An embryonic demethylation mechanism involving binding of transcription factors to replicating DNA

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In vertebrates, transcriptionally active promoters are undermethylated. Since the transcription factor Sp1, and more recently NF-κB, have been implicated in the demethylation process, we examined the effect of transcription factors on demethylation by injecting in vitro methylated plasmid DNA into Xenopus fertilized eggs. We found that various transactivation domains, including a strong acidic activation domain from the viral protein VP16, can enhance demethylation of a promoter region when fused to a DNA binding domain which recognizes the promoter. Furthermore, demethylation occurs only after the midblastula transition, when the general transcription machinery of the host embryo becomes available. Nevertheless, transcription factor binding need not be followed by actual transcription, since demethylation is not blocked by α-amanitin treatment. Finally, replication of the target DNA is a prerequisite for efficient demethylation since only plasmids that carry the bovine papilloma virus sequences which support plasmid replication after the midblastula transition are demethylated. No demethylation is detectable in the oocyte system where DNA is not replicated. These results suggest that, in the Xenopus embryo, promoters for which transcription factors are available are demethylated by a replication-dependent, possibly passive mechanism.

Keywords: bovine papilloma virus (BPV)/Gal4 fusion proteins/Sp1/Xenopus oocytes/Xenopus embryos

Introduction

In vertebrate genomes, CpG methylation can repress promoter activity by directly or indirectly interfering with transcription factor binding to DNA (Iguchi-Arighi and Schaffner, 1989; Bird, 1992; Levine et al., 1992; Hug et al., 1996). A number of examples show that methylated promoters usually become demethylated in the tissues where they are active (Frank et al., 1990; Lichtenstein et al., 1994). Although this inverse correlation between methylation and transcription is widely accepted, whether demethylation plays a primary regulatory role in transcriptional activation remains obscure. In some cases at least however, demethylation has been shown to precede transcriptional activation (Paroush et al., 1990).

Recently a possible molecular link between demethylation and transcription factors was postulated in mammals. The transcription factor Sp1 was implicated in the generation and maintenance of unmethylated DNA, based on the observation that Sp1 binding sites, so-called GC boxes, can enhance demethylation and prevent de novo methylation of the flanking sequences (Brandes et al., 1994; Macleod et al., 1994; Silke et al., 1995). Similarly, a role in B-cell-specific demethylation has been reported for the transcription factor NF-κB (Kirillov et al., 1996). However, it is not known whether these putative demethylating factors harbor specific domains required for the induction of demethylation, or whether the transcriptional activation domains can also act as a ‘demethylation domain’.

The clawed toad, Xenopus laevis, has a CpG methylation system similar to that of mammals (Cooper et al., 1983). Relatively little is known about the overall methylation status and demethylation events in Xenopus. It has been shown, however, that the ribosomal genes remain highly methylated throughout life except for some hypomethylated sites in the spacer–promoter region that are selectively demethylated early in development (Bird et al., 1981; La Volpe et al., 1983). To study demethylation processes in both dividing and non-dividing cells, we microinjected exogenously methylated plasmid DNAs into fertilized eggs and oocytes. Microinjection has several advantages over transfection of DNA into mammalian cultured cells, including ease of introduction of multiple plasmid constructs or purified proteins in defined amounts.

We show, in line with observations in mammals, that Sp1 binding sites induce promoter demethylation of an in vitro methylated plasmid when the plasmid is replicating. We further show that Gal4 binding sites can also induce demethylation, in the presence of exogenous hybrid transcription factors in which different types of activation domains were fused to the DNA binding domain of the yeast Gal4 protein. Such induced demethylation occurs only after the midblastula transition (MBT), when assembly of the transcription complex is no longer repressed and zygotic transcription is restored. However, ongoing transcription is not required since α-amanitin treatment did not inhibit demethylation. Surprisingly, DNA replication is a compulsory condition for promoter-specific demethylation, suggesting the existence of a passive embryonic demethylation mechanism.

Results

Experimental design
To assess the influence of transcription factors on demethylation, we used a target plasmid, OVEC, con-
Demethylation in *Xenopus* embryos

To investigate the role in CpG demethylation of Sp1 binding sites (GC boxes) in *Xenopus* embryos without adding exogenous activators, we first used the marginally replicating OVEC plasmid that carries no eukaryotic origin of replication. Methylated plasmids, Sp-OVEC or Tq-OVEC, were injected into fertilized eggs and the DNA was recovered from stage 12 embryos. Southern analysis of the recovered DNA shows that the 4.1 kb *HindIII* fragment remains resistant to *HpaII*, indicating that all *HpaII* sites remain methylated (Figure 2A). The persistence of methylation in spite of ongoing replication indicates that maintenance methylases are present in the cleaving embryo. However, mock-methylated plasmids yield only the 0.72 kb band, indicating that such plasmids remain unmethylated irrespective of the presence of intact Sp1 binding sites and that no de novo methylation occurs. Stability of the methylation status of injected plasmids has been observed previously (Bendig and Williams, 1983). The *MspI*-resistant 1.7 kb band observed with only methylated Sp-OVEC is due to an idiosyncrasy of the *MspI* enzyme and is not informative.

To test the ability of different transcription factors to maintain the β-globin promoter (Westin et al., 1987). The target promoters used and the strategy for monitoring promoter-specific demethylation by restriction with *HpaII* enzyme are represented in Figure 1A. The binding sites for the transcription factors Sp1 or Gal4, flanked by *HpaII* sites, were inserted into the promoter region. A mutated Sp1 site (Tq) served as a control. The plasmids were methylated *in vitro* with *HpaII* methylase at CpGs in CCGG, including two *HpaII* sites adjacent to the binding motifs for the transcription factors. *HpaII* methylase was chosen because this methylates only 16 out of more than 200 CpGs in the plasmids, and does not interfere with transcriptional activation (data not shown). After injection of *HpaII*-methylated plasmids into fertilized eggs, DNA was recovered from embryos at various stages, usually stage 12 (late gastrula stage, 20 h after injection). Injected oocytes were also incubated for 20 h. The DNA was digested with *HindIII* to release the 4.1 kb globin fragment (Figure 1A) and then cut further with either methylation sensitive (*HpaII*) or insensitive (*MspI*) restriction enzymes. Only CCGG sites that are unmethylated on both strands are cut by *HpaII*. Southern blots were hybridized with a promoter-specific probe fragment. Presence of the 4.1 kb *HindIII* fragment indicates persistence of methylation. Appearance of the 0.72 kb band is indicative of demethylation at both the upstream *HpaII* site and one of the two promoter-linked *HpaII* sites, whereas the 0.82 kb band reflects selective demethylation of the promoter site(s), leaving the upstream *HpaII* site methylated. Formulation of a 1.7 kb band would indicate demethylation in the upstream and downstream sites, but not in the promoter-linked sites.

Figure 1B summarizes important biological parameters of the *Xenopus* embryo system. It is known that any DNA injected into *Xenopus* fertilized eggs replicates during the subsequent cleavages even if they carry no distinct eukaryotic origin of replication (Harland and Laskey, 1980; Rusconi and Schaffner, 1981; Etkin and Balecells, 1985), initiation taking place at numerous sites along the DNA (Harland and Laskey, 1980; Hyrien and Méchali, 1993). After the MBT, which occurs after the 12th division during stage 8, however, normal mitoses take place and replication is initiated at more distant and specific sites. Exogenous DNA, devoid of such replication origins, now disappears gradually with the exception of a few, integrated copies that replicate along with the host genome (Rusconi and Schaffner, 1981). The OVEC plasmids used here contain no distinct origin of replication and stop replicating at the MBT (‘marginally replicating’). This was verified by nucleotide incorporation or quantitative Southern blot experiments after *DpnI* digestion (not shown). To convert the ‘marginally replicating’ OVEC plasmids into ‘continuously replicating’ plasmids, we inserted the 8 kb bovine papilloma virus (BPV) genome that sustains replication in *Xenopus* embryos after the MBT (Schmid et al., 1990).

**Maintenance of methylation status in *Xenopus* embryos**

To investigate the role in CpG demethylation of Sp1 binding sites (GC boxes) in *Xenopus* embryos without adding exogenous activators, we first used the marginally replicating OVEC plasmid that carries no eukaryotic origin of replication. Methylated plasmids, Sp-OVEC or Tq-OVEC, were injected into fertilized eggs and the DNA was recovered from stage 12 embryos. Southern analysis of the recovered DNA shows that the 4.1 kb *HindIII* fragment remains resistant to *HpaII*, indicating that all *HpaII* sites remain methylated (Figure 2A). The persistence of methylation in spite of ongoing replication indicates that maintenance methylases are present in the cleaving embryo. However, mock-methylated plasmids yield only the 0.72 kb band, indicating that such plasmids remain unmethylated irrespective of the presence of intact Sp1 binding sites and that no de novo methylation occurs. Stability of the methylation status of injected plasmids has been observed previously (Bendig and Williams, 1983). The *MspI*-resistant 1.7 kb band observed with only methylated Sp-OVEC is due to an idiosyncrasy of *MspI*, which cuts *HpaII* sites in CCGG only with difficulty (Busslinger et al., 1983; Keshet and Cedar, 1983) and is not informative.

To test the ability of different transcription factors to
induce demethylation, we replaced the two GC boxes in Sp-OVEC with two Gal4 binding sites (Figure 1A). The resultant plasmid GAL4-OVEC was methylated and injected into fertilized eggs, together with plasmids encoding Gal4 fusion activators. The coinjection of activator plasmids allowed us to use transcription factors that are not naturally present in Xenopus embryos, i.e. Gal4 fusion proteins with various transactivation domains (Seipel et al., 1992). As expected, HpaII methylation next to, but outside, the Gal4 binding motif (Figure 1A) does not affect binding by Gal4 fusion proteins in a bandshift assay (data not shown). Figure 2B shows the outcome of the injection experiment. Even though some of the fusion activators such as Gal-VP16 (acidic transactivation domain derived from Herpes simplex virus) and Gal-ITF2 (Ser–Thr rich) drive strong transcription (not shown), HpaII cleavage was not detected. In agreement with the data obtained with Sp1, a plasmid DNA replicating only during cleavages (up to stage 8) does not become demethylated following injection. This experiment indicates that active transcription per se does not cause promoter demethylation of marginally replicating plasmids which cease replicating at the MBT.

**Binding of transcription factors induces demethylation of continuously replicating plasmids**

To see whether replication beyond the MBT could enhance demethylation, we carried out similar experiments with ‘continuously replicating’ target plasmids carrying the BPV fragment. In contrast to the marginally replicating OVEC plasmid, a fraction of such Sp-OVEC–BPV plasmids became demethylated at CpGs in both strands (Figure 3A). It is important to note the selective appearance of the 0.82 kb band, indicating that demethylation occurs preferentially in at least one of the two HpaII sites in the promoter and only rarely at the site located 0.72 kb further upstream (Figure 1A). Formation of the 1.7 kb band, indicative of upstream and downstream demethylation, was not observed. As no exogenous Sp1 was added, endogenous proteins which can bind the GC-box must be responsible for inducing this weak demethylation of the promoter-linked sites. In a control experiment, Tq-OVEC–BPV containing mutated GC boxes without Sp1 binding capacity did not show comparable demethylation (Figure 3A).

The continuously replicating plasmid GAL4-OVEC–BPV is demethylated to various degrees when coinjected with plasmids coding for Gal4 fusion proteins (Figure 3B). Gal-VP16 and Gal-AP2, harboring acidic and Pro-rich domains respectively, show a higher level of demethylating activity than the two Gal4–Sp1 fusion proteins, Gal–Sp1ΔZf (Sp1 lacking the zinc-fingers) or Gal–SpQ (N-terminal Gln-rich domain of Sp1). The extent of demethylation induced by the different Gal4 fusion activators was compared with their capacity to induce transcription from the GAL4-OVEC promoter. On the one hand, the amount of RNA transcribed from the target plasmids, as assessed by S1 mapping analysis (Figure 3C), correlated with the extent of demethylation induced in the target promoter (correlation coefficient, r = 0.951). On the other hand, 82–95% of the GAL4-OVEC–BPV recovered in each sample had become resistant to DpnI digestion (not shown), indicating that the test plasmid replicated to a similar extent.

In Xenopus embryos, endogenous genes (Newport and Kirschner, 1982) as well as injected plasmids (Harland and Laskey, 1980; Bendig and Williams, 1983; Etkin and Balcells, 1985) are substantially transcribed only after the MBT. In the above experiment, the activators were produced from cojected expression vectors indicating that the induced demethylation necessarily occurred after the MBT. After this transition, only the BPV plasmids continue replicating. Since only the BPV plasmids are demethylated, replication appears to be another prerequisite for efficient demethylation in this system.

**Increased amounts of transcription factor enhance demethylation**

In order to increase the efficiency of demethylation, we coinjected bacterially produced purified transcription factors with the methylated target plasmids. As can be seen in the Southern blot analyses of DNA recovered...
from such embryos, increased amounts of the activator protein Gal-VP16 induce more extensive promoter demethylation of the target plasmid GAL4-OVEC–BPV (Figure 4A). On average we obtained 20% demethylation with the low dose of Gal-VP16 (0.1 ng), and 68% demethylation with the high dose (1 ng) (Table I, see below). The observation that the 0.82 kb band is formed almost exclusively, even at the highest levels of demethylation, allows us to rule out the possibility that promoter-specific demethylation is merely the result of a stochastic effect whereby the two \textit{Hpa}II sites in the promoter would be twice as likely to become demethylated than the single one 0.72 kb further upstream.

Since toxic effects caused by high concentrations of injected protein led to heavy losses in some embryonic batches, we used the low protein dose (0.1 ng) in most of the following large injection series. In contrast to Gal-VP16, the Gal4 DNA binding domain alone, Gal(1-93) does not promote detectable demethylation at the low protein dose, confirming that the transcriptional activation domain is involved in this process (Figure 4B). Furthermore, Gal-VP16 failed to induce demethylation of a plasmid (Tq-OVEC–BPV) that does not contain Gal4 binding sites, indicating that DNA binding of the activator is crucial for demethylation (Figure 4B). A further control, in which the GAL4-OVEC plasmid and a BPV-containing plasmid were injected together with Gal-VP16 protein showed no demethylation (Figure 4B, Mix). This clearly excludes the possibility that the BPV genome in \textit{trans} can promote demethylation of the GAL4-OVEC plasmid.

**Transcription is not a prerequisite for promoter demethylation**

To test whether ongoing transcription is essential for demethylation, \textit{α}amanitin was coinjected with the \textit{Hpa}II-methylated plasmid GAL4-OVEC–BPV and the Gal-VP16 protein. Transcription from the test plasmid was undetectable in the presence of \textit{α}amanitin, indicating that RNA polymerase II activity was effectively inhibited (not shown). Moreover, development was slowed after the MBT when the endogenous transcription machinery is activated (Newport and Kirschner, 1982), indicating a global shut down of transcription.

Strikingly, demethylation is strongly induced by co-injected Gal-VP16 protein irrespective of \textit{α}amanitin treatment (Figure 4C). Mean demethylation (triplicates) of GAL4-OVEC–BPV induced by Gal-VP16 protein was 72% in the absence, and 77% in the presence of \textit{α}amanitin. Again, Gal(1-93), the Gal4 DNA binding domain alone, is much less efficient in demethylating GAL4-OVEC–BPV. Only at the higher injected protein dose (1 ng), does limited demethylation induced by Gal(1-93) become detectable. Although demethylation is dependent on the binding of transcriptional activators (Figure 4), and even varies with the type of transactivation domain (Figure 3B), it also occurs in the presence of \textit{α}amanitin and thus ongoing transcription is not required.

**Replication is a prerequisite for efficient promoter demethylation**

We have observed that demethylation takes place only on BPV-containing plasmids that continue replicating after the MBT (Figures 3 and 4) suggesting that demethylation occurs after this stage. To verify this, we measured demethylation of GAL4-OVEC–BPV DNA in the presence of coinjected Gal-VP16 or Gal(1-93) proteins, recovering DNA from different embryonic stages before and after the MBT.

Demethylation is hardly detectable before the MBT (which occurs during stage 8), but quickly reaches a high level after the MBT (stage 10) and thereafter increases only slightly (Figure 5A). The onset of demethylation at the MBT is also demonstrated in a separate, similar experiment (Figure 5B) where demethylation is again undetectable before the MBT, at stage 7, whereas it is induced selectively by Gal-VP16 after the MBT, at stage 12. Injection of Gal(1-93), or the protein injection buffer alone, which contains \textit{β}-mercaptoethanol, does not increase demethylation over background (Figure 5B). Selective promoter demethylation by Gal-VP16 activator after the MBT was reproducibly observed in independent experiments using different DNA–protein mixes and different batches of embryos. Table I summarizes the extent of demethylation obtained under the various experimental conditions.
To assess the role of replication in demethylation more closely, we tried to inhibit DNA synthesis by incubating the embryos from stage 4 (16 cell stage) onwards with aphidicholin that acts on DNA polymerase α-primase which is involved in BPV replication (Park et al., 1994). Although the increase in embryonic DNA during cleavages was efficiently blocked by 90 μM aphidicholin, as reported previously (Rollins and Andrews, 1991), replication of BPV plasmids was only partially inhibited, and no reduction in demethylation was observed. A higher concentration of aphidicholin (350 μM) seemingly inhibited replication of the BPV plasmids which now were lost by DNA turnover and thus, their methylation status could not be monitored (not shown).

Instead of attempting to inhibit replication artificially, we decided to inject the same mixtures of HpaII-methylated GAL4-OVEC–BPV and activator proteins as used for embryo injection, into *Xenopus* oocyte nuclei. *Xenopus* oocytes are used widely as a transcription system because of their huge capacity to transcribe injected DNA. They contain an efficient inhibitor of DNA replication (Zhao et al., 1987). Sp1 family members contain Gln-rich domains that can activate transcription when fused to the Gal4 DNA binding domain (Gerber et al., 1994). The Gln-rich polypeptides can also activate transcription when fused to the Gal4 DNA binding domain (Gerber et al., 1994). The Gln-rich proteins, can bind to their recognition sites even when they are methylated (Höller et al., 1988; Silke et al., 1995; Radtke et al., 1996), and that Sp1 binding sites are often associated with CpG island promoters, which are usually unmethylated (Bird, 1986; Gardiner-Garden and Frommer, 1987). This hypothesis was consistent with the observation that Sp1, and a few other zinc finger proteins, can bind to their recognition sites even when they are methylated (Höller et al., 1988; Silke et al., 1995; Radtke et al., 1996), and that Sp1 binding sites are often associated with CpG island promoters, which are usually unmethylated (Bird, 1986; Gardiner-Garden and Frommer, 1987).

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**Discussion**

By analyzing plasmid DNAs microinjected into *Xenopus* embryos and oocytes, we have shown that:

(i) the binding site for the transcription factor Sp1 can induce demethylation as in mammals,

(ii) various types of activation domain can enhance demethylation,

(iii) methylated sites close to the protein-binding site are selectively demethylated,

(iv) ongoing transcription is dispensable for the induction of demethylation,

(v) demethylation only occurs after the midblastula transition (MBT),

(vi) concomitant replication is a prerequisite for efficient demethylation.

The observation that the binding site for the transcription factor Sp1 was involved in the demethylation of DNA (Brandeis et al., 1994; Macleod et al., 1994; Silke et al., 1995) suggested that Sp1 or its family members such as Sp3, which share DNA binding specificity (Kingsley and Winoto, 1992; Hagen et al., 1994), belonged to a putative group of demethylation-inductive transcription factors (see also Martin et al., 1997). This hypothesis was consistent with the observation that Sp1, and a few other zinc finger proteins, can bind to their recognition sites even when they are methylated (Höller et al., 1988; Silke et al., 1995; Radtke et al., 1996), and that Sp1 binding sites are often associated with CpG island promoters, which are usually unmethylated (Bird, 1986; Gardiner-Garden and Frommer, 1987). Sp1 family members contain Gln-rich domains which can activate transcription, and pure Gln polypeptides can also activate transcription when fused to the Gal4 DNA binding domain (Gerber et al., 1994). The Gln-rich

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**Table I. Extent of demethylation (%)**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Target DNA</th>
<th>Activator protein</th>
<th>Gal(1-93)</th>
<th>Gal-VP16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>none</td>
<td>0.1 ng</td>
<td>1 ng</td>
</tr>
<tr>
<td>Oocyte</td>
<td>GAL4-OVEC–BPV</td>
<td>0.5</td>
<td>1</td>
<td>nd</td>
</tr>
<tr>
<td>Embryo&lt;MBT</td>
<td>GAL4-OVEC–BPV</td>
<td>5</td>
<td>8</td>
<td>nd</td>
</tr>
<tr>
<td>Embryo&gt;MBT</td>
<td>GAL4-OVEC</td>
<td>2</td>
<td>3</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>Tq-OVEC–BPV</td>
<td>8</td>
<td>7</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>GAL4-OVEC–BPV</td>
<td>8 ± 1.2</td>
<td>7</td>
<td>10 ± 3</td>
</tr>
</tbody>
</table>

(n = 3) (n = 3) (n = 9) (n = 8)

nd, not done; *<MBT*, stage 7 before the midblastula transition; >MBT, stage 12 after the MBT; b standard deviation; c n is the number of independent experiments, carried out with different injection mixtures on different batches of embryos. In a Student’s t-test, the differences between the series 1 ng Gal(1-93) and 0.1 ng Gal-VP16 (t = −2.97; p = >0.02) and between the 0.1 ng and 1 ng doses of Gal-VP16 (t = −12; p <0.0001) are significant.

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**Fig. 5. DNA replication after the MBT is a prerequisite for efficient demethylation.**

(A) Southern blot analysis of HpaII-methylated GAL4-OVEC–BPV coinjected with 0.1 ng Gal-VP16 transactivator protein. DNA was recovered at the embryonic stages indicated. Note that the MBT occurs around stage 8. (B) Southern blot analysis of HpaII-methylated GAL4-OVEC–BPV injected into fertilized eggs alone (−) or together with buffer containing β-mercaptoethanol (βM), 0.1 ng of Gal-VP16, or Gal(1-93), and recovered from embryos at stage 7 before the midblastula transition (<MBT), or at stage 12 (>MBT). Oocytes: similar experiment injecting the same DNA–protein mixtures into oocyte nuclei and recovering DNA after 20 h. H and M, as in Figure 2.
domains of Sp1 have been shown to interact with other Sp1 molecules (Mastrangelo et al., 1991; Su et al., 1991) and with a TBP-associated factor, TAFII110 (Hoey et al., 1993), and protein–protein interaction of Gln-rich repeats might be mediated by a ‘polar zipper’ structure (Stott et al., 1995). Thus, a specific interaction between Gln-rich domains of Sp1 and putative ‘demethylase’ might be anticipated. However, our data clearly show that different types of transcription activation domains, most notably an acidic activation domain, can promote efficient demethylation. This is in agreement with the finding that the NF-xB–Rel family of transcription factors, which lack characteristic Gln-rich activation domains, can also induce demethylation (Kirillov et al., 1996).

How then do transcription factors trigger demethylation? In our experiments different classes of activation-domain-induced demethylation and demethylation took place only after the MBT. Although we do not know which of the changes occurring during the MBT creates conditions permissive for demethylation, it is conceivable that a general component(s) of the preinitiation complex, recruited by the transactivation domain of the activator, is needed for efficient demethylation. Likewise, TBP has been shown to become available at the MBT (Prioleau et al., 1995). Thus, a specific interaction between Gln-rich domains of Sp1 and putative ‘demethylase’ might be anticipated. However, our data clearly show that different types of transcription activation domains, most notably an acidic activation domain, can promote efficient demethylation. This is in agreement with the finding that the NF-xB–Rel family of transcription factors, which lack characteristic Gln-rich activation domains, can also induce demethylation (Kirillov et al., 1996).

Conclusively, as summarized in Figure 6A, our results demonstrate that three conditions are required for promoter-specific demethylation in the Xenopus embryo: binding of a functional transactivator, availability of a general transcription factor(s), and DNA replication. Accordingly, demethylation could be carried out by a ‘passive’ mechanism, whereby the binding of a site-specific transcription factor and association of general transcription factor(s) would prevent maintenance methylation from acting on post-replicative, hemimethylated DNA. After a second round of replication, fully unmethylated DNA would be generated (Figure 6B). Such a model resembles the ‘hypomethylated footprint’ hypothesis regarding chromosomal rDNA spacer demethylation in the Xenopus embryo (LaVolpe et al., 1983) but seemingly contrasts with accumulating observations indicating the existence of enzyme, i.e. active, demethylation processes (Paroush et al., 1990; Jost, 1993; Kafri et al., 1993; Jost et al., 1995; Weiss et al., 1996).

We favor the view that fully ‘passive’ demethylation (i.e. postreplicative impediment of maintenance methylase by transcription factors) occurs during Xenopus embryogenesis. However, our observations do not exclude a ‘replication-dependent’ enzymatic demethylation. Yet, we have to point out that most, if not all, published reports on enzymatic demethylation are essentially based on detection of demethylation on one strand of DNA. If on either symmetrically methylated or hemimethylated DNA, but not both, were the preferred substrate for the demethylation enzyme, replication would still be required. Active and passive demethylation might thus be coupled. However, the observation that virtually no demethylation takes place on non-replicating DNA indicates that, in the


**A CONDITIONS INDUCING DEMETHYLATION**

<table>
<thead>
<tr>
<th>TAD</th>
<th>GENERAL TF</th>
<th>REPLICATION</th>
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<tr>
<td>OVO</td>
<td>OVO</td>
<td>MBT</td>
</tr>
<tr>
<td>OVE</td>
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<td>MBT</td>
</tr>
<tr>
<td>OVE/BPV</td>
<td>OVE/BPV</td>
<td>MBT</td>
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**B DEMETHYLATION MECHANISMS**

**PASSIVE DEMETHYLATION**

**ACTIVE DEMETHYLATION**

Fig. 6. Embryonic demethylation mechanism. (A) Summary of observations. Lack (−) or occurrence (+) of demethylation of injected target plasmids (OVEC, OVEC–BPV) in the presence of a Gal4 binding domain only (−TAD) or a functional Gal4 hybrid factor with active transactivation domain (+TAD), in the three different life spans of the host: oocyte, cleaving or mitotically dividing embryonic cells. F, fertilization; nd, not done. (B) Passive and active demethylation mechanisms. A passive mechanism would be sufficient to produce fully demethylated DNA; but combinatorial action of active demethylases may occur.

Xenopus embryo, the enzymatic activities alone are not capable of producing substantial demethylation in the absence of replication. Of course it remains possible that different cell types carry out demethylation by different mechanisms.

Exploiting the sustained genomic replication, the ‘on switch’ of the general transcription machinery and the appearance of specific transcription factors would represent a parsimonious mechanism to carry out massive promoter-specific demethylation during vertebrate embryogenesis.

**Materials and methods**

**Construction and methylation of plasmids**

Target plasmids were constructed by inserting SacI-SalI oligonucleotides (Figure 1) into the plasmid OVEC (Westin et al., 1987). To construct replication competent plasmids, the 8 kb BPV genome from pCGBP9V (Matthias et al., 1983) was inserted into a HindIII site of OVEC. The activator plasmid Gal–Sp1Δzf was constructed similarly to other fusions from pPacSp1 (Couray and Tjian, 1988), by deleting the zinc fingers between BamHI and HaelII. Other activator plasmids have been described previously (Seipel et al., 1992). Target plasmids were methylated with HpaII methylase (New England). Mock methylation was performed by excluding the enzyme. All work with recombinant DNA was carried out according to NIH guidelines and the SKBS–CSSB regulations.

**DNA, protein and α-amanitin injection into fertilized eggs and oocytes**

Manipulation of animals and embryos was in agreement with the Swiss laws on animal experimentation and registered with the Geneva Cantonal Veterinary Office. Target DNA (200 pg) alone, or a mixture of activator plasmids (100 pg) and target DNA (100 pg), in 10 nl of injection buffer was injected into fertilized eggs as described previously (Nichols et al., 1995 and references therein). Alternatively, either 100 pg or 1 ng of bacterially produced activator protein was co-injected with 200 pg of target DNA. Where indicated, α-amanitin (Sigma) was co-injected with the DNA in a 100× concentration (10 nl injection/1 µg egg volume) of the desired final concentration (10 µg/ml). This concentration blocks polymerases II and III. In some experiments, aphidicholin (Boehringer Mannheim), was added to the culture medium to a final concentration of 90 or 350 µM. Embryos were staged according to Nieuwkoop and Faber (1994). Injection into oocyte nuclei with the same DNA–protein mixtures as used in embryos was done as described in detail by Bertrand et al. (1991). Oocytes were incubated for 20 h at 20°C.

**Southern blot analysis**

Total nucleic acids were extracted from 10 host embryos pooled together. A DNA amount equivalent to two embryos was separated per lane in an agarose gel and transferred to Hybond-N (Amersham) with 0.4 N NaOH. In the oocyte experiments, total DNA extracted from 40 oocytes was processed per lane.

**S1 mapping analysis**

An RNA amount equivalent to two embryos was used. S1 mapping was performed using an oligonucleotide probe as described previously (Westin et al., 1987; Xu et al., 1994).

**Quantification of radioactivity**

The intensity of bands from Southern blots or S1 mapping gel was measured by Phosphoimager (Molecular Dynamics). Demethylation = 0.82 kb band intensity/(Intensity of 0.72 kb + 0.82 kb + 4.1 kb bands).

**Acknowledgements**

We thank Walter Schaffner in whose laboratory part of this work was carried out and who initiated this study, Kristina Rother and Max L. Birnstiel for helpful discussions, Lisbeth Muster and André Solaro for technical support, and Fritz Ochsenbein and Slim Chraiti for artwork. This work was supported by the Kanton of Zürich, the Société Académique de Genève, the Sandoz Foundation (Basel), the Georges and Antoine Claraz Foundation (Zürich) and the Swiss National Science Foundation, grants 31-45930.95 and 33-46946.96.

**References**


Demethylation in *Xenopus* embryos


