tkluc by GAL4:VP16 (+GAL4:VP16/-GAL4:VP16 result for each plasmid). Each bar represents the mean luciferase expressed in 108–150 embryos. Panel **E**. Stimulation of ptkluc and pG_9tkluc by butyrate in the presence of GAL4:VP16 (data in panel C/data in panel A).

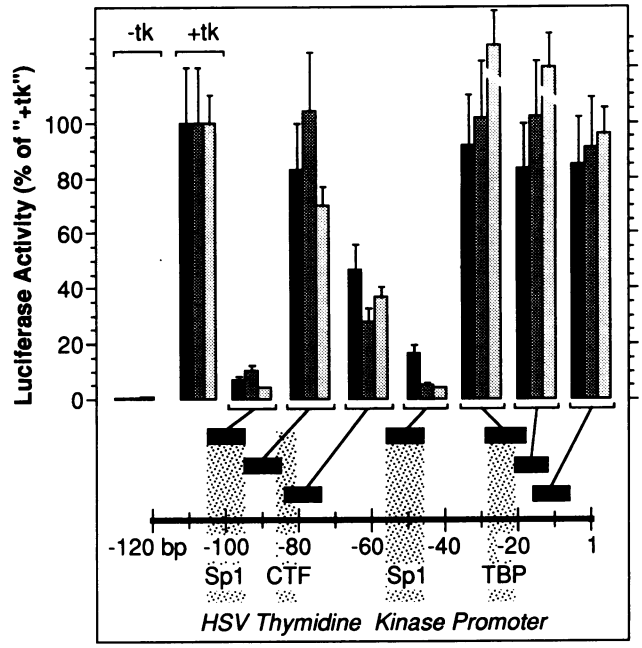

Fig. 5. Effects of linker-scanning mutations on tk promoter activity in mouse embryos and fibroblasts. Plasmid DNA was injected into the paternal pronucleus of 1-cell embryos incubated in aphidicolin (black bar), into one of the zygotic nuclei of 2-cell embryos incubated in aphidicolin (dark grey bar) or electroporated into mouse proliferating NIH/3T3 cells (light grey bar). Positions of linker-scanning mutations are indicated by solid horizontal bars above the scale. From right to left, nucleotide positions of mutations are −16/−6, −21/−12, −29/−18, −56/−46, −84/−74, −95/−85 and −105/−95. Consensus sequences for TATA box binding protein (TBP), Sp1, CAAT box binding protein (CTF) and Sp1 are located at −21/−27, −56/−49, −86/−80 and −105/−98. Experimental conditions for embryos are described in Figure 2. Electroporation was carried out as described by Ustav and Stenlund (1991) and luciferase activity was normalized to total cell protein (Bio-Rad protein assay system). Mutations −29/−18 and −21/−12 produced 200% and 148%, respectively, of wild-type promoter activity in 3T3 cells.

if any, effect in cells with a high transcriptional capacity. This question was evaluated by comparing the activities of different promoters in the presence and absence of the embryo-responsive F101 enhancer. The results show that the transcriptional capacities of 1-cell and 2-cell embryos are equivalent.

Mutations in the tk promoter suggested that Sp1 was an important transcription factor at the beginning of mouse development. Therefore, to create a promoter that would exhibit strong activity in preimplantation embryos, six tandem Sp1 sites from the SV40 T-antigen (T-ag) promoter were placed 30 bp upstream of the adenovirus major late promoter's TATA box (pS_6Tluc). This arrangement of Sp1 DNA binding sites and a TATA box efficiently expresses a reporter gene (Smale *et al.*, 1990) and is functional only in the presence of Sp1 (Pugh and Tjian, 1990). The activity of pS_6Tluc was compared with the activities of a single TATA box (pTluc), the Py T-ag promoter which consists of a TATA box and a CAAT box (pPyluc, Folk *et al.*, 1987) and the HSV tk promoter described above (ptkluc). Each promoter was tested with and without the F101 enhancer cloned into a site 600 bp upstream (Figure 1).

In the presence of the F101 enhancer, each promoter produced similar amounts of luciferase in both 1-cell and 2-cell embryos (Figure 6). Enhancer stimulation was observed only in 2-cell embryos where it was greatest with the weaker promoters. The Py T-ag promoter was stimulated 350-fold while the Sp1 promoter was stimulated only 6-fold.

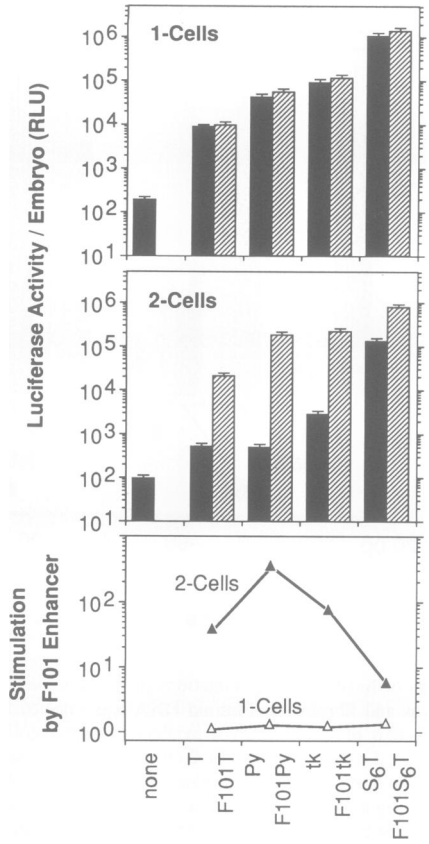

Fig. 6. Effects of different combinations of transcription factor binding sites on promoter activity in 1-cell and 2-cell mouse embryos. Five different promoter constructions, either with (hatched bar) or without (black bar) the F101 enhancer present, were injected into the nuclei of 1-cell and 2-cell embryos under the conditions described in Figure 2. Promoter: 'none' is pluc, 'T' is pTluc, 'Py' is pPyluc, 'tk' is ptkluc and 'S_6T' is pS_6Tluc (Figure 1). Corresponding constructs containing the F101 sequence are also shown. A single TATA box stimulated luciferase in 1-cell embryos 30-fold relative to the absence of a promoter. This basal level of promoter activity was stimulated 5-fold by addition of a CAAT box (pPyluc) had no effect. Addition of two Sp1 sites as well as a CAAT box (ptkluc) stimulated luciferase activity another 6-fold, and six Sp1 sites (pS_6Tluc) stimulated the TATA box activity 260-fold.

Promoter activity increased with increasing numbers of transcription factor binding sites, and the strongest promoter (pS_6Tluc) was almost as active in 2-cell embryos as it was in 1-cell embryos. A single TATA box stimulated luciferase 5-fold (2-cells) to 30-fold (1-cells) (Figure 6), suggesting that TBP is present at the onset of zygotic gene expression even though a TATA box is not required in the presence of other transcription factors (Figure 5). Six tandem Sp1 DNA binding sites stimulated this basal level of activity 134-fold (1-cells) to 260-fold (2-cells), resulting in impressive levels of luciferase activity in both embryos, despite the absence of an enhancer. The strongest promoter was five tandem GAL4 DNA binding sites (pG_5TCAT) that functioned with the same efficiency in both 1-cell and 2-cell embryos (Figure 3). These results demonstrate that enhancers have their greatest effect on weak promoters, and virtually no effect on strong promoters. Thus, both embryos have similar capacities to utilize strong promoters in the absence of an enhancer; the transcriptional capacity of 1-cell embryos is equivalent to that of 2-cell embryos. Therefore, the ability of 1-cell embryos to dispense with enhancers is not due to an unusually high capacity for utilizing promoters.

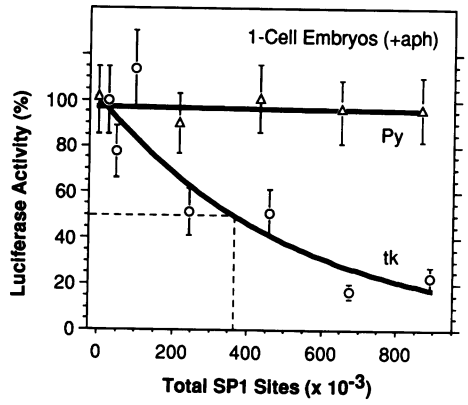

Fig. 7. Titration of Sp1 in 1-cell embryos. Injection conditions were the same as in Figure 2. The concentration of injected DNA was kept constant at 170 μg DNA/ml. Each DNA solution injected consisted of [50 μg/ml ptkluc (○)] + [0−120 μg/ml p[Sp1]$_{12}$] + [120 to 0 μg/ml pML-1]. The same experiment was carried out with pPyluc (△) in place of ptkluc. The number of Sp1 sites indicated includes substrate and competitor plasmids; 26 500 Sp1 sites were represented by ptkluc alone. The error bars indicate the standard error to the mean for 45−55 injected embryos per data point. The dashed line indicates the total fraction of Sp1 sites present when luciferase activity was reduced to 50% of its initial value.

Sp1 activity increases upon formation of a 2-cell embryo

To determine whether or not the general conclusion concerning transcriptional capacity could be demonstrated at the level of a specific transcription factor, advantage was taken of the fact that tk promoter activity was strongly dependent on transcription factor Sp1 in both 1-cell and 2-cell embryos (Figures 5 and 6). This provided a means to measure the relative amounts of Sp1 activity in these two cell types by a competition assay. Plasmid ptkluc was coinjected into 1-cell embryos in the presence of increasing amounts of a second plasmid that contained 12 tandem Sp1 DNA binding sites (pS_{12}). Changes in the amount of pS_{12} were compensated for with pML-1, the bacterial vector that had been used in construction of ptkluc and pS_{12}, in order to maintain the total amount of DNA injected constant. This assay measures the amount of transcription factor activity available to the injected promoter. It is this, rather than the physical amount or subcellular distribution of a transcription factor, that is the critical parameter that determines the level of promoter activity.

As expected, competition between the ptkluc and pS_{12} for binding Sp1 protein resulted in decreased tk promoter activity (Figure 7). Approximately a 15-fold excess of Sp1 binding sites halved the tk promoter activity in 1-cell embryos. Competition was specific for Sp1, because it was not observed when the Py T-ag promoter (pPyluc) was substituted for the HSV tk promoter (Figure 7). The Py T-ag promoter does not depend on Sp1 (Folk *et al.* 1987).

When the same experiment was carried out with 2-cell embryos, about 6 times as many Sp1 DNA binding sites were required to reduce tk promoter activity to half of its initial level (Figure 8, top). There are two ways to account for this difference between 1-cell and 2-cell embryos: 2-cell embryos contain more Sp1 than 1-cell embryos, and 2-cell embryos contain other proteins that also bind to Sp1 DNA binding

sites but do not stimulate transcription. One would expect that competition between Sp1 and other proteins that recognize the Sp1 DNA binding site in ptkluc would repress tk promoter activity in 2-cell embryos. This repression would be relieved by titration with competitor Sp1 DNA binding sites present in pS_{12}. In fact, increasing amounts of pS_{12} first stimulated tk promoter activity ~3-fold and then inhibited it as Sp1 was sequestered by the competitor DNA (Figure 8, top). Under these conditions, a 95-fold excess of Sp1 binding sites was required to reduce tk promoter activity to half of its initial activity. If one assumes that all of the initial 10^6 Sp1 DNA binding sites were occupied by transcriptionally inactive proteins, then a 32-fold excess of Sp1 DNA binding sites was required to reduce tk promoter activity to half of its maximum level. In either case, the amount of Sp1 activity in 2-cell embryos was greater than in 1-cell embryos.

The F101 enhancer eliminated interference of Sp1 binding to the tk promoter, giving results strikingly similar to those observed in 1-cell embryos (Figure 8, bottom). Under these conditions, an ~85-fold excess of Sp1 sites was required to halve pF101tkluc activity. Sp1 sites had no effect on pF101Pyluc. Taken together, these data demonstrate that the amount of Sp1 activity available in 2-cell embryos is at least 2-fold and as much as 6-fold greater than the amount available in 1-cell embryos. Therefore, the ability of an enhancer to stimulate promoters in 2-cell embryos can be explained by a decrease in the amount of a rate-limiting transcription factor that is required to activate the promoter.

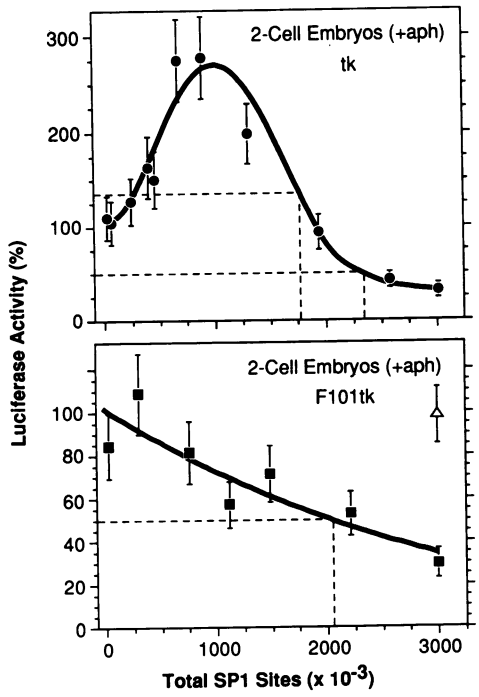

Fig. 8. Titration of Sp1 in 2-cell embryos. Conditions are described in Figure 2, except that the DNA solution injected consisted of [50 μg/ml ptkluc (●)] + [0–410 μg/ml p[Sp1]$_{12}$] + [410 to 0 μg/ml pML-1]. The same experiment was carried out with pF101tkluc (■) or pF101Pyluc (△) in place of ptkluc. In the top panel, dashed lines indicate the total fraction of Sp1 sites present when luciferase activity was reduced to 50% of its initial value (lower line) or 50% of its maximum value (upper line).

Enhancers appear to relieve repression of promoters by altering chromatin structure

The changes that occur with formation of 2-cell embryos affect a diverse range of promoter and origin sequences that bear little homology and that interact with different initiation factors (Figure 6; Wiekowski *et al.*, 1991). Therefore, the repression observed in 2-cell embryos probably results from changes in either nuclear organization or chromatin structure which can affect all DNA sequences. Repression does not appear to result from differences in nuclear organization between paternal pronuclei in 1-cell embryos and zygotic nuclei in 2-cell embryos, because repression of injected promoters occurred in 2-cell embryos whose nuclei were derived entirely from one parent and were either haploid or diploid (Wiekowski *et al.*, 1993). On the other hand, repression does appear to result from chromatin structure.

DNA injected into mouse 2-cell embryos is assembled into nucleosomes (Martínez-Salas *et al.*, 1989). Butyrate can stimulate gene expression in mammalian cells by inhibiting histone deacetylase. This increases the fraction of acetylated core histones in newly assembled chromatin and reduces their association with histone H1, characteristics of transcriptionally active chromatin (Turner, 1991). Repression of tk promoter activity in 2-cell embryos could be relieved either by culturing the embryos with 2.5 mM butyrate or by linking the promoter to the embryo-responsive F101 enhancer, suggesting that enhancers relieve repression resulting from chromatin structure (Wiekowski *et al.*, 1993). Stimulation by either mechanism raised the level of tk promoter activity in 2-cell embryos to that observed in paternal pronuclei of arrested 1-cell embryos. Repression was absent in paternal pronuclei of 1-cell embryos, because neither butyrate nor the F101 enhancer stimulated the injected promoter.

To determine whether or not this conclusion was affected by the presence of enhancer specific activation proteins such as GAL4:VP16, the same experiment was repeated with ptkluc and pG_9tkluc in the presence of GAL4:VP16. Butyrate stimulated the level of tk promoter in 2-cell embryos to that observed in 1-cell embryos, but butyrate did not stimulate promoter activity in 1-cell embryos (Figure 4C, 4E). Butyrate had little, if any, effect on the GAL4-dependent enhancer in either embryo. Thus, butyrate and the enhancer appear to play the same role in stimulating promoter activity. These results suggest that the primary role of enhancers is to prevent general repression of promoter activity, restoring it to levels equivalent to those observed in 'unrepressed' 1-cell embryos.

Discussion

There are essentially two roles that an enhancer can play in the activation of promoters. One is to facilitate formation of an active initiation complex by providing additional transcription factor binding sites, while the other is to prevent either general or site specific repressors from interfering with formation of an active initiation complex (Felsenfeld, 1992). The same is true for the role of enhancers in activating origins of replication. The results presented in this paper demonstrate three important attributes of enhancers that were not previously apparent. First, the function of an enhancer *in vivo* can be distinguished from that of a promoter: 2-cell mouse embryos require enhancers for activation of promoters or origins of replication, whereas 1-cell embryos do not utilize enhancers even when saturating amounts of the

enhancer-specific activation protein are present. Second, the difference between 1-cell and 2-cell embryos in their need for enhancers does not result from differences in the composition or concentration of transcription factor activities because these cells utilize the same promoter elements and have equivalent transcriptional capacities, and 1-cell embryos have less Sp1 activity available to drive the enhancer-responsive tk promoter than do 2-cell embryos. Third, the role of enhancers in activating promoters in 2-cell embryos can be mimicked by butyrate, an agent that alters chromatin structure. Therefore, the primary function of enhancers is to prevent repression of weak promoters, probably by altering chromatin structure.

The primary role of enhancers is distinct from that of promoters

This view of enhancers was first suggested by the existence of a zygotic clock that can initiate transcription in either arrested 1-cell embryos or in 2-cell embryos. This clock implies that DNA injected into either embryo is exposed to the same composition and activity of transcription factors. In fact, 1-cell embryos become transcriptionally competent before mitosis even though they do not initiate transcription (Latham *et al.*, 1992). The four types of experiments described in this paper confirm this assumption and demonstrate that enhancers do, in fact, provide a specific function *in vivo*.

First, the GAL4-dependent enhancer failed to stimulate promoters in 1-cell embryos even when the activity of the appropriate *trans*-acting factor was saturating. Previous studies have shown that a single DNA-binding transcription factor can function in the capacity of either a promoter or an enhancer in mammalian fibroblasts (Schatt *et al.*, 1990). One example is GAL4:VP16 (Guo and DePamphilis, 1992). In the present study, GAL4:VP16 was tested for its ability to function both as a promoter transcription factor and as an enhancer transcription factor in the same cells. GAL4:VP16 was expressed in both 1-cell and 2-cell embryos at sufficient levels to allow a GAL4-dependent promoter (pG_5TCAT) to function equally well in both embryos (Figure 3). However, under the same conditions, a GAL4-dependent enhancer (pG_9tkluc) failed to stimulate the tk promoter in 1-cell embryos whereas it stimulated the tk promoter 75-fold in 2-cell embryos (Figure 4). This was equivalent to the ability of the F101 enhancer to stimulate the tk promoter in 2-cell embryos (Figure 2). Furthermore, the failure of the GAL4-dependent enhancer to stimulate a promoter in 1-cell embryos was not due to the limiting transcriptional capacity of these cells, because the S_6T promoter was at least 10 times more active than with tk promoter in 1-cell embryos (Figure 6). Therefore, 1-cell embryos do not need the function provided by an enhancer, whereas 2-cell embryos do.

Second, each of seven different linker-scanning mutations in the HSV tk promoter had the same relative effect on promoter activity, regardless of whether this promoter was functioning in 1-cell embryos, 2-cell embryos or mouse fibroblasts (Figure 5). Thus, the composition of transcription factor binding sites required for efficient tk promoter activity in 1-cell embryos was the same as in 2-cell embryos or differentiated cells. Although the tk promoter's TATA box sequence was not required in these experiments, and was even slightly stimulatory in 3T3 cells, it was required when the same plasmids were introduced into HSV infected cells (data not shown), as previously reported (Jones *et al.*, 1985). This result reveals that the ability to dispense with an enhancer in 1-cell embryos does not result from a change in the composition of transcription factors required for promoter activity.

Third, comparison of expression from different promoters in 1-cell and 2-cell embryos revealed that these two embryos have equivalent transcriptional capacities (Figure 6). Therefore, the ability of 1-cell embryos to dispense with enhancers cannot be explained by a greater capacity to utilize promoters relative to 2-cell embryos.

Fourth, the activity of Sp1, a transcription factor required for tk promoter function in all cells, was as much as 6-fold greater in 2-cell embryos than in 1-cell embryos (Figures 7 and 8). Thus, one would expect the tk promoter to be more active, instead of less active, when injected into 2-cell embryos. In fact, a promoter consisting of tandem repeats of Sp1 DNA binding sites (pS_6Tluc) was 12-fold more active than the tk promoter in 1-cell embryos and 46-fold more active in 2-cell embryos, consistent with the increased Sp1 activity present in 2-cell embryos. Nevertheless, both of these promoters required an enhancer in 2-cell embryos in order to reach the levels of activity observed in 1-cell embryos (Figure 6). Therefore, the ability to dispense with an enhancer in 1-cell embryos does not result from a change in the activity of transcription factors required for promoter activity. The fact that Sp1 is a major transcription factor in both 1-cell and 2-cell mouse embryos suggests that Sp1 is involved in the activation of zygotic gene transcription.

The role of enhancers appears to involve chromatin structure

The ability of butyrate, like that of the F101 or GAL4-dependent enhancers, to stimulate tk promoter activity in 2-cell embryos but not in 1-cell embryos (Figure 4; Wiekowski *et al.*, 1993) suggests that the role of enhancers is to relieve repression by altering chromatin structure. In general, enhancers have little effect on promoter or origin activity unless the DNA is organized into chromatin. The PyV enhancer stimulates the PyV origin of replication >500-fold in cells but not in cytoplasmic extracts (Prives *et al.*, 1987) incapable of chromatin assembly (Gruss *et al.*, 1990). The immunoglobulin gene κ-chain enhancer stimulates its natural promoter 30-fold *in vivo* (Queen and Stafford, 1984), but fails to stimulate transcription in B-cell extracts (Sen and Baltimore, 1987). The SV40 enhancer stimulates the SV40 T-ag promoter >100-fold *in vivo*, but only 2- to 10-fold in HeLa cell extracts (Sassone-Corsi *et al.*, 1984; Sergeant *et al.*, 1984). The extent of stimulation varied with DNA concentration, competition between transcription and chromatin assembly, and proximity to a TATA box; no effect was observed when the enhancer was distant. Similar results have been reported for other systems (Müller *et al.*, 1989).

The small effects of enhancers observed *in vitro* are consistent with simply changing the strength of the promoter, rather than employing the primary function of enhancers *in vivo* which is to relieve repression from chromatin structure. For example, NF-κB, a protein that binds to the immunoglobulin enhancer, can stimulate transcription *in vitro* but only when acting close to the transcription start site (Kawakami *et al.*, 1988). GAL4:VP16 has been reported to stimulate transcription from a TATA box in cell extracts even when Ga14 DNA binding sites were placed far upstream of the TATA box

(Carey *et al.*, 1990). However, transcription was reduced 27-fold as the distance between Ga14 DNA binding sites and the TATA box was increased from 102 bp to 1318 bp. Moreover, the ability of these distal Ga14 DNA binding sites to stimulate transcription has only been observed by others when the DNA template has been packaged into histone H1-containing chromatin (Laybourn and Kadonaga, 1992). This observation is consistent with results reported here suggesting that Ga14 DNA binding sites 600 bp upstream of a natural promoter stimulate transcription 75-fold only under conditions where a repressive chromatin structure is formed (e.g. 2-cell mouse embryos), and did not stimulate promoter activity under conditions where repression is absent (e.g. 1-cell mouse embryos).

The fact that weak promoters such as pPyluc and ptkluc require enhancers in 2-cell embryos while strong promoters such as pG_5TCAT either do not (Figure 3) or are stimulated to a much lower extent (pS_6Tluc, Figure 6 and Thali *et al.*, 1990) is not inconsistent with our conclusion that the primary function of enhancers is to prevent repression of weak promoters. Tandem arrangements of GAL4:VP16 (Taylor *et al.*, 1991) and Sp1 (Laybourn and Kadonaga, 1991) DNA binding sites are effective at preventing repression of transcription by chromatin structure and therefore do not require the function of an enhancer. In addition, since both of these transcription factors can stimulate transcription from distal as well as proximal locations (Courey *et al.*, 1989), GAL4:VP16 and Sp1 can constitute enhancer as well as promoter elements. This suggests that the distinction of transcription factors based on their ability to prevent chromatin mediated repression could lead to a unified definition of enhancers and promoters. Consistent with this hypothesis, Seipel *et al.* (1992) have distinguished two classes of transcription activation domains, one that functioned only when proximal to the transcription start site and the other that functioned in both proximal and distal positions.

What does this phenomenon suggest about early mouse development? The paternal genome arrives packaged in protamines, which must be replaced with histones provided by the egg (Zirkin *et al.*, 1989; Nonchev and Tsanev, 1990). The process of chromatin remodeling in paternal pronuclei apparently makes promoters accessible to transcription factors. This would explain the presence of a zygotic clock that inhibits transcription during this period in order to ensure that genes are not prematurely expressed. When remodeling is complete, chromatin structure is modified in order to repress expression of promoters prior to initiating zygotic gene transcription in 2-cell embryos. Thus, the need for enhancers at this stage in development would be to provide a specific mechanism to relieve this repression and activate transcription of tissue-specific genes. A similar phenomenon exists in the frog. W.Schaffner and K.Seipel (personal communication) have observed that a GAL4-dependent enhancer functioned in 'late' *Xenopus* embryos but not in *Xenopus* oocytes or 1-cell embryos. Since zygotic gene transcription in *Xenopus* does not begin until the blastocyst stage, the need for enhancers appears to arise at this time.

Materials and methods

Injection and assay of promoters and enhancers in mouse embryos

Isolation and culture of CD-1 mouse embryos were carried out as previously described (DePamphilis *et al.*, 1988; Martínez-Salas *et al.*, 1989; Wiekowski *et al.*, 1991). Embryos were isolated and cultured in 4 μg/ml aphidicolin (Boehringer-Mannheim) to arrest their development at the beginning of S-phase. Since S-phase had not yet begun in 1-cell embryos, 1-cell embryos retained their two pronuclei throughout the experiment. Since 2-cell embryos were isolated after they had undergone DNA replication, they cleaved into 4-cells. In the absence of aphidicolin, injected 1-cell and 2-cell embryos developed up to the morula stage. Where indicated, 2.5 mM butyrate was included (Wiekowski *et al.*, 1993). Plasmid DNA was prepared in 10 mM Tris−HCl (pH 7.6) and 0.25 mM EDTA (DePamphilis *et al.*, 1988) to the concentration indicated, and ~2 pl was injected into 1-cell embryos between 22 and 28 h post-hCG (human chorionic gonadotrophin) and into 2-cell embryos at between 44 and 48 h post-hCG.

Embryos that survived injection were assayed for luciferase as previously described (Miranda *et al.*, 1992; Wiekowski *et al.*, 1991). Each data point represents the mean value of 40−150 oocytes or embryos, and the variation among individual embryos expressed as ± standard error of the mean. While the range of luciferase activities among individual embryos could vary as much as 1000-fold (Miranda *et al.*, 1993), the mean value obtained from several independent experiments was reproducible to within 13−25%. Moreover, the relative activity between different types of embryo and different promoters was always reproducible, even when DNA injection was performed by different people. CAT assays are described in Figure 3.

Plasmids

pluc, ptkluc, pF101tkluc, Pyluc, pF101Pyluc: DNA 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 described by Martínez-Salas *et al.* (1989). Plasmids containing the luc gene linked to the Py T-ag promoter (162 bp *ApaI*−*BstXI* DNA fragment, pPyluc) or the T-ag promoter plus the Py F1001 enhancer (191 bp *PvuII* DNA fragment, pF101Pyluc) were prepared in a similar way by Françoise Melin (Institut de Recherches Scientifiques sur le Cancer, Villejuif, France).

Tk linker scanning mutants. The 256 bp *BglII*−*PvuII* fragment was isolated from each of the seven linker-scanning mutations in the HSV tk promoter (Figure 5) present in pLStkCAT (McKnight and Kingsbury, 1982). The ends of these fragments were filled in using Klenow DNA polymerase, joined to *SacI* linkers, and then inserted in front of the luc gene at the *SacI* site of pluc. All of these subclones were sequenced through their tk promoter region to confirm sequence accuracy.

pTluc and pS_6Tluc. The *HpaI*−*SacI* promoter fragment was isolated from p2025 or p1964 (Smale and Baltimore, 1989; Smale *et al.*, 1990). p2025 contains the adenovirus major late promoter TATA box. p1964 contains the same adenovirus TATA box 30 bp downstream of the six tandem Sp1 DNA binding sites from the simian virus 40 early promoter. Each fragment was inserted into pluc as described above.

$pF101Tluc$ and $pF101S_6Tluc$. The *Hin*dIII fragment from F101tkluc containing the Py F101 enhancer was inserted at the single *Hin*dIII site in pTluc and pS_6Tluc. This places the enhancer 600 bp upstream from the promoter.

pS_{12} contains a tetramer of the *NcoI*−*Eco*RI fragment from $pSV(Sp1)_3$ core (Guo and DePamphilis, 1992). This fragment containing three Sp1 DNA binding sites was ligated together to generate 12 Sp1 DNA binding sites and inserted at the single *Eco*RI site of pML-1. These Sp1 binding sites can replace the original Sp1 binding sites in stimulating the SV40 origin of replication (Guo and DePamphilis, 1992). *pG_5TCAT* is the same as G_5BCAT (Guo and DePamphilis, 1992) and contains five copies of the 17 bp GAL4 DNA binding site 10 bp upstream of the adenovirus type 5 E1B TATA box which was placed 25 bp upstream of the *Hin*dIII−*Bam*HI segment from pSVCAT containing the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene (J.Lillie, personal communication). *pG_9tkluc* was constructed by inserting the *Bam*HI−*Eco*RI fragment from pMA558 into the unique *Hin*dIII and *BglII* sites into the polylinker region of ptkluc after all restriction sites had first been filled in to produce blunt ends (Guo and DePamphilis, 1992). The GAL4 binding sites in pG_9tkluc are located 600 bp upstream of the HSV tk promoter. The expression vector for GAL4:VP16 (*pSGVP*) has been described by Sadowski *et al.* (1988).

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