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DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei

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Nuclear transplantation experiments in amphibia and mammals have shown that oocyte and egg cytoplasm can extensively reprogram somatic cell nuclei with new patterns of gene expression and new pathways of cell differentiation[1–3]; however, very little is known about the molecular mechanism of nuclear reprogramming. Here we have used nuclear and DNA transfer from mammalian somatic cells to analyse the mechanism of activation of the stem cell marker gene *oct4* by *Xenopus* oocytes. We find that the removal of nuclear protein accelerates the rate of reprogramming, but even more important is the demethylation of somatic cell DNA. DNA demethylation seems to precede gene reprogramming, and is absolutely necessary for *oct4* transcription. Reprogramming by oocytes occurs in the absence of DNA replication and RNA/protein synthesis. It is also selective, operating only on the promoter, but not enhancers, of *oct4*; both a putative Sp1/Sp3 and a GGGAGGG binding site are required for demethylation and transcription. We conclude that the demethylation of promoter DNA may be a necessary step in the epigenetic reprogramming of somatic cell nuclei.

During cell differentiation, the activated and repressed states of gene expression are remarkably stable, and are not normally reversed or redirected. Such changes do, however, take place reproducibly under conditions of nuclear transfer to eggs[1–3]. Nuclei transplanted to eggs (in the second meiotic metaphase) are first induced to initiate chromosome replication, and transcription starts only later. However, somatic cell nuclei injected into growing amphibian oocytes (in the first meiotic prophase) undergo profound changes in gene expression that occur in the absence of DNA replication or cell division[4,5]. In oocytes there is a direct switch of gene expression on the same DNA template as the one that transcribes other genes in a somatic cell. Here, we ask if DNA demethylation is part of the process by which somatic cell nuclei are reprogrammed. Under normal conditions, DNA methylation is epigenetically stable and is reversed only during gametogenesis, in early embryo cells[6,7] and in other special circumstances. Previous work has shown a correlation between incomplete DNA demethylation and the lack of nuclear transfer success in mammals[8–10], but no evidence for DNA demethylation has been found in experiments with *Xenopus* oocytes[11–13]. We have re-investigated the ability of *Xenopus* oocytes to demethylate DNA by examining the promoter of murine *oct4* — a gene that is normally expressed only during gametogenesis, in early embryos and in embryonic stem cells[14,15], but is activated in mouse thymus cells injected into *Xenopus* oocytes[5].

We first asked whether the methylated state of somatic cell DNA restrains the ability of nuclei to be reprogrammed. There was a considerable delay (up to 4 days) before *oct4* transcription from mouse thymus nuclei was seen in *Xenopus* oocytes[5]. If mouse thymus nuclei were deproteinized, and if the same amount of genomic DNA (in its natural methylated state) as that of whole nuclei was injected into oocytes, we saw a considerable acceleration of new transcription, *oct4* transcripts being evident within 2 days (Fig. 1). However, when unmethylated DNA (as grown in a bacterial plasmid) was injected, we observed very rapid transcription with no detectable delay (Fig. 1). This indicates that even when all repressive proteins have been removed from nuclei, there is nevertheless a substantial delay before transcription starts. This suggests that DNA may have to be demethylated or modified in some other way for transcription to ensue.

Direct evidence for DNA demethylation comes from examination of the *oct4* gene promoter[16,17]. Several methylated CpG sites have been described in the regulatory region of *oct4* in mouse tail tissue[17]. The methylated state of four of these sites can be determined by the use of methyl-sensitive enzymes (Fig. 2a). With suitable primers, a product is seen if the site is methylated but not if an enzyme cuts at this site when it is unmethylated. Four *Hpa*II and *Hha*I sites in the *oct4* regulatory region were fully methylated in genomic DNA isolated from adult mouse thymus cells (Fig. 2b). When 50–100 mouse thymus nuclei were injected into oocytes as described[5], demethylation of two *Hpa*II and one *Hha*I sites was observed within the *oct4* promoter. This demethylation was evident one day after nuclear injection (Fig. 2c), although it continued for the next few days. Demethylation of the promoter seemed to precede *oct4* transcription in oocytes, as transcription was seen only between 3 and 4 days after injection (Fig. 1). The *Hpa*II site at –1148 in the proximal enhancer of *oct4* was not demethylated, illustrating the selective action of demethylation by oocytes. The demethylating

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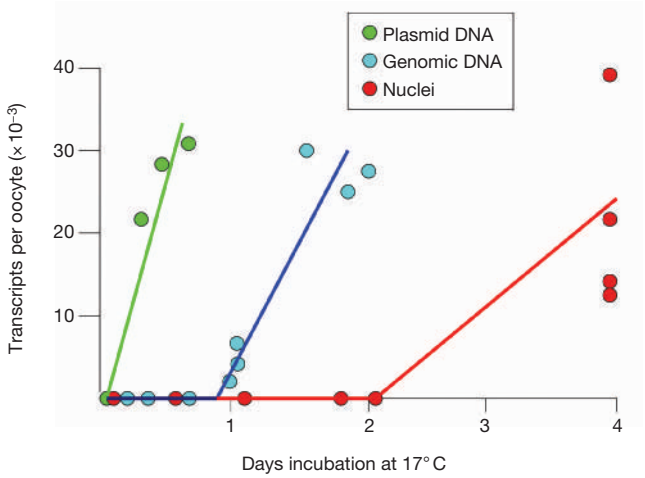

Figure 1 Deproteinization of nuclei and unmethylated DNA accelerate the time when *oct4* transcription starts. Each point represents the analysis of a group of four oocytes injected and then incubated for the number of days indicated. Each oocyte was injected with 50–100 mouse thymus nuclei, with 1.5 ng of mouse thymus genomic DNA, or with 0.03 pg of double-stranded supercoiled *oct4* 476-plasmid DNA (unmethylated). The plasmid DNA, but not the genomic DNA or whole nuclei, was supplemented with 1.5 ng of *Xenopus* genomic DNA per oocyte, as carrier DNA containing no *oct4* genes. The values in the graph have been corrected to show the response elicited by 200 copies of the *oct4* gene, whether injected as nuclei, genomic DNA or plasmid DNA. The numbers of transcripts recovered from oocytes injected with mouse thymus nuclei were determined as described in the Methods section and were similar to those previously reported by us[5]. The results shown were obtained when all three types of samples were injected separately into oocytes of the same female. Although the absolute timing of *oct4* transcription differs with oocytes of different females, the relative timing of transcription of the three preparations used here is similar for the oocytes of the same female. Altogether, 212 oocytes from eight different females were analysed in experiments of the kind shown here.

activity of oocytes was also effective on deproteinized genomic DNA of thymus nuclei (Fig. 2d).

Our evidence for demethylation of DNA is dependent on the use of methyl-sensitive restriction enzymes coupled with PCR amplification. We believe that our assays are quantitative as our results are based on only the linear part of the amplification range (see Methods section). However, we have also assessed our results using bisulphite sequencing (Fig. 2e). This confirms our enzyme-based results, and also shows that demethylation affects many CpGs in the promoter region of *oct4*. Therefore, we conclude that *Xenopus* oocytes have a strong DNA demethylating activity for the promoter region of *oct4*.

To simplify further analysis of the demethylating activity of oocytes, we used plasmid DNA constructs containing different parts of the *oct4* regulatory region to allow methylation of desired sites *in vitro* and to test the effect of these on demethylation and transcription using DNA injection into oocytes[18,19]. The reporter sequences of chloramphenol acetyl transferase (CAT) and/or enhanced green fluorescent protein (EGFP) were included in the plasmid constructs to facilitate transcription assays (Fig. 3a). The plasmid vector BL.CAT.3T was used, which was designed as a no-read-through vector[20,21]. For the purposes of transcription in oocytes, the minimal promoter extends from –52 to –126 (Fig. 3a; also see Methods section for nucleotide numbering). As a negative control, a similar plasmid construct in which the mouse *oct4* sequence is replaced by the regulatory sequence of *Xenopus* cardiac actin was used[22,23]. No transcripts were detected from this muscle-actin-containing plasmid (Fig. 3b). Therefore this plasmid behaves as a no-read-through construct appropriate for transcription assays in DNA-injected oocytes.

To distinguish between global and site-specific demethylation, the 2.7-kilobase (kb) construct (Fig. 3a) was *in vitro* methylated with the bacterial *Sss*I methyltransferase, and the loss of ^{3}H-methyl-labelled DNA recovered from injected oocytes was analysed. Just as no loss of ^{32}P-genomic DNA was observed, there was also no significant loss of ^{3}H-counts per min (cpm) observed (Fig. 3c). This conclusion is in agreement with the results of others using genes not normally transcribed in oocytes[11–13]. Next, we tested demethylation with less than 200 pg of injected DNA. This is about the same amount of DNA as injecting 100 somatic cell nuclei. When 190 or 45 pg of DNA were injected, clear evidence of demethylation was observed: about one third of the ^{3}H-methyl groups were removed (Fig. 3c). The loss of ^{3}H-cpm in the low-dose samples of Fig. 3c were recovered in the phenol phases of the DNA extraction solutions. We conclude that *Xenopus* oocytes have a demethylating activity that is of limited capacity and is able to demethylate only specific methylated sites in a genome.

We next asked whether the *oct4* promoter in a plasmid shows the same demethylation responses as whole thymus nuclei and thymus genomic DNA. We therefore methylated *in vitro* the 2.7-kb and 476-plasmid constructs using *Hpa*II and *Hha*I, as before. Analysis of the results by bisulphite sequencing (Fig. 3d) showed efficient demethylation at –289 (*Hpa*II) and at –166 (*Hha*I), but not in the 2.7-kb plasmid construct at –1148 (or at another *Hpa*II site at –754). This result is in agreement with what we found using whole nuclei or genomic DNA, and validates the use of the *oct4* promoter plasmid for further analysis. It also shows that the demethylating activity of the oocyte can operate locally and independently of the rest of the mouse genome.

We asked at this point how directly the demethylating activity of the oocyte can function. It is widely accepted that the *Xenopus* meiotic prophase oocyte is inactive in DNA synthesis. To confirm that *oct4* demethylation functions in the absence of DNA synthesis, oocytes were injected with 476-plasmid DNA supplemented with aphidicolin, a DNA synthesis inhibitor. Similarly, we tested demethylation in the presence of α-amanitin to suppress RNA synthesis or with cycloheximide to inhibit protein synthesis. In each case, demethylation took place as well as it did without these inhibitors (Fig. 3e). We can conclude that the demethylating activity of the oocyte functions directly by means of nucleic acid and protein molecules already present in the uninjected oocyte.

The successful demethylation of the *oct4* promoter in plasmid DNA permits a further analysis of this demethylating activity by mutating parts of the *oct4* promoter. Figure 4a highlights, within this promoter, a number of sequences that are known to be important for transcription in other systems, and to which known proteins can bind. Each of the mutations made — namely, the A1-like site (–75 to –70), the Sp1/Sp3 site (–113 to –109) and the *Hpa*II site (–290 to –287) — inhibited demethylation of the CpGs at –166, –289 and, as expected, they also inhibited transcription (Fig. 4b). Because the transcription start is at nucleotide –49, all of the inhibitory mutations lie in the promoter region just 5′ to the transcription start site.

We were fortunate to discover that when the *Hha*I site at –166 alone is methylated, the oocyte is not able to demethylate this site or does so only very slowly (Fig. 4c). We can therefore ask whether this promoter construct that cannot be fully demethylated also disallows transcription. We methylated the 476 promoter construct with only the *Hha*I methylase, thereby providing a promoter with a methylated

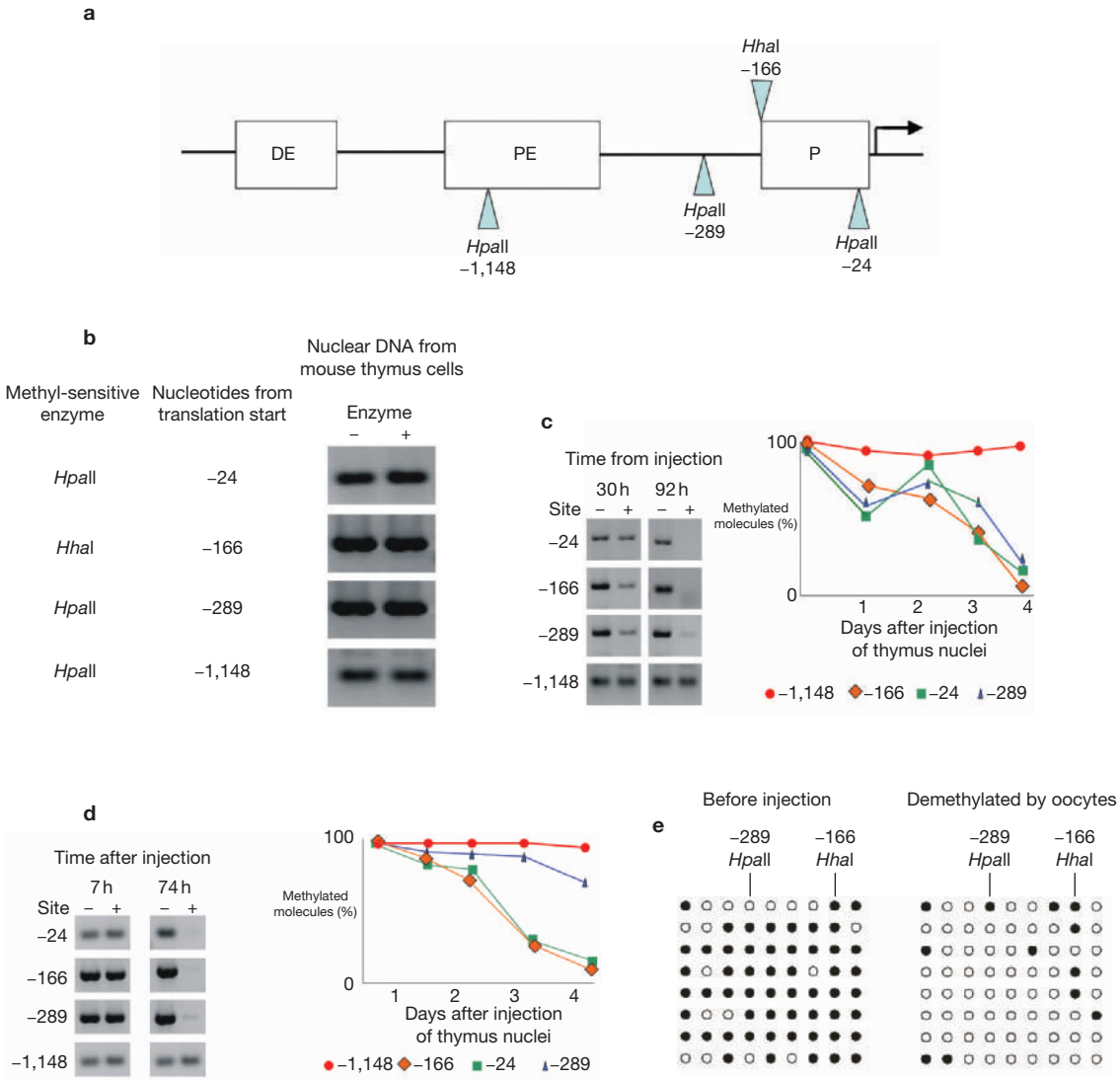

Figure 2 The mouse *oct4* promoter is demethylated by *Xenopus* oocytes. (**a**) Diagram of the regulatory region of mouse *oct4*. The region shown includes 2.3-kb upstream from the translation start site (arrow at nucleotide +1), and contains a distal enhancer (DE), a proximal enhancer (PE) and the promoter (P)[16,17]. Four methyl-sensitive restriction sites are shown. (**b**) The regulatory region of *oct4* is methylated at four methyl-sensitive enzyme sites in adult mouse thymus cells (see Methods section for methyl-sensitive enzyme assays). (**c**) The mouse *oct4* promoter is demethylated in *Xenopus* oocytes injected with permeabilized adult mouse thymocytes. The three sites in the promoter region (**a**) are partially demethylated after 30 h, and fully by 4 days. The –1148 site in the proximal enhancer is not demethylated. Each oocyte received 50–100 permeabilized cells. Each analysis is from a sample of four injected oocytes. (**d**) *Oct4* demethylation is seen in oocytes injected with purified genomic DNA from adult mouse thymus cells. These results are similar to those in **c**, except that demethylation is faster (see Fig. 1). Each oocyte was injected with about 1.5 ng of DNA. (**e**) Bisulphite analysis of *oct4* demethylation (see Methods section). Nearly all CpGs in the *oct4* promoter are methylated in adult mouse thymus cells, and have been demethylated in oocytes injected with genomic DNA as in **d**, and cultured for 3 or 4 days. These results represent the analysis of two groups of four oocytes, with eight clones from each.

site at –166, but no *Hpa*II methylation at –24 or –289 (Fig. 4c). Remarkably, a complete suppression of transcription by the *Hha*I methylated DNA was found, even two days after injection into oocytes (Fig. 4d). We conclude that demethylation, at least of the –166 site in the promoter region of *oct4*, is essential for transcriptional activation. There is therefore a causal connection between demethylation of the *oct4* promoter and transcription.

As the presence of methylated *Hpa*II sites at –24 and –289 enables the oocyte to demethylate the *Hha*I site at –166, its demethylating activity can evidently spread over more than 100 nucleotides. This raises the question of whether the activity can function in a *trans* configuration or only in a *cis* relationship to the –166 site. We made several constructs to test this (Fig. 4e). We found that the two *Hpa*II-methylated sites of –754 and –1148 were unable to cause demethylation of the –166 *Hha*I site in a *trans* configuration, whereas they did so successfully in a *cis* configuration (Fig. 4e). We conclude that demethylation of the *Hha*I CpG at –166 is dependent on the presence of other methylated sites on the same DNA, even if these are several hundred nucleotides away.

DNA methylation is of special importance because of its probable role in maintaining the remarkable stability of cell differentiation and in regulating gene transcription[24,25]. The demethylating

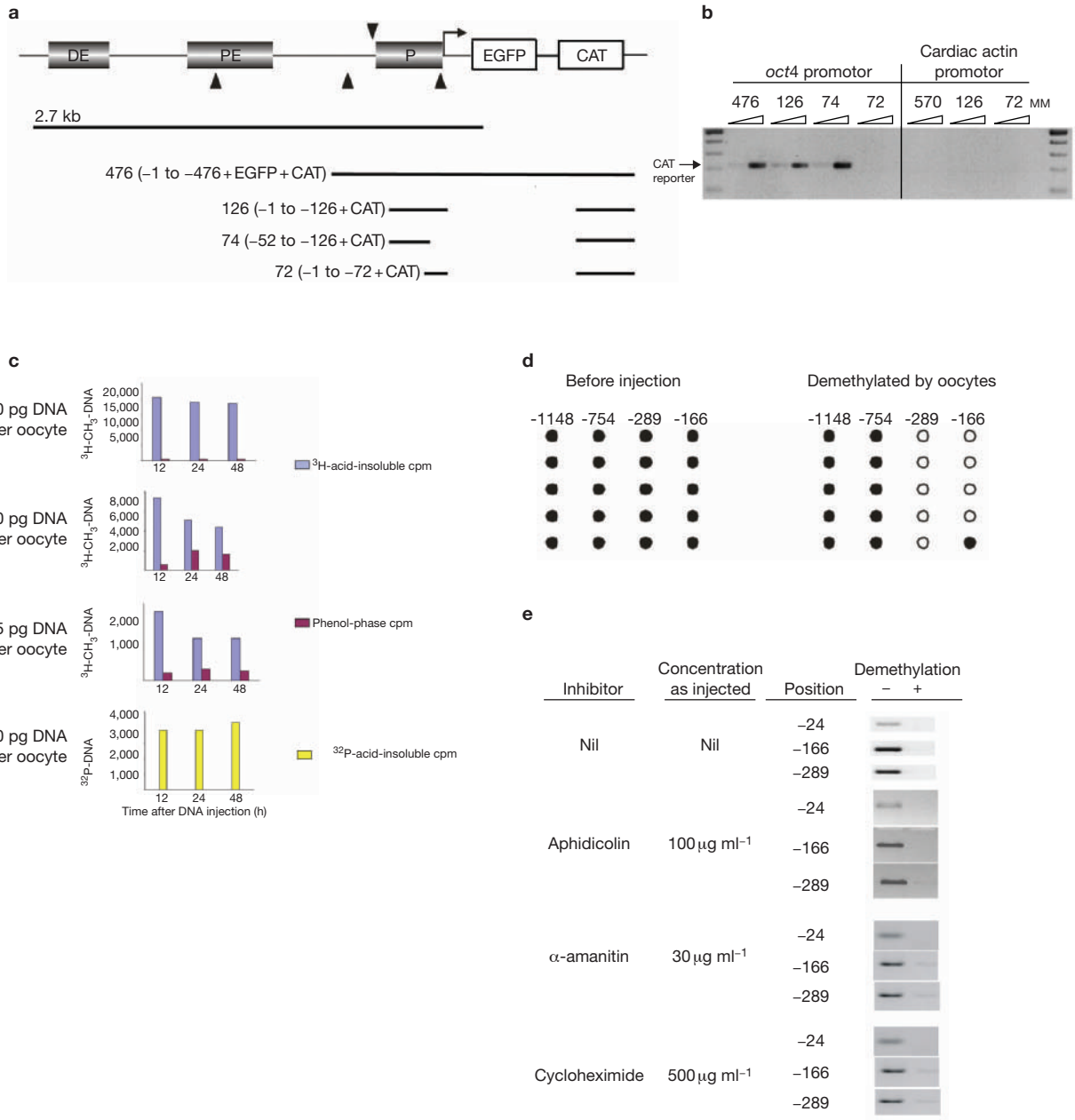

Figure 3 The regulatory region of mouse *oct4* in plasmid DNA can be used to analyse the demethylating activity of *Xenopus* oocytes. (**a**) A diagram of plasmid construction. EGFP and/or CAT have been included as reporter sequences to facilitate transcription assays. The minimal promoter required for transcription in *Xenopus* oocytes extends from nucleotide –52 to –126. The transcription start site is at –49. Transcription results are shown in Fig. 3b. Plasmid constructs are referred to in the text by the number in bold. (**b**) The plasmid BL.CAT3T does not allow read-through transcription from initiation outside the inserted sequence. Transcription from the *oct4* promoter is quantitative from low (7 pg) or high (30 pg) injection amounts. The cardiac actin promoter (also in the plasmid BL.CAT3T), or parts of it, function as negative controls. MM, master mix without DNA sample. (**c**) Demethylation of methylated plasmid DNA. *Oct4*-containing plasmid DNA (2.7-kb) was methylated *in vitro* by *Sss*I DNA methylase; the added methyl groups were labelled with ^{3}H by S-adenosyl-methionine. The aqueous and phenol phases were counted. This series of experiments was performed on the oocytes of the same female. (**d**) Demethylation of the 2.7-kb *oct4* plasmid DNA methylated *in vitro* by *Hpa*II and *Hha*I. Bisulphite sequencing shows that most methyl CpGs in the promoter region, but not in the enhancer region, are demethylated as they are with injected genomic DNA (see Fig. 2e). (**e**) Demethylation occurs in the absence of DNA, RNA or protein synthesis. Each oocyte was injected with 7 pg of 476-methylated plasmid DNA, and cultured for two days. Demethylation was assessed by methyl-sensitive enzyme assays. Inhibitors were mixed with DNA to give the concentrations shown at the time of injection; these concentrations are more than sufficient to suppress DNA, RNA or protein synthesis.

activity described here has several interesting characteristics: it is selective, working on only a limited fraction of the genome; and it operates independently of DNA, RNA and protein synthesis. The significance of this activity in an oocyte may be related to the extensive genomic demethylation that occurs in mammals when germ-cells migrate into the gonad, or perhaps in preparation for the demethylation that occurs at the mid-blastula stage in *Xenopus* embryos[7]. The *Xenopus* genome contains several genes with partial

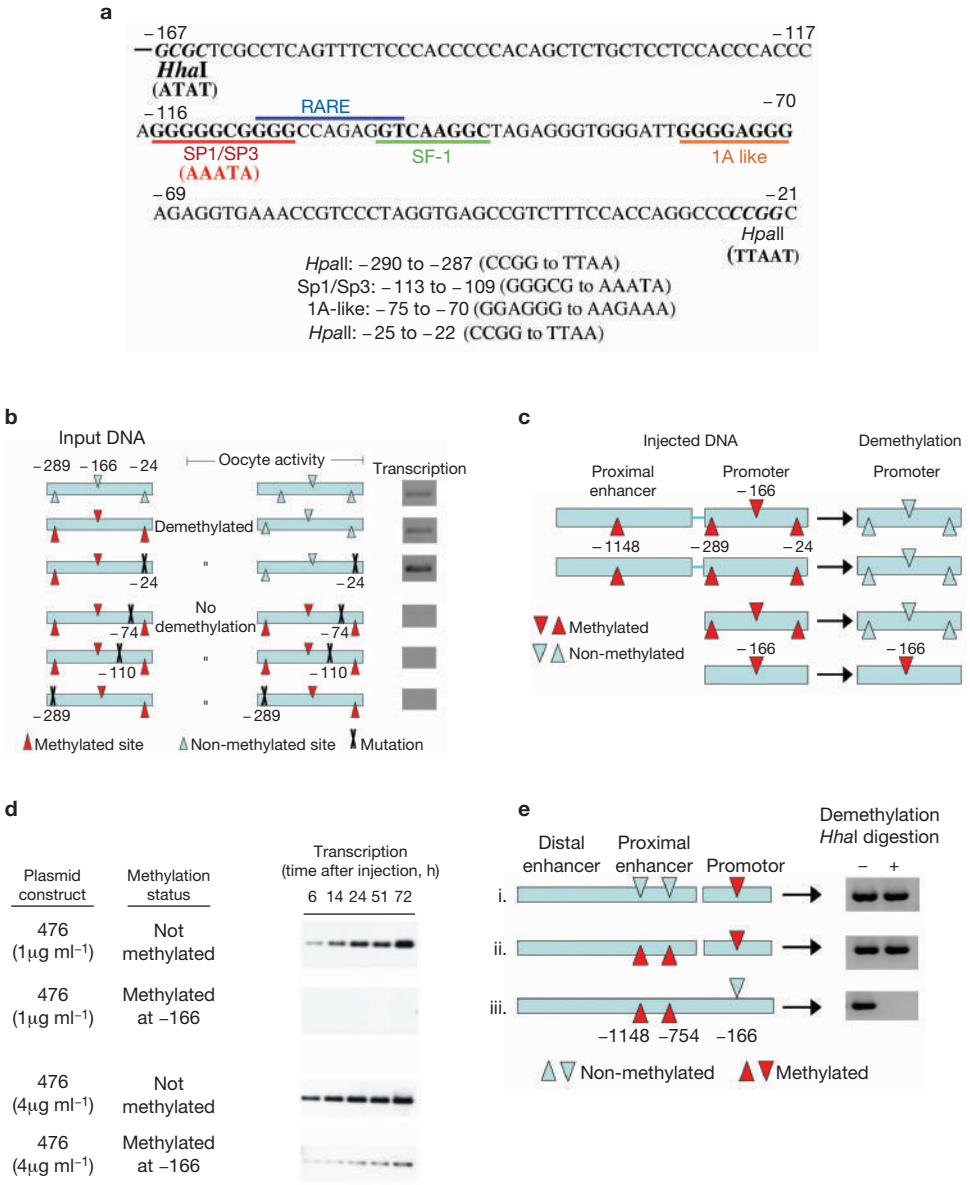

Figure 4 Mutational analysis of *oct4* promoter demethylation. (**a**) The mouse *oct4* promoter sequence, showing regions to which known proteins bind. Using the nucleotide numbering described[16], the transcription start is at –49, and the translation start at +1. The mutations used here (see **b**) are detailed below the promoter sequence. (**b**) The effect of mutations in the mouse *oct4* promoter on demethylation and transcription in oocytes. The blue bars represent the *oct4* promoter from –1 to –300. The triangles represent methyl-sensitive restriction enzyme sites (Fig. 2a), in red when methylated, or in blue when not methylated. Black crosses indicate mutated positions (**a**). DNA is shown as injected on the left (input DNA), and as extracted from injected oocytes after 1 or 2 days on the right (oocyte activity). For methyl-sensitive enzyme and transcription assays, see Methods section. (**c**) Demethylation of the *Hha*I CpG at –166 is dependent on other methylated sites. The design and symbolism is the same as in **b**. Methylation at the positions shown was achieved *in vitro* with *Hpa*II or *Hha*I methylase. (**d**) Promoter methylation at –166 is sufficient to prevent transcription. Oocytes were injected with the 476 construct methylated only at –166 by *Hha*I. Even with four times the normal amount of DNA injected, transcription is minimal. (**e**) Demethylation of the *oct4* promoter at –166 requires methylated CpGs in a *cis* configuration. In i and ii, promoter and enhancer DNAs were physically unconnected and injected as a mixture (each at 1 μg ml^{-1}). In iii, the same sequences were joined together in the same plasmid, and injected at 1 μg ml^{-1}. Demethylation assays were as in previous figures.

sequence-identity to mouse *oct4*, and one, *oct60*, is strongly expressed in *Xenopus* oocytes[26].

Mechanisms of nuclear reprogramming are not well understood. An exchange of proteins between the nucleus and the cytoplasm occurs soon after nuclear transfer[27–29], and a nucleolus-decondensing factor has been described[30]. Whatever the natural significance of the demethylating activity of *Xenopus* oocytes may be, the results described here suggest that the selective demethylation of promoter DNA may be a general mechanism required for the reprogramming of somatic cell nuclei. □

METHODS

Oocytes. Oocytes of mature *Xenopus laevis* females were removed and defollliculated by hand (rather than with collagenase). Oocytes were injected manually using non-centrifuged oocytes, and with a volume of about 7 nl per oocyte, aimed for the germinal vesicle, as described[5]. Injected oocytes were cultured in modified Barth saline at 17 °C. Injected oocytes were frozen before analysis.

Nuclei for injection. Thymus cells were removed from six-week-old mice and a suspension of permeabilized cells prepared as described[5]. Between 50 and 100 nuclei (that is, permeabilized cells) were injected into each oocyte aiming for the germinal vesicle. Donor thymus cells were permeabilized to about 90%, as judged by Trypan blue staining.

DNA. Genomic DNA was prepared by deproteinization of donor nuclei using standard phenol and chloroform methods. DNA was at least 95% deproteinized. Plasmid DNAs were constructed using the vector pBLCAT3T as described[20,22]. In some cases, a construct containing EGFP and CAT sequences was placed after the *oct4* regulatory region. The various promoter constructs tested were created by PCR. A Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to construct the mutated plasmids. All constructs were sequenced. Except where stated otherwise, each oocyte was injected with 1.5 ng of genomic DNA or with 10 pg of plasmid DNA.

Demethylation and transcript assays. For DNA methylation status, standard methods using the methyl-sensitive enzymes *Hpa*II and *Hha*I compared with *Pvu*II were employed as described[17]. DNA was methylated *in vitro* using *Sss*I or *Hpa*II or *Hha*I enzymes. The extent of methylation was judged to be at least 90% effective. RNA was extracted using a kit (Qiagen, Valencia, CA) and analysed by RT–PCR with about 25 cycles for DNA injected oocytes and upto 35 cycles for oocytes injected with nuclei.

Bisulphite modification. DNA bisulphite modification and purification was accomplished using the EZ DNA Methylation Kit (Zymo Research, Orange, CA). Plasmid-2.7 DNA (120 pg) or 5.6 ng of thymus genomic DNA, as extracted from oocytes, was used for the analyses. Bisulphite modified DNA was amplified with primers designed using the program "Meth Primer: designing primers for methylation PCRs: (www.ucsf.edu/urogene/methprimer/index.html) and the forward primer 5′-GGGATTTTAGATTGGGTTTAGAAA-3′ and reverse primer 5′–CCACCCTCTAACCTTAACCTCTAAC-3′. To obtain more products, a second, semi-nested PCR was performed using the forward primer 5′–TGAGGAGTGGTTTTAGAAATAATTG-3′ and in reverse the same as above. The PCR product was then cloned into T vector (Promega, Madison, WI) and 20 colonies of each reaction were purified as minipreps (Qiagen) and screened for correct insertion by restriction enzyme digestion with *EcoR*I, which cuts on both sides of the insert. The individual clones were sequenced. All sequences with more than five cytidines, not followed by a guanidine, and not converted into thymidines, were excluded from the results.

Quantitation of demethylation and transcript levels. DNA was extracted from injected oocytes by treatment with proteinase K at 55 °C overnight and phenol–chloroform. The precipitated DNA was suspended in 100 µl of distilled water. Aliquots of the DNA (1 µl) were taken and cut with *Pvu*II, and then cut with the appropriate methyl-sensitive *Hpa*II or *Hha*I. The digests were then amplified by PCR to the desired cycle number, and then analysed on a 2% agarose gel, which was stained with ethidium bromide. For demethylation assays, the intensity of an ethidium-bromide-stained band was quantified using the BioRad Geldoc quantity program. The results in the figures show a *Pvu*II-only digest on the left with a *Pvu*II and methyl-sensitive enzyme digestion on the right. To ensure that the PCR cycle number used was in the linear range, a dilution series of one of the samples in each experiment was performed.

For quantitative transcript assays, RNA was extracted using the Qiagen RNeasy Mini Kit including DNaseI treatment. The extracted RNA was reverse transcribed by Invitrogen SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). The resulting cDNA was amplified by PCR. To quantify the amount of RNA in the injected oocyte samples, a known amount of plasmid DNA containing *oct4* was run in parallel for the same number of PCR cycles, using the same primers. A dilution series as described above was used to ensure that the PCR cycle number was within the linear range. In the case of Fig. 1, transcript quantitation was performed by adding ^{32}P-dATP to the PCR reactions. The PCR products were resolved on a denaturing sequencing gel and quantified in a phosphoimager as described[5].

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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