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Establishment and relief of CpG-dependent transgene repression during germ line passage and mouse development

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Methylation of genomic DNA at CpG sequences has a repressive effect on gene expression in vertebrates. The level of methylation of the genome varies widely during development in mammals. The DNA of early germ cells and blastocysts is largely hypomethylated but that of implantation embryos is hypermethylated. To test whether these variations might affect gene expression, we have studied the pattern of expression of a CpG-rich and of a CpG-poor LacZ transgene driven by a strong promoter of an ubiquitous gene. We find that the changes of the expression of the CpG-rich LacZ gene directly correlate with variations of methylation and that lowering the level of CpG in the reporter gene decreases dramatically its susceptibility to these variations. Therefore, in association with fluctuations in genome methylation a CpG-dependent system of repression must control positively and negatively gene expression during development and gametogenesis. We also find that the repression of the CpG-rich LacZ reporter can be abolished in 2-cell embryo by inhibitors of histone deacetylases suggesting that, at least at this stage, this CpG-dependent repression acts through histone deacetylation. In addition, we show that the β -globin locus control region and other genomic elements completely reverse the repression established at implantation in embryonic cells. The complex patterns of expression of the CpG-rich and poor LacZ reporter genes during gametogenesis and development-including a sex-dependent expression in the zygotic nucleus before the morula stage-are described. The possible implication of the constraints imposed on gene expression by DNA methylation revealed by this study are discussed.

Key words : CpG, histone deacetylation, LacZ, LagZ, methylation, mouse development, oogenesis, sodium butyrate, spermatogenesis, transgene, trichostatin

¹INTRODUCTION

Numerous studies have demonstrated a repressive effect of methylated CpG (mCpG) on gene expression in differentiated vertebrate cells (Hsieh, 1994; Kudo, 1998; Collas, 1998; Goto and Monk, 1998; Jones et al., 1998). This repressive effect of mCpG is equally efficient when either the promoter or the structural part of the gene (exons and introns) is methylated (Trasler et al., 1990; Komura et al., 1995; Nan et al., 1997; Singal et al., 1997). However, the density of mCpG must reach a threshold value to induce repression (Hsieh, 1994; Komura et al., 1995; Kass et al., 1997; Nan et al., 1997; Goto et al., 1998). Methylated CpGs do not need to be clustered as a dense island to prevent expression. When dispersed on several kilobases, mCpGs are still efficient (Boyes and Bird, 1991; Hsieh, 1994; Nan et al., 1997; Goto et al., 1998). The repressive effects of DNA methylation are probably mediated indirectly since these effects are relieved in trans by methylated oligonucleotides in vitro as in vivo (Boyes and Bird, 1991; Nan et al., 1997) and since the repression appears only after a delay of several hours in transient expression systems (Buschhausen et al., 1987; Kass et al., 1997). Methyl DNA binding proteins (MBD) are likely mediators for the biological effect of mCpG (Hendrich and Bird, 1998; Kudo, 1998). MBDs act as part of multi-protein complexes. For instance, MeCP2 associates with methylated DNA of the somatic genome (Nan et al., 1996) as a complex including Sin3A and histone deacetylases HDAC1 and HDAC2 (Nan et al., 1998; Jones et al., 1998). So, it is conceivable that the mCpG-dependent repression is due, at least in part, to the remodelling of chromatin structure through histone deacetylation (Jones

et al., 1998). In addition, MeCP2 can inhibit gene expression at a distance from the promoter (Nan et al., 1997). Nevertheless, the in vivo implication of this complex in gene expression remains hypothetical since only artificial systems have so far been analysed where MeCP2 is directed towards DNA through a GAL4 DNA binding domain and not by its proper DNA binding domain (Nan et al., 1998). That this system may indeed operate in vivo is suggested by observations of genes repressed by methylation which gain expression after treatment with inhibitors of deacetylases (Boyes and Bird, 1992; Hsieh, 1994; Singal et al., 1997; Jones et al., 1998; Nan et al., 1998). MBD1, another MBD protein is also included in a large complex of 800 kDa (MeCP1) (Boyes and Bird, 1992), the components of which are not yet determined. All these features suggest that mCpG and the associated MBD proteins constitute a general non-specific repressive system of gene transcription in differentiated cells. The pattern of methylation of the genome and of genes is maintained in dividing somatic cells, as best exemplified by the cases of hypomethylated or hypermethylated DNA introduced into cells (Hsieh, 1994; Howell et al., 1998; Kudo, 1998). In contrast, this pattern is dynamic during development and gametogenesis (Monk et al., 1987; Sanford et al., 1987; Trasler et al., 1990; Ariel et al., 1991; Ghazi et al., 1992; Kafri et al., 1992; Martin et al., 1999; Wamecke and Clark, 1999). The global methylation of genome is maximal in the embryo at gastrulation and minimal in cells of the blastocyst. Sperm and oocytes present an intermediate level of methylation and a demethylation is observed during the first cleavages of the embryo (Monk et al., 1987; Sanford et al., 1987; Rougier et al., 1998). The DNA of male and female germ cells at 12.5-14.5 days post coitum (dpc) is hypomethylated (Monk et al., 1987).

The methylation pattern of some endogenous genes also follows this general scheme. In particular, nearly systematically, hypomethylation is observed at the blastocyst stage, stronger methylation is observed at the following stages and in somatic tissues, and hypomethylation at the beginning of male and female gametogenesis (Kafri et al., 1992; Wamecke and Clark, 1999; Martin et al., 1999). However, in addition to this general pattern, numerous variations have been observed at specific genes which illustrate the existence of complex methylation and demethylation

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processes particularly during male gametogenesis and in somatic tissues (Groudine and Conkin, 1985; Trasler et al., 1990; Kafri et al., 1992; Wamecke and Clark, 1999). In some cases, the methylation level seems to be correlated with expression (Trasler et al., 1990; Zhang et al., 1998; Goto et al., 1998; Salvatore et al., 1998; Cameron et al., 1999), but in others, such a correlation is not found (Weng et al., 1995; Zhang et al., 1998; Wamecke and Clark, 1999). In at least one case, the promoter methylation is unchanged whether the gene is expressed or not (Wamecke and Clark, 1999). In another case, the global density rather than specific sites distinguishes expressed from silent alleles, which suggests that the functioning of a gene does not necessarily require demethylation at particular sites (Salvatore et al., 1998). The bisulfite sequencing used for studying DNA methylation of endogenous genes allows the detection of the methylation state of all CpGs of a gene from a single cell. Recent studies, still incomplete, has confirmed these data and reveal an amazingly heterogeneous pattern of gene methylation in different cells for any stage analysed (Salvatore et al., 1998; Wamecke and Clark, 1999). Thus, the concept of tissue-specific gene expression being controlled by a selective demethylation is not completely verified (Trasler et al., 1990; Walsh and Bestor, 1999).

The repressive indirect effect of methylation and the dynamic pattern of methylation during development raise a potential paradox which, if resolved, would have major consequences for our understanding of the evolution of ubiquitous and tissue-specific genes sequence in vertebrates. Indeed, if at a certain density of mCpG, MBD proteins act as an indirect general repressive system on gene expression and if the mCpG density of genes strongly varies at some stages, then expression of genes should be sensitive to these variations. Otherwise, to preserve their spatio-temporal expression tissue-specific or ubiquitous, the sequence of genes would have to differentially adapt to these conditions. It is therefore crucial to test *in vivo* if, indeed, gene expression is sensitive to the fluctuations of the methylation during development, but, so far, no system has allowed this hypothesis to be tested.

We describe here such a system and the first results of the testing of this hypothesis. The experimental system used compares in transgenic mice the expression of a LacZ reporter gene for which the density of CpG sequence is higher (8.6%, 302 sites for 3.5 kb) than that of endogenous genes and the same reporter gene in which the CpG density has been lowered by directed mutagenesis to a percentage close to that of endogenous genes (2.2%, 78 sites for 3.5 kb). To be able to explore different stages during development and gametogenesis, these two genes have been combined to the strong promoter of EF1 α gene (human translation elongation factor), an ubiquitous gene (Uetsuki et al., 1989). We have also studied the expression of the CpG-rich reporter gene controlled by a weak ubiquitous promoter, the HPRT promoter (human hypoxanthine phosphoribosyl transferase), when associated or not with the minilocus control region of β -globin (mini-LCR) (Talbot et al., 1989). Results show that at periods of genome hypomethylation, both CpG-poor and CpG-rich reporter genes associated to ubiquitous promoters are expressed whereas only the CpG-poor reporter is expressed at periods of genome hypermethylation in embryonic and somatic cells after implantation. Moreover, we show that CpG-rich transgenes are repressed at several stages during male and female gametogenesis and, depending on the parental origin, in the early embryo where a strong expression is observed for only CpG-poor transgenes. This is the first proof that gene expression *in vivo* is regulated by the fluctuations of a CpG-dependent negative control system. Finally, this repression of CpG-rich transgenes can be completely reversed by tissue specific trans-activating factors in specialized cells and relieved by treatment with inhibitors of histone deacetylases in 2-cell embryo. This suggest that several of the CpG-dependent repressive effects observed during development and gametogenesis are mediated by histone deacetylation of chromatin.

RESULTS

For this study, the bacterial LacZ reporter gene has been used, because it is CpG-rich according to criteria defined in Larsen et al. (1992) with a G+C content above 50% (% (G+C) = 54.4%) and a ratio of observed versus expected (O/E) CpG above 0.6 (O/E = 1.17), that are potential targets for methyl CpG binding proteins and their associated partners (Hendrich and Bird, 1998; Jones et al., 1998). For comparison, a modified CpG-poor LacZ gene has been constructed, from which 224 CpG sites were replaced by directed mutagenesis to achieve characteristics of non-CpG-rich sequence with a % (G+C) of 48.9% and a O/E of 0.37. This new reporter has been called LagZ and, as with LacZ, was used as a reporter in association with a nuclear localization signal in order to readily identify expression in all tissues and at all stages during embryogenesis (Bonnerot et al., 1987). These two sequences have been fused to a strong promoter from the gene encoding the human translation elongation factor EFl α , whose expression is ubiquitous (Fig. 1) (Hanaoka et al., 1991). To examine a transgene with a different combination of promoter, nlsLacZ was fused to a weaker, though also ubiquitous, promoter from the human hypoxanthine phosphoribosyl transferase gene (HPRT) (Fig. 1) (Bonnerot et al., 1990). Finally, the HPRTLacZ transgene was combined with the mini-locus control region of the (β -globin gene to determine the effect of this strong activating element on a potentially repressed structure in a specific somatic lineage (Bonnerot and Nicolas, 1992).

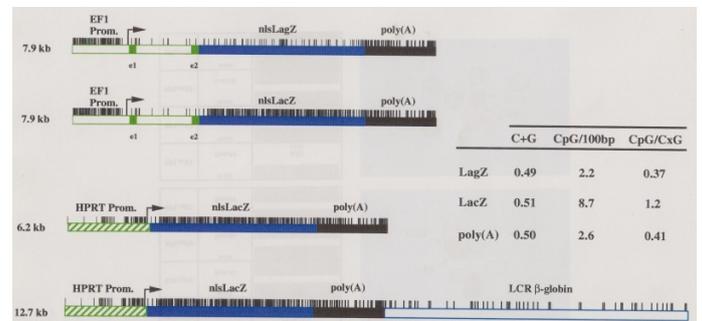


Figure 1. Structure of the transgenes. All constructs contain the nuclear localization signal of SV40 (nls), a reporter, LagZ or LacZ gene and the Moloney murine leukemia virus (MMLV) polyadenylation signal. Each vertical dash above or below the reporter gene indicates a CpG dinucleotide. The size of each DNA insert is indicated in kilobases (kb). EFl α Prom.: promoter of the human translation elongation factor a subunit gene; el : exon 1; HPRT Prom. : promoter of the human hypoxanthine phosphoribosyl transferase gene; LCR β -globin : mini-locus control region of the (β globin locus; Poly A : the MMLV polyadenylation signal. The table at the left side contains parameters used to identify a CpG-rich region according to Larsen et al (1992) for reporter genes and the poly(A) sequences. (G+C) is (number of C + number of G) / number of nucleotides in the sequence, CpG/100bp is (number of CpG per 100 nucleotides) and CpG/CxG is (number of CpG x number of nucleotide in the sequence / number of C x number of G = O/E).

Similar levels of transient expression of the CpG-rich (EFLacZ) and CpG-poor (EFLagZ) transgenes after microinjection into fertilized egg. To test whether the CpG content or other sequence differences between the LacZ and LagZ genes influenced their expression in the absence of methylation, EFLacZ and EFLagZ DNA constructs (depleted of plasmidic sequences) were microinjected into the male pronucleus of fertilized eggs at 20-22 hours post injection of human chorionic gonadotrophin (hphCG). Expression was then analysed by X-gal staining, once eggs had cleaved (46-48 hphCG). Both inserts were expressed in about half of injected eggs (eleven eggs were microinjected for each insert) and their level of expression was comparable (data not shown). Therefore, the EFl α gene promoter is capable of driving expression of both the reporter genes in the cleaved embryo, and all trans elements required for expression are present at this stage. Sequence differences, including their CpG content, do not change the qualitative expression level. However, an analysis of transient expression does not reveal how expression evolves during development, nor the influence of transgene passage through gametogenesis. To study

these questions, transgenic mice containing EFLagZ (EFLagZ 1 to 3) and EFLacZ (EFLacZ 1 to 4) as stable transgenes were generated. In all 7 lines, the transgene was integrated in an autosome.

Expression of EFLacZ and EFLagZ correlates with a variation in the global methylation of the genome

Global methylation of the genome is minimal in blastocysts and maximal in the cells of implanted embryos (Monk et al., 1987; Sanford et al., 1987; Kafri et al., 1992). To check whether LagZ and LacZ are sensitive to these global changes, we first analysed the expression pattern of the transgenic lines at these two stages. At the blastocyst stage, the three EFLagZ and the four EFLacZ lines all expressed the transgene (Fig. 2), demonstrating that whatever the CpG content, the reporter gene is in a permissive state for expression. In addition, the parental origin of the transgene did not affect the expression, except for the EFLacZ 1 line in which the maternally inherited transgene was not expressed.

Line	Parental origin of the transgene	Proportion of β-gal+ embryos (total number of analysed embryos)
Blastocyst (96hphCG)		
EFLagZ1	male	0.25 (8)
	female	0.75 (5)
EFLagZ2	male	0.52 (19)
	female	0.37 (32)
EFLagZ3	male	0.37 (8)
	female	0.55 (18)
EFLacZ1	male	0.46 (28)
	female	0.00 (14)
EFLacZ2	male	0.58 (41)
	female	0.26 (23)
EFLacZ3	male	0.45 (11)
	female	0.50 (14)
EFLacZ4	male	0.41 (17)
	female	0.38 (29)

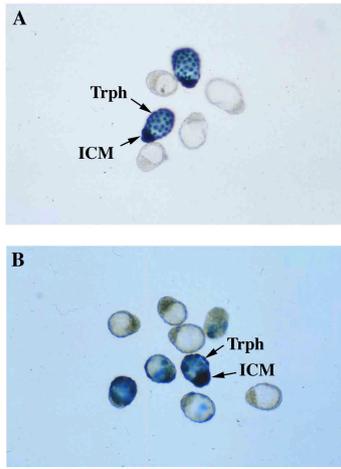
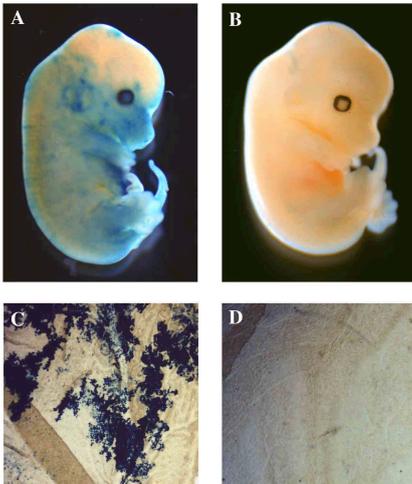


Figure 2. Expression of EFLacZ and EFLagZ correlates with the level of global methylation in the genome : the blastocyst stage. To analyse the expression of paternally or maternally transmitted transgene, embryos were obtained by crossing transgenic males or females with (B6D2) FI females or males respectively. Embryos were recovered at the time indicated (in hours post-injection of human chorionic gonadotrophin : hphCG) and immediately stained with X-gal. (A) Paternal transmission in blastocysts of the EFLagZ1 line. (B) paternal transmission in blastocysts of the EFLacZ2 line. Trph : trophectoderm; ICM : inner cell mass



Line	13.5 dpc Embryos (total number of analysed embryos)	
	Embryo	Yolk Sac
EFLagZ1	+ <21>	+ <21>
EFLagZ2	+ <33>	+ <33>
EFLagZ3	- <6>	+ <6>
EFLacZ1	ε (10)	- (10)
EFLacZ2	- (4)	- (4)
EFLacZ3	- (17)	- (17)
EFLacZ4	nd	nd

Figure 3. Expression of EFLagZ and EFLacZ transgenes in post-implantation embryos. Embryos at 13.5 dpc and adults mice were generated by crossing transgenic males to (B6D2) FI females. (A-B) Paternal transmission in 13.5 dpc embryos of EFLagZ1 and EFLacZ2 lines, respectively. (C-D) Yolk sac of 13.5 dpc embryos from EFLagZ1 and EFLacZ2 lines with paternal transmission. (A) β-gal+ cells were detected in numerous structures but expression was variegated. (C) In the yolk sac, β gal+ cells were distributed in clusters. (B and D) The EFLacZ transgene was not expressed. - : β-gal-; + : (β-gal+; e : only a few cells are (β-gal+; dpc : day post coitum and nd : not determined. Numbers between the arrows indicate the total number of transgenic embryos or animals analysed. Numbers between bracket indicate the number of analysed embryos or animals.

After implantation, the expression pattern of the EFLagZ lines was clearly different from that of the EFLacZ lines. At 13.5 dpc, none of the EFLacZ lines expressed the transgene, neither in extraembryonic tissues such as the yolk sac nor in somatic tissues (Fig. 3B,D). In contrast, we observed a constant level of transgene expression for all EFLagZ lines in the yolk sac and in embryos (Fig. 3A,C). However, in both cases, the expression was variegated, with only a fraction of the cells expressing LagZ. For instance in the yolk sac, the labelling was distributed in clusters of cells which strongly suggests a clonal transmission of the permissive state for expression, since growth in this tissue is known to be coherent (Fig. 3C (Gardner and Lawrence, 1985). In addition, transgene expression was still detected in EFLagZ adults in a variety of tissues (Fig. 4). In contrast, in EFLacZ mice, expression was never observed in adult tissues. Therefore, a differential expression corresponding to the CpG content of the transgene appears after implantation and is subsequently maintained throughout development and into adulthood. From these results we conclude that, even in combination with a strong ubiquitously active promoter, a high CpG content in the structural part of the gene leads to complete gene inactivity whereas a low CpG content leads to gene activity in at least a fraction of cells. Because the comparison between the expression of these two transgenes gave valuable information about the implication of CpG density in gene inactivity, we next analysed the expression of EFLacZ and EFLagZ during gametogenesis and early development, since information concerning the methylation status of these developmental stages is not as clear as in blastocysts and somatic cells.

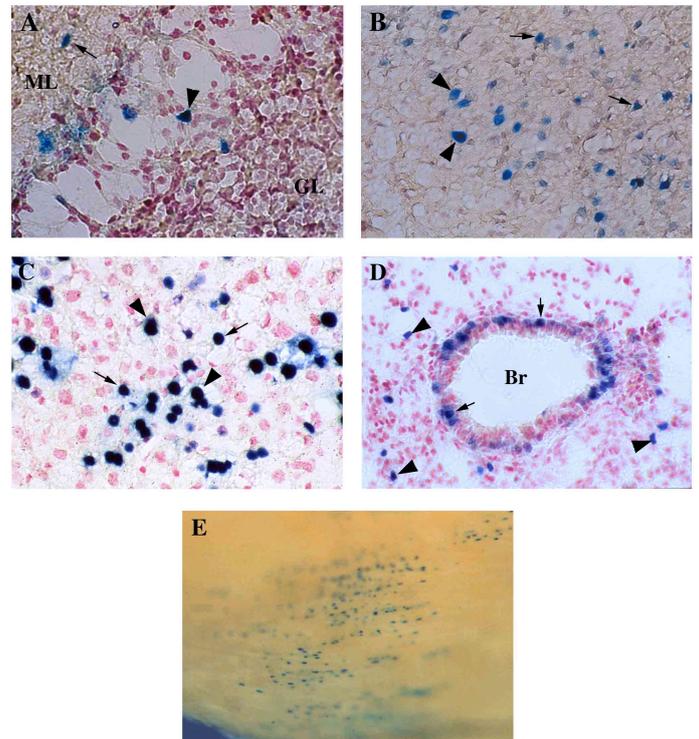


Figure 4. Examples of EFLagZ transgene expression in adult tissues. (A-D): X-gal staining of cryostat sections from adult organs of the EFLagZ 1 line. (E) X-gal staining of an adult heart in toto from the EFLagZ 1 line. (A) Cerebellum. The

EFLagZ transgene was expressed in different types of neuronal cells (arrow and arrow head). ML : molecular layer and GL : granular layer. (B) Cortex. Arrows and arrow heads show different types of neuronal cells. (C) Liver. Arrows and arrows head show tetraploid and diploid hepatocytes, respectively. (D) Lung. Arrow head and arrow show cells of respiratory tissue and cells of bronchiolar epithelium, respectively. Br : bronchiole. (E) EFLagZ transgene was expressed in myotubes.

Differential expression of EFLagZ and EFLacZ transgenes during oogenesis

Global genome methylation fluctuates during oogenesis. The maternal genome is demethylated in primordial germ cells, then further demethylated during Preleptotene-Leptotene-Zygotene (P-L-Z) of prophase I and is finally methylated at an unknown stage, such that a moderate level of methylation is attained at the terminal stages of oogenesis (Monk et al., 1987). In contrast, analysis of the methylation status of individual transgenes during oogenesis has shown that, in general, maternal transgenes remain hypermethylated (Chaillat, 1994).

Transgene expression in the EFLagZ and EFLacZ transgenic lines during oogenesis was examined in female gonads from 12.5 dpc embryos, in which oocytes were at the P-L-Z stages of prophase I, and also in the adult where oocytes were blocked at metaphase II (Fig. 5A). Ovulated oocytes were also analysed and these correspond to the transcriptionally inactive gametes (Schultz, 1986).

In the EFLagZ lines, the transgene is expressed in virtually all female germ cells, as early as the preleptotene stage of prophase I (13.5 dpc) (Fig. 6A). A continuous β -gal activity was also detected in all subsequent stages : at the pachytene stage (15.5-16.5 dpc and birth), during the growth phase at diplotene (starting at 8 dpp) and at metaphase II in the adult gonad (Fig. 6B-D). As expected, β -gal activity was not detected in the transcriptionally silent ovulated oocytes (Fig. 5B). The identical and continuous expression of the EFLagZ transgene observed for different lines during oogenesis confirms that the pattern of expression is independent of the integration site (transgene-dependent) and demonstrates that trans-activators for the EFLa gene promoter are constitutively active in oocytes until the transcriptional arrest that characterizes the terminal stage of oogenesis. The EFLagZ3 line also expressed the transgene during oogenesis but only transiently at 16.5 dpc (Fig. 5B) ; this effect is probably due to a position effect of the transgene.

In contrast, none of the CpG-rich EFLacZ lines continuously expressed the transgene during oogenesis (Fig. 6E-H). In the EFLacZ4 line, the transgene was never expressed and in EFLacZ1, EFLacZ2 and EFLacZ3, the transgene was only transiently expressed at the pachytene stage of prophase I (16.5 dpc for EFLacZ1 and 2 lines) and at birth (EFLacZ3) (Fig. 5B). In the adult ovary, only the EFLacZ2 line expressed the transgene and only in a few gametes at the beginning of growth phase (Fig. 6H). This absence of expression at preleptotene, leptotene, zygotene and then at the diplotene stage is, therefore, transgene dependent. The expression of the EFLagZ transgene at the corresponding stages indicates that the absence of expression is not due to a lack of trans-activators for the EFLa gene promoter. Taken together, these results reveal that the pachytene stage of oogenesis is particular, because both EFLagZ and EFLacZ transgenes are expressed. This period of expression during pachytene is flanked by two periods of repression which are CpG-dependent; notably EFLacZ transgenes are silenced beginning at the diplotene stage in growing oocytes.

Expression of EFLagZ and EFLacZ transgenes during spermatogenesis

Global methylation studies during spermatogenesis indicate that the paternal genome is demethylated in primordial germ cells and is then found methylated in sperm at an intermediate level (Monk et al., 1987). Nearly nothing is known concerning the evolution of global methylation between these two stages of male gametogenesis. The analysis of CpG methylation at specific positions for several genes has shown that both demethylation and methylation events can occur in meiotic cells (Trasler et al., 1990; Ariel et al., 1991; Kafri et al., 1992).

In the male embryo, gonocytes are dividing from 12.5 to 16.5 dpc, then arrest in G1 (Vergouwen et al., 1991). At birth, the first spermatogenic

wave begins with appearance of type A spermatogonies. At 8 dpp type B spermatogonies appear and two days later, primary spermatocytes (the preleptotene, leptotene and zygotene stages) arising from the division of type B spermatogonies (Kluin and de Rooij, 1981). Finally, primary spermatocytes at the pachytene stage appear, along with post-meiotic round spermatids at 14 and 20 dpp respectively. The terminal differentiation stage, involving generation of elongated spermatids and spermatozoa, occurs during the following 15 days (Fig. 5A).

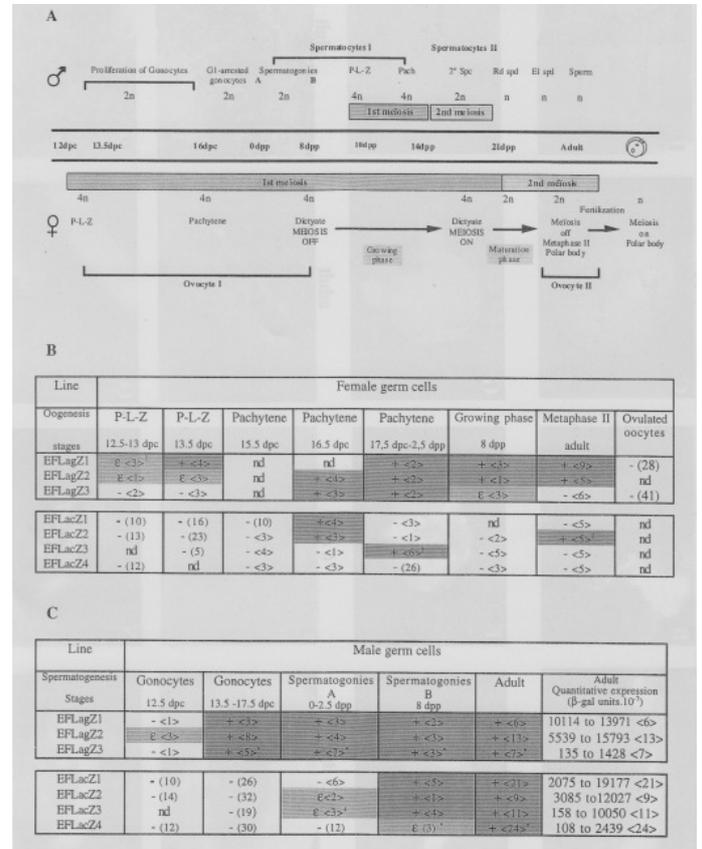


Figure 5. EFLagZ and EFLacZ transgene expression during gametogenesis. (A) Chronology of meiotic events during oogenesis and spermatogenesis. The figure summarizes the timing of important events in oogenesis and spermatogenesis beginning from the stage of colonization of the genital ridges by primordial germ cells to the stage of mature gametes and the first cell cleavage after fertilization, dp refers to the number of days after mating (for embryos), dpp indicates the number of days after birth. P, L, Z : preleptotene, leptotene and zygotene stages of prophase I respectively; Pach : pachytene stage of prophase I; 2° Spc : secondary spermatocyte; Rd spd : round spermatid; El spd : elongated spermatid and n : haploid genome. (B) Expression of maternally transmitted EFLagZ and EFLacZ transgenes during gametogenesis and in the adult gonad. Embryos or animals were obtained by crossing heterozygous transgenic females or males to (B6D2) F1 males or females according to the parental origin of the transgene. Numbers between arrow indicate the total number of transgenic embryos or animals analysed. Number between brackets indicate the number of analysed embryos or animals. - : (β -gal-; + (β -gal+; e : only a few germ cells were β -gal+ ; * : (β -gal+ germ cells were clustered 1 : one transgenic female was (β -gal+ in gonads; 2 : two transgenic females were (β -gal+ in gonads; 3 : four transgenic females were β -gal+ in gonads; 4 : two transgenic males were β -gal+ in gonads; nd : not determined. The last column in (C) refers to : quantitative analysis of paternal transgene expression in adult testis. The β -gal activity was quantified using a fluorogenic substrate of β -n galactosidase (MUG). β gal activity of control testis was 41.5x10⁷ β -gal units (mean value of 12 control teste were analysed).

Analysis of the first wave of spermatogenesis in the EFLagZ lines indicates that their transgene is expressed continuously in male germ cells (Fig. 6I-L). (β -gal activity was detected very early, in gonocytes (12.5-13.5 dpc)

(Fig. 6I, K), as well as in type A spermatogonies at birth (Fig. 6J), and type B spermatogonies at 8 dpp. This pattern was maintained at all stages in adult testis, for which all stages of spermatogenesis were examined including elongated spermatids (Fig. 6K-L).

Similarly, all EFLacZ lines showed β -gal activity in male germ cells (Fig. 6M-P); however the β -gal activity was first detected at birth when type A spermatogonies appeared, as there was no detectable activity in gonocytes. Moreover, the number of β -gal⁺ type A spermatogonies in EFLacZ lines was lower than in EFLagZ lines. From birth to 8 dpp, the number of EFLagZ β -gal⁺ germ cells, which represent type A and B spermatogonies, increased. In adult testis, β -gal activity was observed in type A spermatogonies up to the round spermatid stage (Fig. 6O-P). The identical and continuous expression of the EFLagZ transgene observed for all EFLagZ lines during spermatogenesis (Fig. 5B) confirms that the pattern of expression is transgene-dependent and demonstrate that the trans-activators for the EFla gene promoter are constitutively active in male gametes during all spermatogenesis.

In summary, prior to spermatogenesis, the expression of the EFLagZ gene begins shortly after the transition period between primordial germ cells and gonocytes, at 13.5 dpc, and the expression of EFLacZ is delayed until the first appearance of type A spermatogonies. After this differential timing in activation, expression of both transgenes is detected until the transcriptional arrest at the round spermatid stage. These data suggest that a non-permissive state for expression exists in male gonocytes in relation to the high CpG content of transgenes and that a favourable condition later appears in type A spermatogonies cells, which relieves this repressive state.

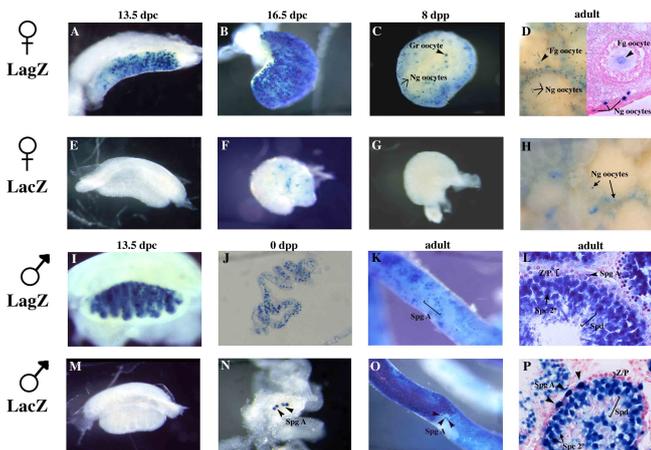


Figure 6. EFLagZ and EFLacZ transgene expression in the gonads during development. (β -gal pattern in the EFLagZ 1 (A-D, I-L) and EFLacZ 2 (E-H, M-P) lines after in toto X-gal staining of female gonads (A-H) and male gonads (I-P) at different stages of development : 13.5 dpc (A, E, I, M); 16.5 dpc (B, F); birth (0 dpp) (J, N); 8 dpp (C, G) and adult (D, H, K, L, O, P). (C) shows β -gal⁺ oocytes before the growth phase (immature oocytes : (i) oocytes) and during the growth phase (growing oocyte : Gr oocyte). In the right part of D, a 20 μ m cryostat section of the in toto ovary showing β -gal⁺ oocytes at the beginning of growth (immature : (i) oocyte, arrows), surrounded by follicular cells, up to the end of the growth phase (fully grown : Fg oocyte, arrow head) inside the follicular cavity. (I) shows β -gal⁺ gonocytes. (J,N) β -gal⁺ type A spermatogonies (arrow head). (K, O) type A spermatogonies at the surface of an adult seminiferous tubules (arrow head). (L, P) 20 μ m cryostat sections of adult seminiferous tubule showing the XII stage of spermatogenesis : EFLagZ and EFLacZ transgenes were expressed in type A spermatogonies (arrow head, Spg A) and secondary spermatocytes (arrow, Spc 2°). Elongated spermatids (Spd) were expressing only in the EFLagZ lines.

The Sex-dependent transgene expression during gametogenesis persists in the zygotic nucleus before the morula stage

There is a differential expression of the EFLacZ transgene in male and female germ cells, persisting until the transcriptional arrest in both types of mature gametes. During gametogenesis, a sex-dependent expression of the

EFLacZ transgene is shown by repression of maternal transgene expression at the diplotene stage in relation to its high CpG content. To determine if this sex-dependent expression of the EFLacZ transgene is maintained in the embryo after fertilization, transgene expression in EFLagZ and EFLacZ mice was analysed (Table 1). To study the expression of transgene of paternal and maternal origin, embryos were obtained from the progeny of both male and female transgenics crossed with (B6D2) F1 animals.

Lines	Parental origin of the transgene	Proportion of β -gal ⁺ embryos			
		(total of analysed embryos)			
		2-cell (48hphCG)	4-cell (56hphCG)	Morula (72hphCG)	Blastocyst (96hphCG)
EFLagZ1	male	0,17 (17)	0,38 (18)	0,40 (15)	0,25 (8)
	female	0,90 (29)	0,38 (18)	0,44 (43)	0,75 (8)
EFLagZ2	male	0,00 (17)	0,36 (11)	0,29 (17)	0,52 (19)
	female	0,00 (35)	0,55 (9)	0,40 (50)	0,37 (32)
EFLagZ3	male	0,00 (16)	0,67 (28)	0,90 (11)	0,37 (8)
	female	0,00 (14)	0,14 (14)	0,43 (23)	0,55 (18)
EFLacZ1	male	0,00 (14)	0,44 (19)	0,33 (66)	0,46 (28)
	female	nd	0,00 (23)	0,00 (33)	0,00 (14)
EFLacZ2	male	0,00 (13)	0,43 (7)	0,37 (54)	0,58 (41)
	female	0,00 (13)	0,00 (8)	0,14 (35)	0,26 (23)
EFLacZ3	male	0,00 (34)	0,07 (12)	0,50 (42)	0,45 (11)
	female	0,00 (34)	0,00 (40)	0,01 (66)	0,50 (14)
EFLacZ4	male	0,00 (65)	0,00 (16)	0,66 (18)	0,41 (17)
	female	0,00 (65)	0,00 (48)	0,19 (36)	0,38 (29)

Table 1. Sex-dependent expression of EFLacZ but not EFLagZ during early development. Embryos were obtained by crossing heterozygous transgenic males or females with (B6D2) F1 animals to analyse paternal and maternal transgene expression. Embryos were recovered at the time indicated (in hours post-injection of human chorionic gonadotrophin : hphCG) and immediately stained with X-gal.

At the blastocyst stage, the three EFLagZ and the four EFLacZ lines all expressed the transgene (Fig. 2), demonstrating that whatever the CpG content, the reporter gene is in a permissive state for expression. In addition, the parental origin of the transgene did not affect the expression, except for the EFLacZ 1 line in which the maternally inherited transgene was not expressed.

After implantation, the expression pattern of the EFLagZ lines was clearly different from that of the EFLacZ lines. At 13.5 dpc, none of the EFLacZ lines expressed the transgene, neither in extraembryonic tissues such as the yolk sac nor in somatic tissues (Fig. 3B,D). In contrast, we observed a constant level of transgene expression for all EFLagZ lines in the yolk sac and in embryos (Fig. 3A,C). However, in both cases, the expression was variegated, with only a fraction of the cells expressing LagZ. For instance, in the yolk sac, the labelling was distributed in clusters of cells which strongly suggests a clonal transmission of the permissive state for expression, since growth in this tissue is known to be coherent (Fig. 3C) (Gardner and Lawrence, 1985). In addition, transgene expression was still detected in EFLagZ adults in a variety of tissues (Fig. 4). In contrast, in EFLacZ mice, expression was never observed in adult tissues. Therefore, a differential expression corresponding to the CpG content of the transgene appears after implantation and is subsequently maintained throughout development and into adulthood. From these results we conclude that, even in combination with a strong ubiquitously active promoter, a high CpG content in the structural part of the gene leads to complete gene inactivity whereas a low CpG content leads to gene activity in at least a fraction of cells. Because the comparison between the expression of these two

transgenes gave valuable information about the implication of CpG density in gene inactivity, we next analysed the expression of EFLacZ and EFLagZ during gametogenesis and early development, since information concerning the methylation status of these developmental stages is not as clear as in blastocysts and somatic cells.

Differential expression of EFLagZ and EFLacZ transgenes during oogenesis

Global genome methylation fluctuates during oogenesis. The maternal genome is demethylated in primordial germ cells, then further demethylated during Preleptotene-Leptotene-Zygotene (P-L-Z) of prophase I and is finally methylated at an unknown stage, such that a moderate level of methylation is attained at the terminal stages of oogenesis (Monk et al., 1987). In contrast, analysis of the methylation status of individual transgenes during oogenesis has shown that, in general, maternal transgenes remain hypermethylated (Chaillat, 1994).

Transgene expression in the EFLagZ and EFLacZ transgenic lines during oogenesis was examined in female gonads from 12.5 dpc embryos, in which oocytes were at the P-L-Z stages of prophase I, and also in the adult where oocytes were blocked at metaphase II (Fig. 5A). Ovulated oocytes were also analysed and these correspond to the transcriptionally inactive gametes (Schultz, 1986).

In the EFLagZ lines, the transgene is expressed in virtually all female germ cells, as early as the preleptotene stage of prophase I (13.5 dpc) (Fig. 6A). A continuous β -gal activity was also detected in all subsequent stages: at the pachytene stage (15.5-16.5 dpc and birth), during the growth phase at diplotene (starting at 8 dpp) and at metaphase II in the adult gonad (Fig. 6B-D). As expected, β -gal activity was not detected in the transcriptionally silent ovulated oocytes (Fig. 5B). The identical and continuous expression of the EFLagZ transgene observed for different lines during oogenesis confirms that the pattern of expression is independent of the integration site (transgene-dependent) and demonstrates that trans-activators for the EFla gene promoter are constitutively active in oocytes until the transcriptional arrest that characterizes the terminal stage of oogenesis. The EFLagZ3 line also expressed the transgene during oogenesis but only transiently at 16.5 dpc (Fig. 5B); this effect is probably due to a position effect of the transgene.

In contrast, none of the CpG-rich EFLacZ lines continuously expressed the transgene during oogenesis (Fig. 6E-H). In the EFLacZ4 line, the transgene was never expressed and in EFLacZ1, EFLacZ2 and EFLacZ3, the transgene was only transiently expressed at the pachytene stage of prophase I (16.5 dpc for EFLacZ1 and 2 lines) and at birth (EFLacZ3) (Fig. 5B). In the adult ovary, only the EFLacZ2 line expressed the transgene and only in a few gametes at the beginning of growth phase (Fig. 6H). This absence of expression at preleptotene, leptotene, zygotene and then at the diplotene stage is, therefore, transgene dependent. The expression of the EFLagZ transgene at the corresponding stages indicates that the absence of expression is not due to a lack of trans-activators for the EFla gene promoter. Taken together, these results reveal that the pachytene stage of oogenesis is particular, because both EFLagZ and EFLacZ transgenes are expressed. This period of expression during pachytene is flanked by two periods of repression which are CpG-dependent; notably EFLacZ transgenes are silenced beginning at the diplotene stage in growing oocytes.

Expression of EFLagZ and EFLacZ transgenes during spermatogenesis

Global methylation studies during spermatogenesis indicate that the paternal genome is demethylated in primordial germ cells and is then found methylated in sperm at an intermediate level (Monk et al., 1987). Nearly nothing is known concerning the evolution of global methylation between these two stages of male gametogenesis. The analysis of CpG methylation at specific positions for several genes has shown that both demethylation and methylation events can occur in meiotic cells (Trasler et al., 1990; Ariel et al., 1991; Kafri et al., 1992).

In the male embryo, gonocytes are dividing from 12.5 to 16.5 dpc, then arrest in G1 (Vergouwen et al., 1991). At birth, the first spermatogenic wave begins with appearance of type A spermatogonies. At 8 dpp type B

spermatogonies appear and two days later, primary spermatocytes (the preleptotene, leptotene and zygotene stages) arising from the division of type B spermatogonies (Kluin and de Rooij, 1981). Finally, primary spermatocytes at the pachytene stage appear, along with post-meiotic round spermatids at 14 and 20 dpp respectively. The terminal differentiation stages, involving generation of elongated spermatids and spermatozoa, occur during the following 15 days (Fig. 5A).

Analysis of the first wave of spermatogenesis in the EFLagZ lines indicates that their transgene is expressed continuously in male germ cells (Fig. 6I-L). (β -gal activity was detected very early, in gonocytes (12.5-13.5 dpc) (Fig. 6I, K), as well as in type A spermatogonies at birth (Fig. 6J), and type B spermatogonies at 8 dpp. This pattern was maintained at all stages in adult testis, for which all stages of spermatogenesis were examined including elongated spermatids (Fig. 6K-L).

Similarly, all EFLacZ lines showed (β -gal activity in male germ cells (Fig. 6M-P); however the (β -gal activity was first detected at birth when type A spermatogonies appeared, as there was no detectable activity in gonocytes. Moreover, the number of (β -gal+ type A spermatogonies in EFLacZ lines was lower than in EFLagZ lines. From birth to 8 dpp, the number of EFLagZ β -gal+ germ cells, which represent type A and B spermatogonies, increased. In adult testis, β -gal activity was observed in type A spermatogonies up to the round spermatid stage (Fig. 6O-P). The identical and continuous expression of the EFLagZ transgene observed for all EFLagZ lines during spermatogenesis (Fig. 5B) confirms that the pattern of expression is transgene-dependent and demonstrate that the trans-activators for the EFla gene promoter are constitutively active in male gametes during all spermatogenesis.

In summary, prior to spermatogenesis, the expression of the EFLagZ gene begins shortly after the transition period between primordial germ cells and gonocytes, at 13.5 dpc, and the expression of EFLacZ is delayed until the first appearance of type A spermatogonies. After this differential timing in activation, expression of both transgenes is detected until the transcriptional arrest at the round spermatid stage. These data suggest that a non-permissive state for expression exists in male gonocytes in relation to the high CpG content of transgenes and that a favourable condition later appears in type A spermatogonic cells, which relieves this repressive state.

The Sex-dependent transgene expression during gametogenesis persists in the zygotic nucleus before the morula stage

There is a differential expression of the EFLacZ transgene in male and female germ cells, persisting until the transcriptional arrest in both types of mature gametes. During gametogenesis, a sex-dependent expression of the EFLacZ transgene is shown by repression of maternal transgene expression at the diplotene stage in relation to its high CpG content. To determine if this sex-dependent expression of the EFLacZ transgene is maintained in the embryo after fertilization, transgene expression in EFLagZ and EFLacZ mice was analysed (Table 1). To study the expression of transgene of paternal and maternal origin, embryos were obtained from the progeny of both male and female transgenics crossed with (B6D2) FI animals.

In all EFLagZ lines, the transgene was expressed independently from its parental origin as early as the 2 or 4-cell stage until the blastocyst stage. In EFLacZ lines, the transgene was expressed from the 4-cell stage to the blastocyst stage but only when it was transmitted by a male. In contrast, when the EFLacZ transgene derived from a female its expression was always detected later and not before the morula stage. Therefore, a parental origin-dependent expression, also related to its high CpG content, characterizes the EFLacZ transgene during the first embryonic cleavages of embryo. These results strongly suggest that the permissive or non-permissive transcriptional state of transgenes in differentiating gametes is maintained during the first cleavages of the embryo.

Persistence of the gametic transcriptional permissivity in the preimplantation embryo

Our results suggest that the regulation exerted on EFLacZ and EFLagZ transgenes in the early embryo is previously determined during

gametogenesis. The following observations argue for this gameto-zygotic continuity. In some EFLacZ lines, in particular EFLacZ 1, a variegated expression was observed in the germ cells contained in the adult seminal tube from one male to another in the same line (Fig. 5C, adult quantitative expression). Therefore, during gametogenesis, the transition between gonocytes and spermatogonies is not followed by relief of a non-permissive transcriptional state in all germ cells. If we postulate that a gameto-zygotic continuity indeed exists, then a correlation should be observed between the level of transgene expression in the adult testis of a male and the proportion of β -gal⁺ preimplantation embryos that have inherited their transgene from this same male. This comparison has been made using two EFLacZ1 males selected on the basis of the β -gal activity measured in one of their surgically removed testis. EFLacZ1 males expressing a high or low β -gal activity were crossed with non transgenic females to generate 4-cell embryos (Fig. 7A, top table). The transgenic male with a very low β -gal activity in germ cells generated β -gal⁻ embryos only while the one with a high β -gal activity in germ cells generated β -gal⁺ embryos. These results establish a correlation between the transcriptional state of the EFLacZ transgene in male gametes and that of the preimplantation embryos, supporting the concept of a gameto-zygotic continuity of this transcriptional state.

The morula-blastocyst period : A general relief from all gametic repressive states

We have already reported that the EFLacZ transgenes are expressed at the blastocyst stage (Fig. 2). Because maternally transmitted transgenes are repressed during the first cleavages, we have investigated in more details at which stage this sex-dependent repression is released. At the morula stage, a certain fraction of the embryos carrying the maternal transgene were already β -gal⁻ and this fraction increased further at the blastocyst stage. Release from the repression of the maternal transgene begins at the morula stage and seems to be progressive (Table 1).

Several EFLacZ lines and one EFLagZ line (EFLagZ3) were characterized by a variegated expression of their transgene during spermatogenesis. (β -gal⁺ germ cells were arranged in clusters along the seminal cord and the overall β -gal activity (MUG) was low (Fig. 5B). Therefore, only a fraction of gonocytes (EFLagZ3) and type A and B spermatogonies (EFLacZ lines) were relieved of the non-permissive state for transgene expression. The most obvious example of this was seen for EFLacZ4. Strikingly, in this line, the paternally transmitted transgene was only active at the morula stage (Table 1). Since the morula stage is also the period at which repression of maternal transgenes is relieved (see Table 1), the morula-blastocyst stage appears to correspond to a developmental period when all gametic repressions, applied to both male and female EFLacZ transgenes, are released.

A similar regulated expression for the HPRT promoter

To test whether regulatory mechanisms described for EFLacZ were specific to this promoter, other promoters were fused with the LacZ gene and used to generate transgenic mice (Fig. 1). The weak promoter of the ubiquitously expressed human hypoxanthine phosphoribosyl transferase (HPRT) gene was used for these studies and a construct combining the HPRT promoter with hematopoietic specific enhancers derived from the (β -globin LCR (HPRT-DCR) was also analysed. Two transgenic lines containing the HPRT insert (HPRTLacZ1 and HPRTLacZ3 lines) were analysed, along with seven lines containing the HPRT-DCR insert (DCR1 to 7 lines). The transgene expression patterns during gametogenesis and the first cleavages of embryo were examined in the same way as for the EFLagZ and EFLacZ lines described above. First, as previously reported, none of the HPRTLacZ and HPRTLacZDCR lines ubiquitously expressed the transgene in postimplantation embryo, which confirms a general repression of the transgene in somatic cells (see in Bonnerot et al. (1990, 1993a)). Second, the male germ cells in all HPRTLacZ lines and six of the seven DCR lines expressed the transgene, at least in the pachytene spermatocytes (Table 2A and Fig. 8A-B). None of the DCR lines expressed their transgene in gonocytes. Rather expression began at different times

according to the line : at birth for DCR1 and DCR6, at 8 dpp for DCR4 and 7 and at 10 dpp for DCR2 and DCR3. In adult testis, expression was also readily detected at the pachytene stage and at all stages up to the development of round spermatids. However, we observed variations in the staining intensity from line to line. In particular, the staining in HPRTLacZ mice was lower than in DCR mice (data not shown). Quantitative analysis of the (β -galactosidase activity in adult testis confirmed this result (Table 2A).

Line		Male germ cells							Adult	Adult Quantitative expression (β -gal units 10^7)
Spermatogenesis stage	Gonocytes (13.5 dpc)	Gonocytes (15.5 dpc)	Gonocytes (16.5 dpc)	Gonies A (0-2.5 dpp)	Gonies B (8 dpp)	P-L-Z (10 dpp)	Adult	Adult	Adult	
DCR1	- <5>	- <3>	- <3>	e <7>	e <7>	nd	+ <44>	123 to 748	<44>	
DCR2	- <7>	- <2>	nd	e <2>	+ <2>	+ <2>	+ <59>	103 to 5698	<59>	
DCR3	- <9>	- <2>	- <7>	- <8>	- <7>	e <4>	+ <30>	90 to 858	<30>	
DCR4	nd	- <4>	- <5>	- <2>	e <2>	e <5>	+ <55>	1416 to 2160	<55>	
DCR5	- <9>	- <2>	- <7>	- <8>	- <7>	e <4>	- <4>	96 to 261	<4>	
DCR6	- <4>	- <5>	nd	e <7>	+ <3>	+ <2>	+ <47>	283 to 1767	<47>	
DCR7	- <4>	- <4>	nd	- <2>	+ <3>	+ <1>	+ <40>	90 to 6711	<40>	

Line		Female germ cells						Adult
Oogenesis stages	Gonocytes (13.5 dpc)	Pachytene (15.5 dpc)	Pachytene (16.5 dpc)	Pachytene (0-2.5 dpp)	Diplotene (8 dpp)	Growing phase (10 dpp)	Adult	
DCR1	nd	- <1>	- <4>	- <5>	- <13>	nd	-	
DCR2	+ <6>	+ <7>	nd	+ <2>	- <3>	- <4>	-	
DCR3	- <5>	+ <4>	e <2>	e <14>	- <4>	- <1>	-	
DCR4	+ <6>	+ <1>	+ <2>	+ <7>	- <3>	- <1>	-	
DCR5	- <5>	- <1>	- <4>	- <5>	- <13>	- <4>	-	
DCR6	e <5>	+ <3>	nd	e <6>	- <4>	- <3>	-	
DCR7	- <1>	- <1>	nd	+ <1>	nd	- <5>	-	

Table 2. HPRTLacZDCR transgene expression in gonads during development.

Embryos or animals were obtained by crossing transgenic males or females with (B6D2) F1 animals to analyse paternal (bottom table) and maternal (top table) transgene expression. Gonads were recovered at different stages of development and stained with X-gal. Expression in germ cells is depicted as follows : - : β -gal⁻ cells; + : β -gal⁺ cells and e : few β -gal⁺ cells. Numbers between arrows represent the total number of male or female embryo examined. 1 : five transgenic females were β -gal⁺ in gonads; 2 : one transgenic male was β -gal⁺ in gonads; 3 : one male was β -gal⁺ in gonads; 4 : five transgenic males were β -gal⁺ in gonads and nd : not determined. The last column of the bottom table refers to quantitative expression of the paternal transgene in adult testis. The β -gal activity was quantified with the fluorogenic substrate of β -galactosidase, MUG (see materials and methods). β -gal activity of control testis was 41.5×10^7 β -gal units measured with a mean value of 12 control testes. For details on germ cell stages, see figure 3.

Third, in female germ cells, a transient transgene expression was detected between 12.5 dpc and 2.5 dpp during the pachytene stage in five DCR lines (Table 2B and Fig. 8C-D). For all DCR lines, this period of expression was followed by a period of repression starting at the diplotene stage and continuing up to the full grown stage of the oocyte in the adult ovaries. Therefore, together with the observations made during spermatogenesis, these data indicate that the sex-dependent expression observed during gametogenesis for the EFLacZ transgene also occurs when the CpG-rich LacZ gene is controlled by the HPRT promoter.

To determine whether the sex-dependent gametic expression of the HPRTLacZ and DCR transgenes is correlated with a parental effect in the cleavage stage embryo (as for EFLacZ lines), expression of paternal and maternal transgenes was tested. Probably because of the weakness of the HPRT promoter, LacZ expression was only detected by X-gal staining in aphidicolin arrested eggs, in which the signal is amplified (see Material and Methods for a more detailed description of this technique).

Fertilized eggs recovered at 24 hphCG were stained 24 hours later, at a time when control embryos reached the late 2-cell stage (Table 3). All lines that expressed the transgene during spermatogenesis also expressed the transgene in arrested 1-cell embryos. Strikingly, none of these lines expressed the maternally transmitted transgene and this parental effect was still observed when embryos were cultured at the 2-cell and 4-cell stage

from the DCR6 and DCR7 lines. Therefore, the sex-dependent transgene expression persists through several cleavages after fertilization.

Line	Parental origin of the transgene	Proportion of β -gal+ embryo (total number of analysed embryos)		
		arrested 1-cell	arrested 2-cells	arrested 4-cells
		I	II	III
Protocols				
DCR1	male	0,03 (70)	0,00 (37)	0,00 (18)
	female	0,00 (44)	0,00 (43)	0,00 (7)
DCR2	male	0,32 (66)	0,38 (81)	nd
	female	0,00 (16)	0,00 (25)	nd
DCR3	male	0,18 (62)	0,14 (62)	nd
	female	0,00 (45)	0,00 (14)	nd
DCR4	male	0,16 (75)	0,43 (46)	nd
	female	0,00 (17)	nd	nd
DCR5	male	0,00 (101)	0,00 (14)	nd
	female	0,00 (16)	0,00 (9)	0,00 (8)
DCR6	male	0,78 (60)	0,36 (11)	0,25 (4)
	female	0,02 (138)	0,00 (33)	0,00 (21)
DCR7	male	0,32 (63)	0,40 (25)	0,37 (54)
	female	0,00 (79)	0,04 (21)	0,00 (39)
HPRTLacZ1	male	0,17 (148)	0,00 (19)	nd
	female	0,00 (43)	0,00 (27)	nd
HPRTLacZ3	male	0,00 (94)	0,00 (14)	0,00 (6)
	female	0,00 (11)	0,00 (40)	0,00 (16)

Table 3. Expression of paternally and maternally transmitted DCR and HPRTLacZ transgenes during early development. Transgenic males or females were mated with (B6D2) F1 animals to analyse paternal and maternal transgene expression. All the transgenic mice used were homozygous except for DCR4 mice. Embryos were collected and cultured as described in materials and methods.

The idea of a gameto-zygotic continuity for the transcriptional state was tested in FIPRTLacZ

and DCR lines by comparing expression in preimplantation embryos of transgenes inherited from two males of the same line (DCR4, DCR7 and HPRTLacZ1) (Fig. 7A), selected for differences in their β -gal activity in adult germ cells. In all cases, the transgene transmitted by males with a high (β -gal activity in testis was also expressed in cleavage stages embryos (Fig. 7B (a-b)); whereas embryos derived from males showing a low β -gal activity in testis did not express the transgene (Fig. 7B (c-d)).

Finally, we studied expression of LacZ transgenes containing tissue-specific promoters, Hoxb-7 (Kress et al., 1990) or AchRa (Klarsfeld et al., 1991). Expression of the paternal transgene was not detected in germ cells of the adult testis nor in 2-cell embryo blocked by aphidicolin (data not shown). In contrast, the tissue-specific expression of these two promoters was, as expected, observed in post-implantation embryos (Kress et al., 1990; Klarsfeld et al., 1991).

Taken together, these results demonstrate that the regulation described for the EFLacZ transgene in somatic cells, during gametogenesis and at first cleavages after fertilization (parental differential expression, gameto-zygotic continuity), are not specific to the EFla promoter, but also applies to the association of a CpG-rich LacZ with the weaker HPRT promoter. However, the LacZ gene needs to be combined with promoter sequences of a ubiquitous gene in order to be expressed in germ cells and the embryo. The minimal promoter sequences (TATA and CAAT box) contained in the tissue-specific Hoxb-7 and AchRa promoters seem unable to drive a detectable level of expression in these cells.

Line	Adult testis Quantitative expression (Unité β -gal .10 ⁻⁷)	Preimplantation embryos Proportion of β -gal+ embryos (total number of analysed embryos)	
		late 4-cells	
EFLacZ1	19177	0,28 (7)	
	2075	0,00 (18)	
		arrested 1-cell	arrested 2-cells
Protocols		I	II
DCR4	937	0,60 (5)	0,17 (23)
	187	0,00 (37)	0,00 (7)
DCR7	828	0,09 (22)	0,29 (17)
	140	0,00 (21)	0,00 (21)
HPRTLacZ1	436	0,08 (35)	0,00 (2)
	213	0,00 (51)	0,00 (16)

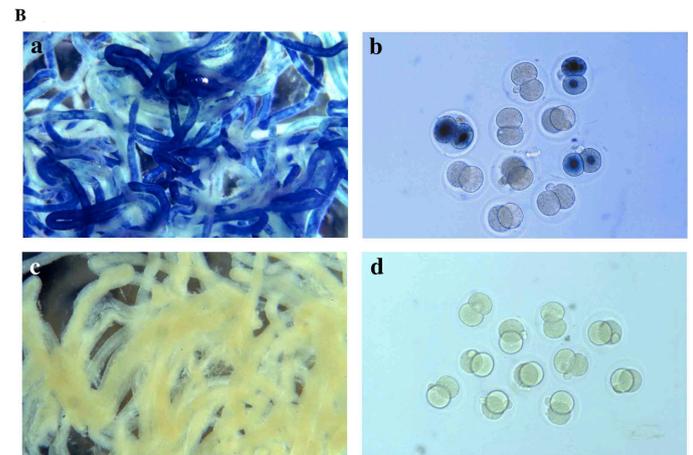


Figure 7. Expression of paternally transmitted LacZ transgenes in embryos obtained from animals with low and high β -gal+ activity in their testis.

In each of the four lines analysed, two males were selected on the basis of a high or a low β -gal activity (quantified using the MUG assay) measured in one surgically removed testis. Qualitative transgene expression in testis and in arrested 1-cell, or 2-cell embryos and 4-cell embryos. EFLacZ 1, DCR4 and DCR7 males were hemizygous for the transgene and HPRTLacZ1 males were homozygous. Each male was mated with a (B6D2) F1 female to generate 1-cell, 2-cell or 4-cell embryos. 1- and 2-cell embryos were collected and allowed to develop in medium supplemented with aphidicolin (2 μ g/ml) as described in Material and Methods. Late 4-cell embryos were immediately stained with X-gal after recovery at 56 hphCG. In toto X-gal staining of (a) seminal tubes from male n° 1 DCR7 adult testis (b) 2-cellembryos obtained after crossing of this male with B6D2 female (c) seminal tubes from male n° 2 DCR7 adult testis and (d) 2-cell embryos obtained after crossing of this male with B6D2 females.

Repression of the maternal and paternal LacZ transgenes in embryos before the morula stage is mediated by histone deacetylase complexes

It is becoming more and more evident that at least part of the transcriptional repression dependent on methylated CpG islands is mediated by histone deacetylation. Indeed, the MeCP2/Sin3A/histone deacetylase complex has been shown to bind to methyl CpG (Nan et al., 1998; Jones et al., 1998) and a large fraction of the deacetylases of the cells are complexed with MeCP2 (Bestor, 1998). To test whether this mechanism could be responsible for the non-permissive transcriptional state established during gametogenesis and inherited by the embryo, cleavage stage embryos were treated with sodium butyrate (NaB) and

trichostatin A (TSA), two inhibitors of histone deacetylases (Yoshida et al., 1995).

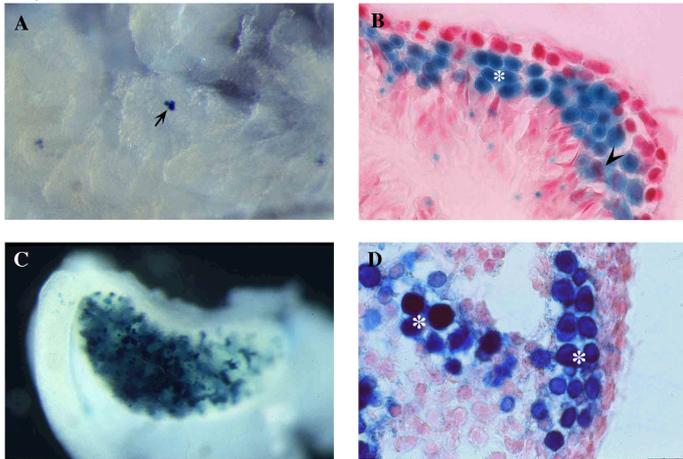


Figure 8. HPRTLacZDCR transgene expression in gonads. X-gal staining of male (A, B) and female (C,D) DCR2 gonads. (A) seminiferous tubules at birth (0 dpp) with rare (β -gal⁺ type A spermatogonies (arrow head). (B) a 20 μ m cryostat transverse section of adult seminiferous tubules showing the XII stage of spermatogenesis with (β -gal⁺ secondary spermatocytes (white asterisks) and (β -gal⁺ spermatogenic cells at meiosis I or II (white arrow). (C) ovary from a 15.5 dpc embryo. (D) a 20 μ m cryostat transverse section of a 15.5 dpc ovary showing (β -gal⁺ pachytene oocytes type I (white asterisk).

LacZ transgenes from the DCR6 and DCR7 lines were studied since the transgene of both parental origin is either expressed in a small number of 2-cell embryos or not at all. In both cases, a release from repression was obtained in embryos treated with either NaB or TSA (Fig. 9A). This strongly suggests that the mechanism of repression of the maternal LacZ transgene is mediated by histone deacetylases at the chromatin level. Since we have shown that this repression is also related to the high CpG content in LacZ, it implies that histone deacetylases act through methylated DNA.

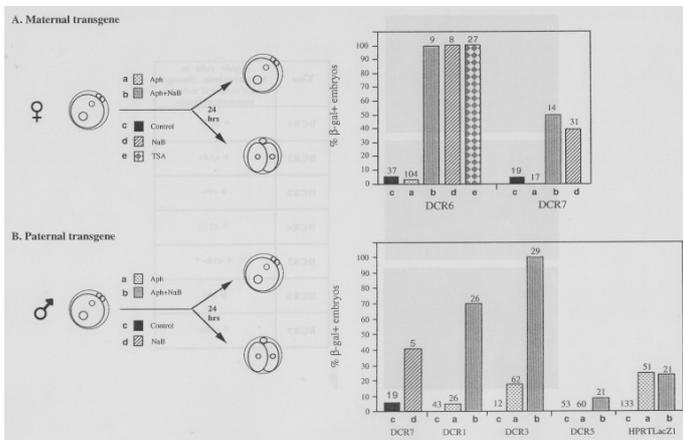
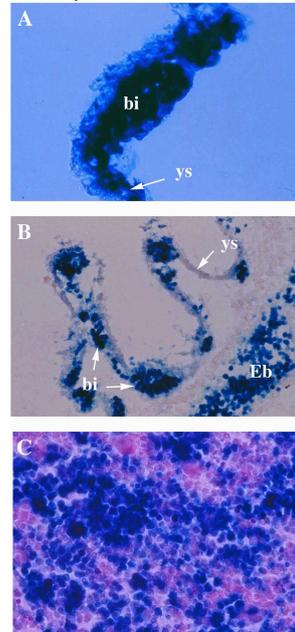


Figure 9. Inhibitors of histone deacetylases relieve the repressed state of maternally and paternally transmitted LacZ transgenes in 2-cell embryos. 1-cell embryos from different lines, carrying a maternal (A) or paternal (B) of origin transgenes were recovered at 24 hphCG and allowed to develop in the absence (control) or presence of sodium butyrate (NaB) (2.5 mM) or trichostatin A (TSA) (66 nM) for 24 hours. Aphidicolin, an inhibitor of DNA polymerases, was used alone (Aph) (2 μ g/ml) or in combination with sodium butyrate (Aph + NaB).

The effect of NaB was also tested on the repressed paternal transgenes which characterize certain transgenic lines. For instance, in the DCR3, DCR1 and DCR5 lines, expression of the transgene was repressed in 82, 97 and 100% of arrested 1-cell embryos, respectively (Table 3). In all three lines, relief from repression was observed in a fraction of the NaB treated embryos (Fig. 9B) and seemed to be related to the percentage of β -gal⁺

untreated embryos : the greater the (β -gal⁺ percentage (18%, 3% and 0% for DCR3, DCR1 and DCR5, respectively) in untreated embryos the greater the proportion of β -gal⁺ embryos after NaB treatment (100%, 70% and 10% respectively). Therefore, as for the maternal transgenes, these data suggest that paternal transgenes may be locally repressed at the chromatin level by histone deacetylases in some embryos. Moreover, the correlation between the percentage of β -gal⁺ embryos before and after NaB treatment suggests that quantitative and not qualitative differences in the level of inhibition account for the observed differences between transgenic lines and between embryos in the same line. These quantitative differences may result from the relative degree of CpG methylation and may determine the relative dependance on histone deacetylase activity.



Line	β -gal ⁺ cells in hematopoietic lineage <total number of analysed transgenic embryos>
DCR1	+ <12>
DCR2	+ <14>
DCR3	+ <9>
DCR4	+ <11>
DCR5	+ <16> *
DCR6	+ <8>
DCR7	+ <10>

Figure 10. The repression on LacZ transgene in embryonic cells can be relieved by lineage-specific activators. Embryos were obtained by crossing transgenic DCR males with (B6D2) F1 females. The HPRTLacZDCR transgene was expressed during each step of differentiation of the hematopoietic lineage cells, in the blood islands of the yolk sac in embryos at 8.5 and 10.5 dpc (A and B), in nucleated erythrocytes in liver of embryos at 15.5 dpc (C). (A) a section of the yolk sac (ys) of an embryo at 8.5 dpc showing β -gal⁺ nucleated erythrocytes in blood islands (bi). (B) a section of an embryo at 10.5 dpc showing (β -gal + nucleated erythrocytes in blood islands (bi) of the yolk sac (ys) and in embryo (Eb). (C) a section of liver of an embryo at 15.5 dpc showing (β -gal⁺ nucleated erythrocytes. The table indicates (β -gal⁺ cells detected in hematopoietic lineage for all DCR lines. * : This DCR line expresses the transgene only in blood islands of embryos at 10.5 dpc. It was the DCR line for which no HPRTLacZDCR expression was detected in preimplantary stages (see table 3).

Repression of LacZ transgenes in somatic tissues can be relieved by lineage-specific activators

To determine whether the repressive state of HPRTLacZ in embryonic cells can be reversed when a lineage specific activator, LCR, is developmentally switched on, we expanded a previous observation (Bonnerot and Nicolas, 1993a) by examining nucleated erythrocytes for the presence of β -gal activity in the yolk sac at 8.5 and 10.5 dpc and in the fetal liver at 15.5 dpc (Fig. 10A-C). All DCR lines expressed the transgene in erythrocytes, including those that exhibited incomplete release from the gametic repressive state during early development (DCR1, DCR3, DCR4 and DCR5 see table in Fig. 10). In addition, we have already shown that both HPRTLacZ and DCR transgenes are activated by integration site-dependent elements and these elements probably function in an analogous manner to the LCR. Because site-dependent expression involves many cell types, the repressive state clearly can be completely relieved by activators in many, if not all, somatic tissues.

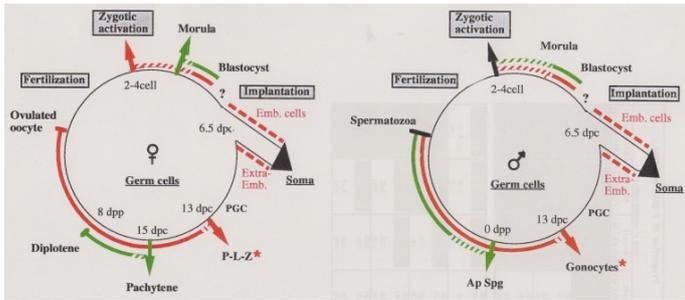


Figure 11. Summary of the expression of EFLagZ, EFLacZ, HPRTLacZ and HPRTLacZDCR transgenes during gametogenesis and early development of the embryo. EFLagZ and LacZ transgenes expression during gametes and embryo development is indicated as red and green draws respectively. In the left part is indicated transgenes expression through a paternal genome and in the right part, transgenes expression through the maternal genome. Gametogenesis is shown at the bottom of the cycle, the cleavage period of the embryo is shown at the top of the cycle and the post-implantation embryo is shown to the right. Periods of development at which the transcriptional permissiveness of transgenes changes is indicated by the arrows. Stages of gametogenesis and the embryo at preimplantation corresponding to the relief of transgene inhibition (red, green and black arrows) or the establishment of inhibition (red, green and black vertical bars) are indicated outside the cycle. Red and green dashed lines indicate that the relief of transgene inhibition is progressive. Black vertical bars and arrows indicate that the two EFLagZ and EFLacZ transgenes were inhibited and become expressed in the same cell type or preimplantation period, dpc : day post-coitum; dpp : day post-partum; PGC : primordial germ cells; Ap Spg : type A spermatogonies; P-L-Z ; preleptotene, leptotene and zygotene stages of prophase I; * : the X-gal staining becomes clear at 13 dpc once germ cells are well established in genital ridges; before that stage we did not detect X-gal staining in cells corresponding to gonocytes or oogonia.

DISCUSSION

This comparison between expression of the LagZ and LacZ transgenes is the first work to demonstrate *in vivo* the influence of CpG density of the structural part of a gene on its expression, to show that variations of the global methylation during development and gametogenesis influence gene expression and to chronicle variations of the repression at specific stages of development. It offers new insight into: (1) the capacity of the CpG-dependent regulatory systems to induce a non-permissive transcriptional state for genes *in vivo*, (2) the relief of this state in gametes and embryonic cells, (3) the occurrence of cyclic demethylation at the level of individual genes.

A system to explore CpG-dependent regulatory mechanisms

Because the activity of promoters used in this study depends on ubiquitous transcriptional factors, which remain relatively constant in the cell at all developmental stages (Kim et al., 1990; Hanaoka et al., 1991), the fluctuations in transgene expression must result primarily from modifications of elements responsible for negative control, such as the mCpG-dependent repressor complexes that modify chromatin structure (Boyes and Bird, 1991; Boyes and Bird, 1992; Hendrich and Bird, 1998; Jones et al., 1998; Nan et al., 1998). Several arguments implicate the CpG-dependent-negative systems, and in particular CpG methylation, in the regulation of most of the variations in transgene expression (summarized in figure 11) rather than other variations bypassing these systems. First, transitions between periods of gene inactivity and expression for the LagZ and LacZ transgenes were never sharply defined but rather spread across several gametic or embryonic stages (Fig. 11) implying a progressive mechanism rather than a rapid qualitative phenomenon. Second, a remarkable parallel is observed between the CpG-rich LacZ reporter expression pattern and the changes in genomic methylation during gametogenesis and early development. Indeed, the two periods of maximal hypomethylation correspond to the blastocyst and pachytene stages of oogenesis (Monk et al., 1987; Kafri et al., 1992; Rougier et al., 1998; Wamecke and Clark, 1999). At these two stages, our transgenes EFLacZ,

EFLagZ and FIPRTLacZDCR were expressed in most of the lines (Fig. 11). Similarly just after embryo implantation, a period of maximal methylation, we observed the expression of only the CpG-poor transgene (EFLagZ) (Fig. 11). Third, in the cleavage-stage embryo, a crucial period of transition between a non-permissive and permissive state for LacZ transgenes, inhibitors of histone deacetylases almost completely relieve the repression of maternal transgenes, and that of still repressed paternal transgenes. It demonstrates that both examples of repression result from the deacetylation of chromatin. Taken together with the differential expression of LagZ and LacZ transgenes at these stages, these findings strongly suggest that this deacetylation is CpG-dependent. Therefore, it may involve the MeCP2/Sin3A/HDAC complexes. Another indication that a repressive mCpG system is active in early embryo comes from the observation that methylated genes are repressed at these early stages of development in mice (Goto et al., 1998) and in *Xenopus laevis* (Jones et al., 1998).

A CpG-dependent repression is active in relation to the CpG density

In *in vitro* systems and in differentiated cells respectively, it has been shown that artificially methylated DNA is indirectly repressed by MBD proteins (Jones et al., 1998; Nan et al., 1998) and that this repression is only effective when a certain level of methylation is reached (Hsieh, 1994; Komura et al., 1995; Kass et al., 1997; Nan et al., 1997; Goto et al., 1998). Our results demonstrate that the presence in the structural part of a gene containing a high density of CpG can create a non-permissive transcriptional state. In cells of the embryo at about 7.5 dpc and in somatic cells, almost all CpGs, except those in CpG islands of promoters, are methylated (Monk et al., 1987; Bird, 1992; Kafri et al., 1992). Therefore, the non-permissive state of LacZ transgenes in embryonic cells just after implantation and later in somatic cells can be attributed to its initiation to a CpG-dependent repressive system. Among the four combinations of sequences tested, only the one containing a strong promoter (E1Fa) and the poor CpG density (LagZ) escapes, although partially, this repression. Therefore, in addition to be a control which demonstrates the implication of CpGs in the regulation of expression, it also shows that even a sequence with a low CpG density can repress. This suggests that *in vivo* the repressive system is determined by a critical threshold of mCpGs.

This leads to the suggestion that it is the global balance between activators and this CpG-dependent repression which controls the activity of a gene. For a gene controlled by the EFla promoter, the threshold for inactivation in somatic cells seems to be close to 2% of CpG in the structural region. This is supported by the fact that the structural region of the ubiquitous human EFla gene contains 1.3% of CpG with a %(C+G) of 41% and a O/E of 0.29 and the LagZ reporter gene contains 2% of CpG with a %(G+C) of 49% and a O/E of 0.37. The use of a reporter gene lacking more CpGs than LagZ and associated with promoters of different strength should allow us to define the threshold at which the expression of a gene become insensitive to these negative regulatory effects.

Capacity of tissue specific trans-activators to relieve the CpG-dependent non-permissive state of embryonic cells

The HPRTLacZDCR transgene which combines a relatively weak promoter to a CpG-rich sequence is in a non-permissive transcriptional state in embryonic cells after implantation. However, remarkably, this repression is completely relieved by the mini-LCR in embryonic and foetal hematopoietic lineages, as well as, by activator elements at the integration site, which confer to transgenes the position-dependent expression pattern also observed in HPRTLacZ lines (Bonnerot et al., 1990; Bonnerot and Nicolas, 1992). These results indicate that the CpG-dependent repressive state does not prevail over tissue-specific activation. Similarly, it has been shown that enhancers can relieve the inhibition of methylated DNA in *in vitro* system and in differentiated cells (Boyes and Bird, 1991).

Following the idea that gene activity is controlled by the global balance between activators and the CpG-dependent repression acting on chromatin structure, then the relief of repression by the LCR and activators of the non-permissive state of LacZ would be achieved by targeting elements

capable of counteracting the action of MBDs complexes. From this point of view, it is remarkable to note that several of the factors associated with RNA polymerase II and several transcription factors are histone acetylases (Brownell and Allis, 1996; Struhl, 1998). These elements have, therefore, the potential to counteract the deacetylation of histones by the MBD-HDAC complexes and thereby to change chromatin structure.

Therefore, the artificial combination of an ubiquitous promotor, a CpG-rich region and a lineage specific activator mimics remarkably the fundamental properties of a tissue-specific gene. It is notable that as with the LacZ transgenes, nearly all tissue-specific genes also contain a CpG-rich sequence in their transcribed region (Larsen et al., 1992). If these CpG islands worked as inhibitory elements in somatic tissues using the process described here for the LacZ gene, then the function of transcriptional activators would be to relieve an active repression. As this does not necessitate demethylation of the CpGs but rather targeting of elements capable of counteracting the action of MBD proteins (such as acetylases), the apparent paradox of activation of a gene in the absence of demethylation would be resolved.

Cycles of methylation/demethylation of the genome during development and gametogenesis.

Is there a developmental control for the establishment of the non-permissive transcriptional state of transgenes? Ours results suggest that, at the morula-blastocyst stage, this repression has not yet been established (or is not yet effective on gene expression). Later, prior to cellular differentiation, between blastocyst stage and 7.5-10.5 dpc, the specific disappearance of LacZ expression indicates that repression is effective in both embryonic and extra-embryonic tissues. It is important to note that since the process is intrinsic to cells, each cell can respond individually. This may explain the heterogeneity observed between cells in EFLagZ embryos. Again these observations are reminiscent of the methylation status of genes and of the genome observed in the embryonic and extraembryonic tissues (Monk et al., 1987; Salvatore et al., 1998; Wamecke and Clark, 1999).

Clearly, in the progenitors of the gonocytes and of the oogonia, the non-permissive transcriptional state is also established since in both male (gonocytes) and female (P-L-Z stages) prior the entry in gametogenesis the CpG-rich transgenes are found repressed while the CpG-poor transgenes are active (see stars in Fig. 11). This CpG-dependent repression of transgenes is reminiscent of the one established at implantation of the embryo, and suggests a sufficient level of methylation for repression of the LacZ transgenes. However, since the expression of LagZ transgenes is higher in germ cells than in embryonic and somatic cells, the activation/repression balance in germ cells may incline more towards a genic activity than a repressive state.

This repression is relieved later in type A spermatogonies, the number of those LacZ (β -gal⁺ cells increasing between 0 and 8 dpp. A more detailed study is necessary to learn whether this relief of non-permissiveness is specific to type A spermatogonies or whether it also concerns subsequent stages, especially post-meiotic stages. But clearly, this relief does not occur in stages preceding A spermatogonies and particularly in the stem cells (type As spermatogonies). Indeed, if this was the case, the heterogeneity of expression observed during gametogenesis for certain transgenic lines would be erased with aging in males. This heterogeneity is strictly maintained for long periods, as shown by the same expression level in gametes of the same male after a period of six months (C.Mariette, S. Capras, unpublished results).

What mechanism does relieve the non-permissive transcriptional state in type A spermatogonies? A strong candidate is DNA methylation because the LacZ transgenes which are still repressed at the entry of male gametogenesis have been shown to be repressed at the 2-cell embryo and are activable by inhibitors of histone deacetylases. It suggests that their CpGs are methylated and that those of the active transgenes are unmethylated (if not, they should be repressed). If this is actually the case, then, a first demethylation of the LacZ gene would occur at the entry of cells in spermatogenesis and later at the morula-blastocyst stage.

During oogenesis, at the pachytene stage, both LagZ and LacZ transgenes are active, even though the relief of their non-permissive state begins at different times in each transgenic lines. Since the genome in pachytene oocytes is minimally methylated (Monk et al., 1987), it is tempting to attribute this activity to the demethylation of CpGs. Later, at the diplotene stage, all LacZ transgenes are again in a non-permissive state, which is only relieved at the morula-blastocyst stage. This relief can be obtained earlier during the first cleavages of the embryo through the inhibition of histone deacetylases whereas the maternal LagZ transgenes are already active. All these observations suggest that the non-permissive state in oocytes is due to methylation of CpGs. Many studies indicate that the DNA of mature oocytes is methylated (Monk et al., 1987; Kafri et al., 1992). Our study suggests that at the level of individual genes, a maximal demethylation occurs in oocytes at the pachytene stage, followed by an active remethylation at the diplotene stage, and finally demethylation of maternal transgenes occurs in the embryo at the morula-blastocyst stage. Although more complex than during spermatogenesis, the situation described here for maternal transgenes globally corresponds again to a cycle of demethylation/methylation.

Biological significance

Our data indicate that transgenes are subject to negative control in embryonic and somatic cells in relation to their high CpG content and that positive control elements relieve the repressive state upon cell differentiation when present. This is compatible with the concept of a global negative control of the genome (Bird, 1995), even for tissue-specific genes, through the methylation of CpGs, as well as with the concept of the control of gene activation by the balance between this global negative control and activators, both acting on chromatin structure.

If the repression of the CpG-rich LacZ gene reflects a global negative control of the genome, then, in addition to embryonic and somatic cells, other stages also undergo this control including: the extra-embryonic cells, the stem cells of male germ cells (both gonocytes and spermatogonies) and the female germ cells at P-L-Z and diplotene stages and some of their precursors. Consequently, the negative control of the genome would be always associated with the activity of specialized cells, excluding only multipotential cells of cleavage embryos.

At two periods during the life of the organism, cells seem not to enforce this negative control: at the morula-blastocyst stage, at the pachytene stage during oogenesis and the corresponding stage during spermatogenesis. However, the relief from negative control in these cells does not appear to be mediated by modifications of the trans-acting elements of the repressive complex, but through the demethylation of CpGs. It would seem easier and more efficient to relieve the genic repression at these stages by temporally inhibiting the expression of one or more components of the repressor complex than to modify all CpGs of the genome. Therefore, cyclic demethylation of the genome is probably necessary for more than merely the specific gene activation in cells at these stages of development.

This raises the hypothesis that both the maintenance of a global negative regulatory mechanism and the maintenance of a periodic demethylation are crucial for the organism. The maintenance of a repressive mechanism based on DNA methylation represents a heavy genetic and epigenetic load for both the genome (through germ cells) and the organism (through somatic cells). The properties of EFLagZ illustrates this point since even though the CpG density of this transgene is close to that of CpG-poor endogenous genes, it was still inhibited, particularly in somatic cells, suggesting that this repression can still act on CpG-poor endogenous genes. Similarly, tissue-specific genes containing CpG-rich regions, would also be particularly susceptible to this repressive mechanism. Because the methylation pattern is clonally transmitted, repression of these genes would be maintained and accumulated in daughter cells. Therefore, the general hypomethylation of the genome at the beginning of embryogenesis may serve to counteract gene repression. The consequence of this hypomethylation is an immediate gain for the embryo and the organism and a genetic gain, through germ cells, for the next generation. The general

methylation which follows the demethylation occurs in individual cells. This polyclonal event is also advantageous to the organism, since potentially inappropriate inactivation caused by this remethylation will not affect every cell of the embryo, and cells with an incorrect pattern of methylation can be ultimately eliminated. This may explain the occurrence of demethylation-methylation precisely at this period of development.

On the other hand, an extended period of genomic hypomethylation could potentially cause cellular disorders (Li et al., 1992; Foss et al., 1993; Finnegan et al., 1996; Kakutani et al., 1996) and this could explain why a rapid methylation follows demethylation at the blastocyst stage and why the female genome methylates at the diplotene stage prior to the growth phase. In this latter case, methylation might also be needed to prevent the inappropriate expression of genes whose products could accumulate in the egg and be maternally transmitted to the embryo. The repressive indirect effect of methylation and the dynamic pattern of methylation during development raise a paradox. To preserve their spatio-temporal pattern of expression, genes may have adapted to these conditions. Ubiquitous genes may have evolved towards a lower CpGs content to escape this global, CpG-dependent, negative control system. In support of this idea is the fact that 82% of genes with a broad expression lack CpG islands outside of their promoters (Larsen et al., 1992). Tissue-specific genes have probably evolved towards a more refined activating mechanism, involving cis and trans-activators, to overcome this CpG-dependent repression. Indeed, it has been shown that one function of the transcriptional machinery is to modify chromatin into an active conformation (Struhl, 1996). It fits with the concept of gene activity based on a balance between global negative control and activators acting on chromatin structure. It appears illogical, therefore, that most tissue-specific genes have preserved at least one CpG-rich region, usually located outside of the promoter (Larsen et al., 1992). Our observations suggest that this CpG-rich region could be used to inhibit tissue-specific gene activity through a general mechanism, particularly at developmental stages where negative control of such genes is essential, such as the period of tissue diversification at about 8 dpc. In this regard it is worth noting that the null mutants for methyltransferase (*dnmt1*) or MeCP2 exhibit lethality at this stage (Li et al., 1992; Tate et al., 1996).

To conclude, all these observations suggest that the mammalian genome is not simply controlled by activation above basal levels but is also actively repressed. Such a system may permit more discrete regulation and a large range of gene activity levels through the combined activity of activators and repressors. Such a fine tuning mechanism with respect to gene activity could, in turn, result in elaboration of more complex regulatory networks. Other functions generally associated with methylation in mammals, such as the control over the spreading of repeated sequences or transposons and genomic imprinting (Walsh and Bestor, 1999) might constitute some secondary uses of this more fundamental mechanism.

MATERIALS AND METHODS

DNA Inserts

Constructs of the HPRTrnslacZ and HPRTrnslacZDCR inserts were previously described in (Bonnerot et al., 1990) and (Bonnerot and Nicolas, 1993a). They contain the promoter of the human hypoxanthine phosphoribosyl transferase (HPRT) gene that drives expression of a nuclear targeted (β -galactosidase (nlsLacZ). HPRTrnslacZDCR contains the four DNaseI hypersensitive sites (mini-LCR) of the human LCR (β -globin gene (GenBank Accession Number U01317) (Talbot et al., 1989). The 7.9 kb EFnslacZ insert was isolated from the plasmid, pBSEFnlacZdenh, as a XhoI-NotI fragment (partial digestion for XhoI). pBSEFnlacZ6enh was derived from pEF321-CAT kindly provided by D.W. Kim (Kim et al., 1990). The 2.3kb HindIII-ScaI fragment of pEF321-CAT containing the (+1) to (+1561) portion of the human EFla gene (promoter, exon 1 and intron 1), plus 730 bp of 5' untranslated sequence (Uetsuki et al., 1989) was ligated (after klenow fill-in) to a 9.5 kb Sail fragment of pBSGA5LTRnslacZ (Bonnerot et al, unpublished), containing the nlsLacZ reporter gene and the polyadenylation signal of Moloney murine leukemia virus on a pBluescript plasmid backbone.

Mutagenesis of the LacZ gene and generation of the EFLagZ insert

The CpG content of LacZ was lowered from 9.2% to 2.2%, a percentage close to that of the mammalian genome by mutagenesis. A Polymerase Chain Reaction (PCR) technique was used, in which the mutagenic oligonucleotide primers spanned the whole LacZ sequence but contained less CpGs sequence. These mutagenic primers were designed to preserve integrity of the amino acid sequence of the (β -galactosidase reporter protein. The DNA sequence of the mutated LacZ was verified by sequencing.

For construction of pBEFnlacZdenh, nlsLacZ was replaced by nlsLagZ after ligation of the 3.5 kb AvrII-BamHI LagZ fragment of pPytknslagZ to the 3.5 kb AvrII-BamHI fragment of pBEFnlacZsenh, lacking nlsLacZ. The resulting plasmid pBEFnlacZsenh was digested by XhoI-NotI (XhoI partial) to obtain the 7.9 kb insert EFnslagZ.

Transgenesis

Plasmids were digested to remove vector DNA sequences and inserts were purified on glass beads. Transgenic mice were generated as described in Forlani et al. (1998)

Recovery of preimplantation embryos

Preimplantation embryos were recovered from crosses between (C57BL/6JxDBA2) FI (B6D2) females or males and transgenic males or females, respectively, as described previously in Vernet et al. (1993) and Forlani et al. (1998).

Recovery of embryonic and adult organs and cryosectioning

Ovaries and testes were dissected from embryos at different ages according to protocols described in (Hogan et al., 1986). Embryonic testes were identified by the presence of seminal cords. After dissection of organs, X-gal staining and cryosectioning were performed as described in (Bonnerot and Nicolas, 1993).

Qualitative analysis of (β -galactosidase activity

For analysis of transgene expression in preimplantation embryos, freshly harvested or cultured embryos were recovered at the appropriate times and immediately analysed by X-gal staining overnight at 30°C (Vemet et al., 1993). Embryonic and adult organs were stained for two days at 30°C and cryosections overnight at 30°C (Bonnerot et al., 1990).

Quantification of (β -galactosidase activity in transgenic adult testis

In some experiments, quantification of β -galactosidase activity was used to screen adult males according to their transgene expression in testis. A single testis was surgically removed such that transgenic males could be subsequently mated with (B6D2) FI females to generate preimplantation embryos.

The removed testis was cut into two parts. The first half was fixed in 4% paraformaldehyde in PBS and X-gal stained. The second half was used to recover proteins and to measure β -gal activity using an assay that measures cleavage of the fluorogenic substrate, 4-methylumbelliferyl 3-D galactoside (Forlani and Nicolas, 1996).

REFERENCES

- Ariel, M., McCarrey, J. and Cedar, H. (1991). Methylation patterns of testis-specific genes. *Proc Natl Acad Sci USA* 88, 2317-2321.
- Bestor, T. H. (1998). Gene silencing. Methylation meets acetylation [news; comment]. *Nature* 393, 311-312.
- Bird, A. (1992). The essentials of DNA methylation. *Cell* 70, 5-8.
- Bird, A. P. (1995). Gene number, noise reduction and biological complexity [see comments]. *Trends Genet* 11, 94-100.
- Bonnerot, C., Rocancourt, D., Briand, P., Grimber, G. and Nicolas, J. F. (1987). A beta galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. *Proc. Natl Acad. Sci.* 84, 6795-6799.
- Bonnerot, C., Grimber, G., Briand, P. and Nicolas, J. F. (1990). Patterns of expression of position dependent integrated transgenes in mouse embryo. *Proc Natl Acad Sci USA* 87, 6331-6335.
- Bonnerot, C. and Nicolas, J.-F. (1992). Manipulation of expression of a position-dependent transgene by the b-globin locus control region. Submitted to *Nature*.
- Bonnerot, C. and Nicolas, J. F. (1993a). Lineage control of an integration site-dependent transgene combined with the b-globin locus control region. *C. R. Acad. Sci. Paris* 316, 352-357.
- Bonnerot, C. and Nicolas, J.-F. (1993b). Application of LacZ gene fusions to post-implantation

- development. In *Methods in Enzymology : Guide to techniques in mouse development*, vol. 225 (ed. A. Press), pp. 451-469. San Diego (California): Wassarman, P. M. and DePamphilis, M. L.
- Boyes, J. and Bird, A. (1991). DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. *Cell* 64, 1123-1134.
- Boyes, J. and Bird, A. (1992). Repression of genes by DNA methylation depends on CpG density and promoter strength : evidence for involvement of a methyl-CpG binding protein. *EMBO J* 11, 327-333.
- Brownell, J. E. and Allis, C. D. (1996). Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. *Current Opinion in Genetics & Development* 6, 176-184.
- Buschhausen, G., Wittig, B., Graessmann, M. and Graessmann, A. (1987). Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene. *Proc Natl Acad Sci USA* 84, 1177-1181.
- Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G. and Baylin, S. B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 21, 103-107.
- Chaillet, J. R. (1994). Genomic imprinting: lessons from mouse transgenes. *Mutat Res* 307, 441-449.
- Collas, P. (1998). Modulation of plasmid DNA methylation and expression in zebrafish embryos. *Nucl Acids Res* 26, 4454-4461.
- Finnegan, E. J., Peacock, W. J. and Dennis, E. S. (1996). Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. *Proc Natl Acad Sci USA* 93, 8449-8454.
- Forlani, S. and Nicolas, J. (1996). Quantification of minute levels of beta-galactosidase. The example of individual 2 to 16-cell stage mouse embryos. *Trends Genet* 12, 498-500.
- Foss, H. M., Roberts, C. J., Claeys, K. M. and Selker, E. U. (1993). Abnormal chromosome behavior in *Neurospora* mutants defective in DNA methylation [published erratum appears in *Science* 1995 Jan 20;267(5196):316]. *Science* 262, 1737-1741.
- Gardner, R. L. and Lawrence, P. A. (1985). Single cell marking and cell lineage in animal development. *Phil Trans R Soc Lond B312*, 1-187.
- Ghazi, H., Gonzales, F. A. and Jones, P. A. (1992). Methylation of CpG-island-containing genes in human sperm, fetal and adult tissues. *Gene* 114, 203-210.
- Goto, T., Christians, E. and Monk, M. (1998). Expression of an Xist promoter-luciferase construct during spermatogenesis and in preimplantation embryos: regulation by DNA methylation. *Mol Reprod Dev* 49, 356-367.
- Goto, T. and Monk, M. (1998). Regulation of X-chromosome inactivation in development in mice and humans. *Microbiol Mol Biol Rev* 62, 362-378.
- Groudine, M. and Conkin, K. F. (1985). Chromatin structure and de novo methylation of sperm DNA: implications for activation of the paternal genome. *Science* 228, 1061-1068.
- Hanaoka, K., Hayasaka, M., Uetsuki, T., Fujisawa-Sehara, A. and Nabeshima, Y. (1991). A stable cellular marker for the analysis of mouse chimeras: the bacterial chloramphenicol acetyltransferase gene driven by the human elongation factor I alpha promoter. *Differentiation* 48, 183-189.
- Hendrich, B. and Bird, A. (1998). Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Mol Cell Biol* 18, 6538-6547.
- Hogan, B., Costantini, F. and Lacy, E. (1994). *Manipulating the mouse embryo: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Howell, C. Y., Steptoe, A. L., Miller, M. W. and Chaillet, J. R. (1998). cis-Acting signal for inheritance of imprinted DNA methylation patterns in the preimplantation mouse embryo. *Mol Cell Biol* 18, 4149-4156.
- Hsieh, C. L. (1994). Dependence of transcriptional repression on CpG methylation density. *Mol Cell Biol* 14, 5487-5494.
- Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J. and Wolffe, A. P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet* 19, 187-191.
- Kafri, T., Ariel, M., Brandeis, M., Shemer, R., Urven, L. and McCarrey, J. (1992). Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. *Genes Dev* 6, 705-714.
- Kakutani, T., Jeddeloh, J. A., Flowers, S. K., Munakata, K. and Richards, E. J. (1996). Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. *Proc Natl Acad Sci USA* 93, 12406-12411.
- Kass, S. U., Landsberger, N. and Wolffe, A. P. (1997). DNA methylation directs a time-dependent repression of transcription initiation. *Current Biol* 7, 157-165.
- Kim, D. W., Uetsuki, T., Kaziro, Y., Yamaguchi, N. and Sugano, S. (1990). Use of the human elongation factor I alpha promoter as a versatile and efficient expression system. *Gene* 91, 217-223.
- Klarsfeld, A., Bessereau, J.-L., Salmon, A.-M., Triller, A., Babinet, C. and Changeux, J.-P. (1991). An acetylcholine receptor alpha-subunit promoter conferring preferential synaptic expression in muscle of transgenic mice. *EMBO J* 10, 625-632.
- Kluin, P. M. and de Rooij, D. G. (1981). A comparison between the morphology and cell kinetics of gonocytes and adult type undifferentiated spermatogonia in the mouse. *Int J Andrology* 4, 475-493.
- Komura, J., Okada, T. and Ono, T. (1995). Repression of transient expression by DNA methylation in transcribed regions of reporter genes introduced into cultured human cells. *Biochim Biophys Acta* 1260, 73-78.
- Kress, C., Vogels, R., de Graaff, W., Bonnerot, C., Hameleers, M., Nicolas, J. F. and Deschamps, J. (1990). Hox-2.3 upstream sequences mediate spatio-temporally restricted LacZ expression in intermediate mesoderm derivatives of transgenic mice. *Development* 109, 775-786.
- Kudo, S. (1998). Methyl-CpG-binding protein MeCP2 represses Spl-activated transcription of the human leukosialin gene when the promoter is methylated. *Mol Cell Biol* 18, 5492-5499.
- Larsen, F., Gundersen, G., Lopez, R. and Prydz, H. (1992). CpG islands as gene markers in the human genome. *Genomics* 13, 1095-1107.
- Li, E., Bestor, T. H. and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69, 915-926.
- Martin, C. C., Laforest, L., Akimenko, M. A. and Ekker, M. (1999). A role for DNA methylation in gastrulation and somite patterning. *Dev Biol* 206, 189-205.
- Monk, M., Boubelik, M. and Lehnert, S. (1987). Temporal and regional changes in DN/methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryonic development. *Development* 99, 371-382.
- Nan, X., Campoy, F. J. and Bird, A. (1997). MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell* 88, 471-481.
- Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N. and Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex [see comments]. *Nature* 393, 386-389.
- Nan, X., Tate, P., Li, E. and Bird, A. (1996). DNA methylation specifies chromosomal localization of MeCP2. *Mol Cell Biol* 16, 414-421.
- Rougier, N., Bourc'his, D., Gomes, D. M., Niveleau, A., Plachot, M., Paldi, A. and Viegas Peignon, E. (1998). Chromosome methylation patterns during mammalian preimplantation development. *Genes Dev* 12, 2108-2113.
- Salvatore, P., Benvenuto, G., Caporaso, M., Bruni, C. B. and Chiariotti, L. (1998). High resolution methylation analysis of the galectin-1 gene promoter region in expressing and nonexpressing tissues. *FEBS Letters* 421, 152-158.
- Sanford, J. P., Clark, H. J., Chapman, V. M. and Rossant, J. (1987). Differences in DN/methylation during oogenesis and spermatogenesis and their persistence during early embryogenesis in the mouse. *Genes Dev* 1, 1039-1046.
- Schultz, R. M. (1986). In *Experimental approaches to mammalian embryonic development*. J. Rossant and R. A. Pedersen Ed. Cambridge University Press. , 195-227.
- Singal, R., Ferris, R., Little, J. A., Wang, S. Z. and Ginder, G. D. (1997). Methylation of the minimal promoter of an embryonic globin gene silences transcription in primary erythroid cells. *Proc Natl Acad Sci USA* 94, 13724-13729.
- Struhl, K. (1996). Chromatin structure and RNA polymerase II connection: implications for transcription. *Cell* 84, 179-182.
- Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev* 12, 599-606.
- Talbot, D., Collis, P., Antoniou, M., Vidal, M., Grosveld, F. and Greaves, D. R. (1989). A dominant control region from the human beta-globin locus conferring integration site-independent gene expression. *Nature* 338, 352-355.
- Tate, P., Skarnes, W. and Bird, A. (1996). The methyl-CpG binding protein MeCP2 is essential for embryonic development in the mouse. *Nat Genet* 12, 205-208.
- Trasler, J. M., Hake, L. E., Johnson, P. A., Alcivar, A. A., Millette, C. F. and Hecht, N. B. (1990). DNA methylation and demethylation events during meiotic prophase in the mouse testis. *Mol Cell Biol* 10, 1828-1834.
- Uetsuki, T., Naito, A., Nagata, S. and Kaziro, Y. (1989). Isolation and characterization of the human chromosomal gene for polypeptide chain elongation alpha. *J Biol Chem* 264, 5791-5798.
- Vergouwen, R. P., Jacobs, S. G., Huiskamp, R., Davids, J. A. and de Rooij, D. G. (1991). Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. *J Reprod Fert* 93, 233-243.
- Vernet, M., Bonnerot, C., Briand, P. and Nicolas, J.-F. (1993). Application of LacZ gene fusion to preimplantation development. In *Methods in Enzymology: Guide to techniques in mouse development*, vol. 225 (ed. 434-451): Wassarman, P. M., DePamphilis, M. L.
- Walsh, C. P. and Bestor, T. H. (1999). Cytosine methylation and mammalian development. *Gene Dev* 13, 26-34.
- Warnecke, P. M. and Clark, S. J. (1999). DNA methylation profile of the mouse skeletal alpha actin promoter during development and differentiation. *Mol Cell Biol* 19, 164-172.
- Weng, A., Engler, P. and Storb, U. (1995). The bulk chromatin structure of a murine transgene does not vary with its transcriptional or DNA methylation status. *Mol Cell Biol* 15, 572-579.
- Yoshida, M., Horinouchi, S. and Beppu, T. (1995). Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. *Bioessays* 17, 423-430.
- Zhang, L. P., Stroud, J. C., Walter, C. A., Adrian, G. S. and McCarrey, J. R. (1998). A gene specific promoter in transgenic mice directs testis-specific demethylation prior to transcriptional activation *In vivo*. *Biol Reprod* 59, 284-292.

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