Epigenetic Control of Mouse Oct-4 Gene Expression in Embryonic Stem Cells and Trophoblast Stem Cells*

Received for publication, August 14, 2003, and in revised form, December 18, 2003
Published, JBC Papers in Press, February 4, 2004, DOI 10.1074/jbc.M309002200

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The first cell differentiation event in mammalian embryogenesis segregates inner cell mass lineage from the trophectoderm at the blastocyst stage. Oct-4, a member of the POU family of transcription factors, is necessary for the pluripotency of the inner cell mass lineage. Embryonic stem (ES) cells, which contribute to all of embryonic lineages, express the Oct-4 gene. Trophoblast stem (TS) cells, which have the ability to differentiate into trophoblast lineage in vitro, never contribute to embryonic proper tissues in chimeras and differentiate only into trophoblastic cells in the placenta. Expression of the Oct-4 gene was undetectable and severely repressed in trophoblastic lineage, including the stem cells. We found that the culture of TS cells with 5-aza-2′-deoxycytidine or trichostatin A caused the activation of the Oct-4 gene. Analysis of the DNA methylation status of mouse Oct-4 gene upstream region revealed that Oct-4 enhancer/promoter region was hypomethylated in ES cells but hypermethylated in TS cells. Furthermore, in vitro methylation suppressed Oct-4 enhancer/promoter activity in reporter assay. In the placenta of Dnmt1−/− mutant mice, most of the CpGs in the enhancer/promoter region were unmethylated, and Oct-4 gene expression was aberrantly detected. Chromatin immunoprecipitation assay revealed that Oct-4 enhancer/promoter region was hyperacetylated in ES cells compared with TS cells, thus demonstrating that DNA methylation status is closely linked to the chromatin structure of the Oct-4 gene. Here we propose that the epigenetic mechanism, consisting of DNA methylation and chromatin remodeling, underlies the developmental stage- and cell type-specific mechanism of Oct-4 gene expression.

In mammalian embryogenesis the first cellular differentiation begins at the end of the third cleavage, which leads to compaction and formation of the blastocyst. The inner cell mass (ICM) of the blastocyst is known to generate all fetal somatic cells and germ cells, whereas the outer cell layer, the trophoectoderm, gives rise to the trophoblastic components of the placenta. The explant culture of ICM or epiblast cells produces pluripotent embryonic stem (ES) cell lines, which contribute to all of the ICM lineages in chimeric embryos (1, 2). Trophoblast stem (TS) cell lines, which have the ability to differentiate into trophoblast lineage in vitro, have also been established from blastocyst or early postimplantation trophoblastic tissue (3). TS cells do not contribute to the embryo proper in chimeras but differentiate only into the trophoblastic cells of the placenta.

We previously investigated the genome-wide DNA methylation status of CpG islands by RLGS (restriction landmark genomic scanning) of mouse stem cells, i.e. ES, embryonic germ, and TS cells, before and after differentiation, as well as germ cells isolated from testis and some somatic tissues (4). These analyses revealed that genes having tissue-dependent, differentially methylated regions (T-DMRs) were numerous. The methylation pattern of T-DMRs was specific but varied according to cell lineage and tissue type (4, 5). We have also shown that individual cloned mice have different methylation aberrations, mainly at tissue-specific methylated loci (6). Considering that DNA methylation is involved in various biological phenomena, such as tissue-specific gene expression, cell differentiation, X-chromosome inactivation, genomic imprinting, changes in chromatin structure, and tumorigenesis (7–10), it is conceivable that the formation of specific DNA methylation patterns is one of the principal epigenetic events underlying mammalian development.

In mice, Oct-4, a member of the POU transcription factors, is expressed in the oocyte and preimplantation embryo but is later restricted to only the ICM of the blastocyst (11–13), indicating that expression is restricted to totipotent and pluripotent cells. In Oct-4-deficient embryos, the ICM loses pluripotency and the trophoblast cells no longer proliferate to form the placenta (14). Expression of the Oct-4 gene is found in ES cells but not in TS cells (3, 15). Reduction in Oct-4 gene expression leads to the trans-differentiation of ES cells into TS cells under adequate culture conditions (16), demonstrating that suppression of the Oct-4 gene is a critical step for the determination of the potency of these stem cells. The Oct-4 gene has a GC-rich and TATA-less minimal promoter (17), and several trans-activators and repressors have been reported as the regulators of Oct-4 expression (17–25). However, specific regulatory mecha-
DNA Methylation Regulates Oct-4 Gene Expression

nisms conferring the developmental stage- and cell type-specific expression of the Oct-4 gene have not been conclusively revealed to date.

In this report, to determine whether DNA methylation is involved in the regulation of Oct-4 gene expression, we investigated 1) the effect of a DNA methylation inhibitor on Oct-4 expression, 2) the methylation status of Oct-4 enhancer/promoter regions in both ES and TS cells, 3) the effect of in vitro methylation on Oct-4 enhancer/promoter activity, 4) Oct-4 expression in the DNA methyltransferase-1 (Dnmt1)-deficient conceptus, and 5) the chromatin structure of Oct-4 enhancer/promoter region in both ES and TS cells.

EXPERIMENTAL PROCEDURES

Reagents, Cell Culture, and Tissue Preparation—All reagents were purchased from Wako Pure Chemicals (Osaka, Japan) unless stated otherwise.

The ES cell line MS12, derived from C57BL/6 mice (26), was kindly provided by Dr. H. Suehori and cultured in a standard condition (27). The cells were kept undifferentiated by the addition of leukemia inhibitory factor (Chemicon, Temecula, CA (28)). TS cells were derived from C57BL/6NCrj mice according to the methods described previously (3). To maintain the undifferentiated state of these TS cells, they were cultured on plate-coated cells in the presence of FGF-4 and heparin (3). TS cells from ICR mice were maintained in the undifferentiated state by culturing in 70% embryonic fibroblast-conditioned medium supplemented with FGF-4 and heparin on gelatin-coated culture dishes. NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum.

We analyzed expression in the DNA methyltransferase-1 (Dnmt1)-deficient conceptus, and 5) the chromatin structure of Oct-4 enhancer/promoter region in both ES and TS cells.

Prior to treatment with 5-aza-2'-deoxycytidine (5-aza-dC) (DNA methylation inhibitor, Sigma), TS cells were pre-cultured for 48 h and then cultured for 2 days in medium containing 0, 1, or 5 μM 5-aza-2'-dC with or without 200 μM trichostatin A (TSA; inhibitor of histone deacetylate 1, Wako). NIH/3T3 cells were similarly cultured in medium supplemented with 0, 1, or 5 μM 5-aza-dC for 3 days following pre-culture. For treatment with TSA, NIH/3T3 cells were incubated for 24 h and then exposed to 200 or 400 μM TSA with or without 5 μM 5-aza-2'-dC for 72 h. Dmnt1 heterozygote mice (Dmnt1+/−) were purchased from The Jackson Laboratory. They were maintained on a 14-h light/10-h dark schedule and allowed free access to food and water. Noon of the day on which estrus was detected was designated as day 0 of the pregnancy. The mice were injected with 5 IU PMSG on day 1 of pregnancy (day 0) and 5 IU hCG on day 7 of pregnancy (day 7) and maintained until day 15 of pregnancy (day 15). Mice were killed by cervical dislocation, and the uterus was isolated. Both the conceptus and the uterus were fixed in 4% paraformaldehyde for 1 h, dehydrated in a graded series of ethanol, and embedded in paraffin. The total RNA was extracted as described previously (6). Briefly, each sample was treated with TRIzol reagent (Invitrogen) to evaluate the methylation status of Oct-4 gene, and stored at −80 °C until used.

Summary of the protocols of the protocol for the analysis of the expression of Oct-4 gene in the DNA methyltransferase-1 (Dnmt1)-deficient conceptus, and 5) the chromatin structure of Oct-4 enhancer/promoter region in both ES and TS cells.

Prior to treatment with 5-aza-2'-deoxycytidine (5-aza-dC) (DNA methylation inhibitor, Sigma), TS cells were pre-cultured for 48 h and then cultured for 2 days in medium containing 0, 1, or 5 μM 5-aza-2'-dC with or without 200 μM trichostatin A (TSA; inhibitor of histone deacetylate 1, Wako). NIH/3T3 cells were similarly cultured in medium supplemented with 0, 1, or 5 μM 5-aza-dC for 3 days following pre-culture. For treatment with TSA, NIH/3T3 cells were incubated for 24 h and then exposed to 200 or 400 μM TSA with or without 5 μM 5-aza-2'-dC for 72 h. Dmnt1 heterozygote mice (Dmnt1+/−) were purchased from The Jackson Laboratory. They were maintained on a 14-h light/10-h dark schedule and allowed free access to food and water. Noon of the day on which estrus was detected was designated as day 0 of the pregnancy. The mice were injected with 5 IU PMSG on day 1 of pregnancy (day 0) and 5 IU hCG on day 7 of pregnancy (day 7) and maintained until day 15 of pregnancy (day 15). Mice were killed by cervical dislocation, and the uterus was isolated. Both the conceptus and the uterus were fixed in 4% paraformaldehyde for 1 h, dehydrated in a graded series of ethanol, and embedded in paraffin. The total RNA was extracted as described previously (6). Briefly, each sample was treated with TRIzol reagent (Invitrogen) to evaluate the methylation status of Oct-4 gene, and stored at −80 °C until used.

In the case of Oct-4, PCR reactions were performed under the following conditions: 95 °C, 30 s; 35 cycles of 94 °C, 30 s; 62 °C, 1 min; 72 °C, 1 min; final extension 72 °C, 5 min. For β-actin, PCR reactions were performed under the following condition: 95 °C, 30 s; 35 cycles of 94 °C, 30 s; 65 °C, 1 min; 72 °C, 1 min; final extension 72 °C, 5 min.

PCR products were analyzed by 6% polyacrylamide gels (15:1 acrylamide/bisacrylaldehyde) with 5 μl of the PCR product. The gels were stained with SYBRGold and visualized under UV light. The amounts of bands were evaluated with Gel Doc 2000 (BioRad) and NIH Image 1.61 software. The following primers were used to amplify the PCR products: Dmnt1 forward (5′-CAGTGCTAGTAGGATTTGTT-3′), reverse (5′-ATGGAAAAGGAACTTGTG-3′), and β-actin forward (5′- GGTTTCGCTTTGGAAA-3′), reverse (5′-GGCGAGCGCTATCTGCCTGTG-3′), and reverse (5′-CTGGAAACCATCTCCTTCT-3′) primers. In the case of Oct-4, PCR reactions were performed under the following conditions: 95 °C, 30 s; 35 cycles of 94 °C, 30 s; 65 °C, 1 min; final extension 72 °C, 5 min. For β-actin, PCR reactions were performed under the following condition: 95 °C, 30 s; 35 cycles of 94 °C, 30 s; 65 °C, 1 min; final extension 72 °C, 5 min.

Luciferase reporter assays—Luciferase reporter assays were performed with forward (5′-GTCGAGAGGAGCTTATGCA-3′) and reverse (5′-CTGGAAACCATCTCCTTCT-3′) primers. In the case of Oct-4, PCR reactions were performed under the following conditions: 95 °C, 30 s; 35 cycles of 94 °C, 30 s; 65 °C, 1 min; final extension 72 °C, 5 min. For β-actin, PCR reactions were performed under the following condition: 95 °C, 30 s; 35 cycles of 94 °C, 30 s; 65 °C, 1 min; final extension 72 °C, 5 min.

RESULTS

Breakdown of Oct-4 Gene Silencing by Treatments with Reagents Affecting Epigenetic Status—The Oct-4 gene is expressed only in restricted types of cells. The expression of Oct-4 was not detected in TS cells or in NIH/3T3 cells, even by RT-PCR analyses (Fig. 1). Similarly, the Oct-4 gene was silenced in other somatic tissues and cells (data not shown).

Oct-4 mRNA becomes detectable in TS cells following treatment with 5-aza-dC, an inhibitor of DNA methylation, in a concentration-dependent manner. In addition, the Oct-4 ex-
expression was also observed in TS cells treated with an inhibitor of histone deacetylase 1, TSA. This suggests that Oct-4 gene activity is kept in the suppressed state in TS cells by DNA methylation and chromatin remodeling (Fig. 1A). The expression of Oct-4 in NIH/3T3 cells, however, was not attained by treatment with either 5-aza-dC or TSA alone. Interestingly, the combined treatment with 5-aza-dC and TSA effectively activated the Oct-4 gene in NIH/3T3 cells (Fig. 1B). Thus, DNA demethylation or histone acetylation alone was not sufficient to evoke Oct-4 transcription in NIH/3T3 cells. Nonetheless, the data suggests that the repression mechanism of the Oct-4 gene in Oct-4 non-expressing cells involves DNA methylation. If this was the case, then the 5′-flanking region of the Oct-4 gene is likely to be hypermethylated in cells or tissues in which the Oct-4 gene is silenced.

DNA Methylation Status of the 5′-flanking Region of the Oct-4 Gene—The Oct-4 gene has two distinct enhancers, a distal enhancer (DE) and proximal enhancer (PE), which alter the expression in preimplantation embryos, germ cells, ES and embryonic germ cells (31, 32). There is no CpG island at the 5′-region of the Oct-4 gene, although the promoter region is relatively rich in CpG dinucleotide sequences (Fig. 2A). There is one HpyCH4IV recognition site (at −4401 (Site 1)) and three TaqI sites (at −4028 (Site 2), −3030 (Site 3), and −2823 (Site 4)) in the DE, whereas PE has a site each for HpyCH4IV and TaqI at −303 (Site 6), respectively. In the promoter region, there is one HpyCH4IV site at −202 (Site 7) (Fig. 2A). We analyzed the DNA methylation status of the Oct-4 regulatory region by restriction mapping analysis, focusing on these seven recognition sites in ES cells, TS cells, and adult liver.

In ES cells, PCR products generated for Site 1 were not digested with HpyCH4IV, indicating that the genomic DNA of ES cells was unmethylated at Site 1 (Fig. 2B). Similarly, PCR products including Site 2, Site 6, and Site 7 remained intact after restriction enzyme treatment in ES cells. Thus, it is clear that the Oct-4 locus was hypomethylated over a wide range of regulatory regions in ES cells (Fig. 2C). In contrast, in TS cells all PCR products were sensitive to enzymatic digestion. The overall percentage of CpG methylation was 37–91% (Fig. 2B). This clearly shows that the Oct-4 upstream region of TS cells was more highly methylated than that of ES cells (Fig. 2C). Likewise, in the liver, in which the Oct-4 gene is also silenced (data not shown), all recognition sites of restriction enzymes were digested, indicating that the Oct-4 gene is highly methylated over the regulatory regions in this tissue (54–94%) (Fig. 2, B and C).

Thus, there is a T-DMR covering the promoter, proximal, and distal enhancer regions of the Oct-4 gene (Fig. 2C). Importantly, the T-DMR was hypomethylated in ES cells that express the Oct-4 gene, whereas TS cells and the liver, which do not express Oct-4, have methylated T-DMR.

DNA Methylation Profile of the Oct-4 Promoter Region—We further investigated the methylation status of each CpG immediately upstream of the Oct-4 gene containing the promoter region up to −470 bp from the translation start site (designated as +1) by sodium bisulfite genomic sequencing (Fig. 3A). There are 16 CpGs in this region, and the CpG density is higher than in other regions upstream of the Oct-4 gene (see diagram in Fig. 2A). In ES cells, the overall percentage of CpG methylation was 5%, and the region is almost unmethylated. In contrast, the region was heavily methylated in TS cells, exhibiting 74% methylation. Similarly, the liver showed 74% methylation of CpGs. Thus, we confirmed that the promoter region of Oct-4 is hypomethylated in ES cells but hypermethylated in TS cells and liver. Taking these results together with those of the 5-aza-dC treatment, it is highly probable that Oct-4 gene expression is regulated through DNA methylation-mediated gene silencing.

Repression of Oct-4 Regulatory Region Activity by DNA Methylation—The promoter activity of Oct-4 without the enhancer region was low (31). We constructed the reporter plasmid with the 5′-upstream region of Oct-4 containing the PE and promoter regions (−1527 to −30, Fig. 3B). The Oct-4 reporter construct showed 4.3-fold activity relative to that of the empty vector. The transcriptional activity of Oct-4 was dramatically reduced by in vitro DNA methylation. Thus, DNA methylation at the Oct-4 regulatory region elicits strong repression in Oct-4 transcription. Co-transfection of the reporter plasmid with MeCP2 expression vector also caused severe suppression. These data clearly demonstrated that the transcription of the Oct-4 gene was regulated through CpG methylation.

Expression of Oct-4 in Dnmt1-deficient Mouse Placenta—Genome-wide demethylation has been observed in Dnmt1<sup>−/−</sup> mice, which exhibit ~30% Dnmt1 activity compared with wild type (33). We assumed that the Oct-4 gene might be activated in Dnmt1-deficient mice if the Oct-4 promoter region is also demethylated in these mutants. We therefore analyzed the methylation status of the Oct-4 promoter region and the expression of Oct-4 in the placenta of Dnmt1<sup>−/−</sup> mice at 10.5 dpc (33). As expected, methylated CpGs in the Oct-4 promoter region comprise only 5% of the total CpGs in the placenta of Dnmt1<sup>−/−</sup> mice, whereas methylated CpGs comprise 43% in the placenta of wild type littermates (Fig. 4A). Importantly, the expression of Oct-4 mRNA was detected in the Dnmt1<sup>−/−</sup> placenta but not in wild type placenta (Fig. 4B). Expression of the Oct-4 gene was detectable in wild type embryos due to the presence of primordial germ cells that express the Oct-4 gene. Greatly reduced expression of Oct-4 in the mutant embryo may be due to retarded growth, particularly in the caudal region.
Thus, aberrant DNA demethylation results in the ectopic expression of Oct-4 gene.

Chromatin Structure of the Oct-4 Gene Enhancer/Promoter Region in ES and TS Cells—Methylation of CpG dinucleotide interferes with the binding of transcription factors (34) and binding of MeCP2 and other methyl CpG-binding domain proteins physically obstructs the basal transcription machinery (35). Alternatively, DNA methylation induces chromatin condensation following deacetylation by histone deacetylases associated with MeCP2 and methyl CpG-binding domain proteins (36). Therefore, we examined the histone acetylation status in ES cells and TS cells at the PE and promoter region of the Oct-4 gene by chromatin immunoprecipitation (ChIP) assay using antibodies that recognize acetylated histones H3 and H4 (Fig. 5).

We designed two sets of primers from the Oct-4 PE (Primer A) and promoter (Primer B) regions (Fig. 5A). By ChIP assay using Primer A, enrichment of histone H3 acetylation in ES cells was observed.
cells was more than 2-fold that of TS cells (Fig. 5, B and C), revealing that the acetylation status of histone H3 in the Oct-4 PE region was higher in ES cells than in TS cells. Furthermore, using Primer B, the acetylation status of histone H3 in ES cells was revealed to be more than three times that of TS cells, indicating that the Oct-4 promoter region was highly acetylated in ES cells compared with TS cells (Fig. 5, B and C). Similarly, the extent of histone H4 acetylation at both the Oct-4 PE and promoter was higher in ES cells than in TS cells. Furthermore, inhibition of DNA methylation by 5-aza-dC and histone deacetylation (TSA) allowed TS cells and NIH/3T3 cells to express the Oct-4 gene de novo. Furthermore, in vitro methylation severely suppressed the Oct-4 promoter activity in the reporter assay. In accordance with these results, Oct-4 mRNA was detected in the placenta of Dnmt1−/− mice, which have minimal activity of DNA methyltransferase (33). Thus, a clear link between Oct-4 expression and epigenetic control by DNA methylation and chromatin modification was demonstrated in the present study. During mammalian development almost all cells differentiate without changes in DNA sequence; yet the differentiation of a given cell type is associated with the activation of a particular set of genes and the inactivation of others. Our findings suggest the Oct-4 gene is a strong candidate for regulation via DNA methylation during early embryogenesis.

In the present study, the data indicated that the Oct-4 gene has T-DMRs and that DNA methylation is critical for the regulation of Oct-4 gene expression. There are numerous T-DMRs, and DNA methylation pattern is cell-type specific as reported.
previously (5). Cell differentiation always associates with the demethylation and methylation of the genomic DNA to form the required cell- or tissue type-specific methylation pattern (5, 6). The Oct-4 gene is one of the target genes regulated by DNA methylation, and, as such, the expression of Oct-4 is severely suppressed in certain cells and tissues. Aberrant Oct-4 gene expression may cause perturbations in cell differentiation. In addition, our findings further support the concept that gene regulatory mechanisms involving DNA methylation and changes in chromatin structure are crucial for normal development.

The Oct-4 enhancer/promoter region was hypomethylated in the placenta of Dnmt1<sup>-/-</sup> mice. To date, several Dnmts (Dnmt1, -3a, -3b, and -3L) have been reported in mammals. They are grouped into either de novo or maintenance DNA methyltransferase based on their in vivo enzyme activities (37). The null mutation of Dnmt1, however, leads to embryonic lethality after gastrulation (33), whereas the null mutation mice of Dnmt3a, -3b, and -3L survive for a longer period of time (37–39), suggesting that enzyme activity is more complex and the enzyme cannot simply be categorized into maintenance and de novo.

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** Chromatin immunoprecipitation assay of the Oct-4 proximal enhancer/promoter region in ES and TS cells. **A**, the left panel is a schematic diagram of the Oct-4 gene PE/promoter region. The numbers indicate the positions of CpG sites from the translation start site (+1), and the black boxes indicate the primer positions (Primer A and Primer B) for PCR of a ChIP assay. The right panel shows the PCR performed using the Input (see below) template of gradient concentration (1/2, 1/4, and 1/8). The intensity of PCR band was reduced in a concentration depending manner, indicating that this condition of amplification was useful for the analysis of ChIP assay. B, acetylation status of histone H3 and H4 in the Oct-4 upstream region in ES and TS cells. ES cells and TS cells were subjected to a ChIP assay using antibodies of acetylated histone H3 (AcH3) and H4 (AcH4). Normal rabbit IgG (IgG) was used as a negative control for the specificity of the immunoprecipitation. Aliquots of the chromatin were also analyzed before immunoprecipitation (Input). The PCR of mouse β-actin was performed as a positive control for the antibodies. The fold-enrichment values are shown below the respective lanes. The values for both acetylated H3 and H4 were higher in ES cells than in TS cells at both the Primer A and Primer B regions. C, histograms of the histone acetylation status of the Oct-4 gene enhancer/promoter region in ES and TS cells. Black and gray bars indicate ES cells and TS cells, respectively. The Oct-4 gene enhancer/promoter region was highly acetylated in ES cells but not in TS cells.
DNA methylation patterns through stepwise interaction during DNA replication (41). Therefore, it is probable that the maintenance system of DNA methylation patterns is affected in Dnmt1 mice.

The consensus sequence of Sp1 binding sites with clustered GC-boxes has been postulated to function as the insulator for protecting CpG sites from methylation (42). In this context, Sp1 and Sp3 have been reported to block the epigenetic inactivation at the Aprt gene promoter in mice (43, 44). A putative Sp1 binding site is present at the edge of the T-DMR of the sphingosin kinase-1 gene (4). Sp1 and Sp3 transcription factors activate Oct-4 gene expression (20, 23), and Sp1 transcription factor is expressed during mouse embryogenesis (45). We noticed that there was one Sp1 site in the Oct-4 promoter region and that this site contained completely unmethylated CpG dinucleotides (∼110) in TS cells. Taken together, the regulatory region of the Oct-4 gene displays quite a unique DNA methylation pattern regulated by specific cis-elements such as Sp1 or Sp3 binding sites.

Oct-4 transcript was increased by treatment with 5-aza-dC, and that this site contained completely unmethylated CpG dinucleotides (∼110) in TS cells. Taken together, the regulatory region of the Oct-4 gene displays quite a unique DNA methylation pattern regulated by specific cis-elements such as Sp1 or Sp3 binding sites.

Acknowledgments—We thank Drs. Benjamin K. Tsang and Kazuhiko Imakawa for proofreading this paper and Dr. Hirofumi Suemori for the critical reading of this manuscript. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (08238204, 08238208, and 14054001) from the Ministry of Education, Science, and Culture, Japan. The human embryonic stem cell line 69C was obtained from the Brain Research Institute, University of Tokyo. The murine ES cells were kindly provided by Dr. John H. W. Rossant, University of California, San Francisco. The mouse spermatogonia was kindly provided by Dr. Y. H. Shiota, Research Center for Experimental Mammalian Breeding, National Institute of Health, Tokyo, Japan.

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J. Biol. Chem. 2004, 279:17063-17069.
doi: 10.1074/jbc.M309002200 originally published online February 4, 2004

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