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DNA Methylation Is a Critical Cell-Intrinsic Determinant of Astrocyte Differentiation in the Fetal Brain

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Summary

Astrocyte differentiation, which occurs late in brain development, is largely dependent on the activation of a transcription factor, STAT3. We show that astrocytes, as judged by glial fibrillary acidic protein (GFAP) expression, never emerge from neuroepithelial cells on embryonic day (E) 11.5 even when STAT3 is activated, in contrast to E14.5 neuroepithelial cells. A CpG dinucleotide within a STAT3 binding element in the GFAP promoter is highly methylated in E11.5 neuroepithelial cells, but is demethylated in cells responsive to the STAT3 activation signal to express GFAP. This CpG methylation leads to inaccessibility of STAT3 to the binding element. We suggest that methylation of a cell type-specific gene promoter is a pivotal event in regulating lineage specification in the developing brain.

Introduction

Specification of cell lineages in the developing brain is thought to be regulated by cell-external cues and cell-intrinsic programs (Edlund and Jessell, 1999). The cues include various cytokines whose signals are transduced into the nucleus by transcription factors that bind to and activate their specific target gene promoters. The programs are, in part, regulated by the epigenetic modification of the cell type-specific genes.

Fetal telencephalic neuroepithelial cells contain neural precursors that give rise to neurons, astrocytes, and oligodendrocytes (Anderson, 2001; Gage, 2000; McKay, 1997). We have previously demonstrated that leukemia inhibitory factor (LIF), a member of the interleukin-6 (IL-6) family of cytokines that share gp130 as a signal-transducing receptor component (Taga and Kishimoto, 1997), and bone morphogenetic protein 2 (BMP2), which belongs to the transforming growth factor β super-family, act in synergy to induce astrocytogenesis of fetal mouse telencephalic neuroepithelial cells (Nakashima et al., 1999b). The synergistic action of LIF and BMP2 is mediated by a complex formation of respective downstream transcription factors, signal transducer and activator of transcription 3 (STAT3), and Smads, bridged by a transcriptional coactivator, p300 (Nakashima et al., 1999b). We have also shown that all the gp130-stimulating cytokines are expressed in the fetal mouse telencephalon and have the potential to induce astrocyte differentiation of neuroepithelial cells through the activation of gp130 (Ochiai et al., 2001; Yanagisawa et al., 1999, 2000). In addition, genetic ablation of gp130 resulted in a significant reduction in the number of astrocytes, assessed by expression of their marker, glial fibrillary acidic protein (GFAP), in the developing brain, proving an essential role for gp130-stimulating cytokines in the differentiation of astrocytes in vivo (Nakashima et al., 1999a). STAT3 recognition sequences have been identified within a regulatory region of the GFAP gene promoter which confer cell-specific expression (Bonni et al., 1997; Nakashima et al., 1999b). Furthermore, within these potential STAT3 binding sites, a particular site between −1518 and −1510 is evolutionarily conserved between human, rat, and mouse GFAP genes and is important for GFAP expression by gp130-stimulating cytokines (Bonni et al., 1997; Nakashima et al., 1999b). A point mutation in this STAT3 recognition sequence or forced expression of a dominant-negative form of STAT3 in neuroepithelial cells abolishes the GFAP expression induced by gp130-stimulating cytokines (Bonni et al., 1997; Nakashima et al., 1999b; Ochiai et al., 2001; Rajan and McKay, 1998; Takizawa et al., 2001; Yanagisawa et al., 1999, 2000). Thus, the expression of GFAP is largely dependent on the activation of STAT3 and the presence of its particular recognition sequence in the promoter.

It should be noted, as shown in this paper, that even when STAT3 is activated, not all cells express GFAP and that the responsiveness of the GFAP gene promoter to gp130 cytokines appears to be developmentally regulated. Furthermore, neurons in the developing brain and cells outside the nervous system do not express GFAP in response to STAT3 activation. This suggests the presence of a mechanism by which GFAP expression is repressed in nonspecific cells. In addition, during mouse development, neuronal differentiation starts at a very early stage and continues throughout brain development, while astrocytes appear just before term (Qian et al., 2000). The mechanism by which astrocytes are prevented from differentiating at earlier stages in the developing brain has long been of interest. All of these observations suggest that there is a cell-intrinsic program, most likely an epigenetic status, that determines whether or not cells respond to astrocyte-inducing cell-external cues.

Methylation of genomic DNA at CpG dinucleotides is

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a major epigenetic modification of mammalian genomes which has been shown to be implicated in the regulation of cell- or tissue-specific gene expression (Bird and Wolffe, 1999). It also functions in some other aspects of gene expression, such as genomic imprinting, X chromosome inactivation, aging, and tumorigenesis (Bird and Wolffe, 1999; Razin, 1998; Robertson and Jones, 2000). In these situations, CpG methylation contributes generally to transcriptional suppression by preventing transcriptional regulators from binding to their target gene promoters, or by triggering the formation of inactive chromatin. The former mechanism is known to occur for transcriptional factors such as cMyb (Klempnauer, 1993), cMyc, E2F (Campanero et al., 2000), CREB (Weih et al., 1991), AP2 (Comb and Goodman, 1990), NF-κB (Kirillov et al., 1996), and ETS, which are incapable of binding to methylated forms of their recognition sequences. The latter mechanism is active suppression mediated by proteins which have a methylated-CpG binding domain (MBD) (Bird and Wolffe, 1999; Li, 1999). They preferentially bind to methylated CpG and repress gene transcription by recruiting histone deacetylases (HDACs).

The methylation status of CpG dinucleotides within the GFAP gene promoter in rat brain cells has been shown to be lower during the developmental window in which astrocytogenesis is predominantly occurring (Condorelli et al., 1997; Teter et al., 1996). Highly frequent methylation of seven CpG sites located between −1464 and −1170 in the rat GFAP promoter, where the sequence is well-conserved between rat, mouse, and human, was observed in nonneural tissues lacking GFAP expression (Teter et al., 1994). Furthermore, methylation frequency of a CpG site at −1176 in the GFAP promoter was reported to be higher in the neuronal nuclei than the glial nuclei of adult brain, as assessed using an incidentally applicable methylation-sensitive restriction enzyme to this site (Barresi et al., 1999). However, in that paper, any relationship between this methylation and transcription factors was not discussed. These data provided evidence that the methylation status of the GFAP promoter varies among cell types and during development, but the mechanism whereby CpG methylation regulates expression of GFAP remains to be elucidated.

In the present paper, we have analyzed the mechanisms underlying cell type- and developmental stage-dependent responsiveness of the GFAP gene promoter to the gp130-STAT3 signal with respect to DNA methylation. We show that a CpG site in a particular STAT3 recognition sequence between −1518 and −1510 in the GFAP promoter, which has been shown to be critical for GFAP expression, is methylated in neurons but not in astrocytes, the former of which do not express GFAP even though STAT3 is activated. This site is also methylated in cells outside the nervous system which do not normally express GFAP. We further show that, in neuroepithelial cells, this particular site becomes demethylated at the stage when cells are responsive to the STAT3 activation signal and express GFAP. We further demonstrate that methylation of this CpG site in the STAT3 recognition sequence within GFAP promoter abolishes the accessibility of STAT3 and transcriptional activation.

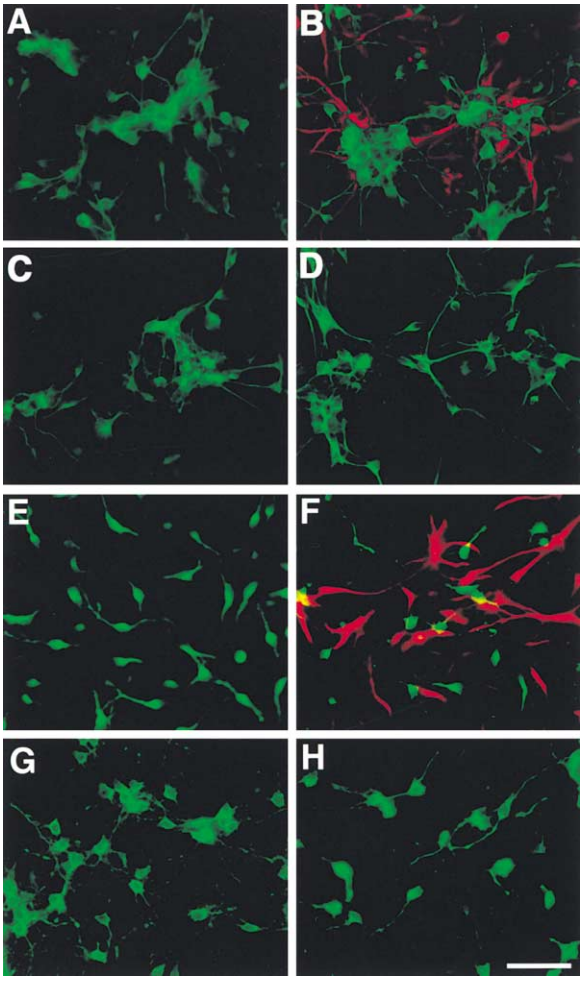


Figure 1. Failure of LIF-Induced GFAP Expression in E11.5 Neuroepithelial Cells and Postmitotic Neurons

(A–D) Neuroepithelial cells prepared from E14.5 (A and B) or E11.5 (C and D) mouse telencephalons cultured in the absence (A and C) or presence (B and D) of LIF (80 ng/ml) for 4 days.

(E–H) Neuroepithelial cells prepared from E14.5 cultured in the absence (E and F) or presence (G and H) of AraC (1 μM) for 4 days, followed by incubation without (E and G) or with LIF (80 ng/ml) (F and H) for 4 days.

Cells were stained with antibodies against GFAP (red) or MAP2 (green). Bar = 50 μm.

The observations in this study suggest how this astrocyte-specific protein is exclusively expressed in this cell type, and provide insight into the mechanism underlying lineage specification during brain development.

Results

Developmental Stage-Dependent, Cell Type-Specific Expression of GFAP in LIF-Stimulated Brain Cells

In the course of mouse development, neurons appear from nearly the beginning of neuroectoderm formation whereas astrocytes start to appear at a much later stage in brain development (Qian et al., 2000). We and others have previously reported that astrocyte differentiation can be induced in cultured fetal mouse neuroepithelial

cells prepared from, for example, E14.5 brain by gp130-stimulating cytokines (Bonni et al., 1997; Nakashima et al., 1999c; Rajan and McKay, 1998). Figures 1A and 1B show that GFAP-positive astrocytes were induced by LIF in a culture of E14.5 neuroepithelial cells, as has been shown previously. In marked contrast, no astrocytes appeared from E11.5 neuroepithelial cells cultured under the same conditions (Figures 1C and 1D). This result suggests that the cellular status of E11.5 neuroepithelial cells did not allow them to differentiate into astrocytes in response to LIF. It should be noted in Figure 1B that cells expressing a neuronal marker protein, MAP2 (microtubule-associated protein 2), did not express GFAP, and vice versa, suggesting that the expression of MAP2 and GFAP are mutually exclusive.

We therefore wanted to discover whether differentiated neurons do not respond to LIF by expressing GFAP. In the telencephalon of E14.5 mice, neurogenesis is active but gliogenesis has not yet started (Ghosh and Greenberg, 1995), indicating that cells which have withdrawn from the mitotic cycle at E14.5 are only neurons and not glial cells. Postmitotic neurons were enriched by incubation of E14.5 telencephalic neuroepithelial cells with 1 μM of 1-β-D-arabinofuranosylcytosine (AraC) for 4 days to eliminate proliferating cells. In contrast to the results without AraC treatment (Figures 1E and 1F), postmitotic neurons did not express GFAP (Figures 1G and 1H), even in the presence of LIF. This result suggests the existence of as yet unknown mechanisms by which the expression of the astrocyte-specific gene, GFAP, is prohibited in neurons.

Activation of the gp130-STAT3 Pathway Is Not Sufficient to Induce GFAP Expression in E11.5 Neuroepithelial Cells and Postmitotic Neurons

In the mouse, rat, and human GFAP gene promoters, a STAT3 binding element (TTCCGAGAA, −1518 to −1510 for mouse; see Figure 2A) is perfectly conserved (Bonni et al., 1997; Nakashima et al., 1999b). Introduction of nucleic acid substitution in this site (CCAAGAGAA; GF1L-SBSPM) abolishes the LIF-induced GFAP promoter activation as shown in Figure 2A. We then generated reporter constructs containing either the minimal promoter region of the GFAP gene alone (−73 to +12; GFMP) or 8 repeats of the STAT3 binding element appended to the minimal promoter (SBS8GFMP) (Figure 2A). Although the minimal promoter showed no response to LIF stimulation, 8 repeats of the STAT3 binding element made it responsive to LIF (see SBS8GFMP in Figure 2A). Taken together with the previous finding that a dominant-negative form of STAT3 ($STAT3^{Y705F}$) (Minami et al., 1996) abolished LIF-induced GFAP expression (Nakashima et al., 1999b), this suggests that not only the activation of the STAT3 protein but also its binding to the particular site (i.e., −1518 to −1510) in the GFAP gene promoter are prerequisites for LIF-induced GFAP expression in neuroepithelial cells.

The importance of the gp130-STAT3 signaling pathway in GFAP expression was then confirmed by infecting neuroepithelial cells with a recombinant adenovirus generated to express $STAT3^{Y705F}$. Staining with an antibody raised against GFAP after 4 days of LIF stimulation showed that $STAT3^{Y705F}$ expression resulted in a dramatic reduction in GFAP expression, while the expression of the control protein (LacZ) did not change (Figures 2B–2G).

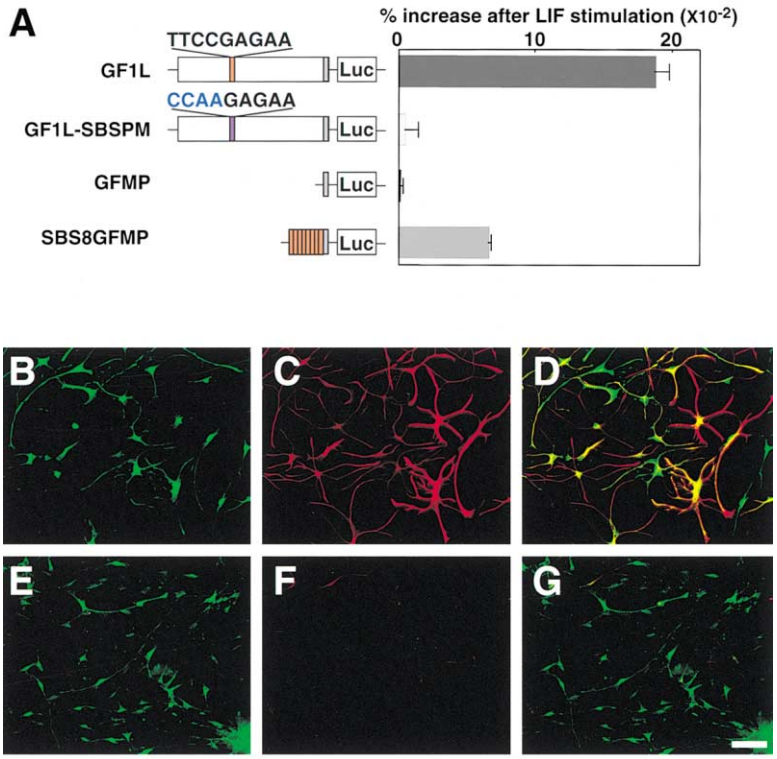
As we showed in Figure 1, E11.5 neuroepithelial cells and postmitotic neurons did not express GFAP even in the presence of LIF. Given that GFAP expression is dependent on STAT3 activation, it was possible that STAT3 may not be activated in these cell populations upon LIF stimulation. However, as depicted in Figure 2H, STAT3 protein is expressed and activated in response to LIF stimulation in E11.5 neuroepithelial cells and postmitotic neurons. This result suggests that a mechanism other than failure of STAT3 activation underlies the impairment of GFAP expression in these cells in response to signals activating gp130 and STAT3.

The Critical STAT3 Site in the GFAP Gene Promoter Is Methylated in E11.5 Neuroepithelial Cells and Postmitotic Neurons

As mentioned above, one particular STAT3 binding sequence (−1518 to −1510) in the GFAP promoter is essential for cells to express GFAP. We found a CpG dinucleotide, a potential target for DNA methylation in mammals, within this sequence. We thus postulated that this STAT3 binding sequence, which is critical for GFAP expression, may be methylated in E11.5 neuroepithelial cells and postmitotic neurons, since this type of epigenetic modification in genomic DNA has been shown to confer transcriptional suppression, resulting in tissue-specific regulation of gene expression (Bird and Wolffe, 1999). In light of these findings, we sought to determine whether methylation of the STAT3 binding element correlated with the regulation of GFAP expression.

We prepared postmitotic neurons from mouse E14.5 telencephalic neuroepithelial cells by elimination of proliferating neural precursors with AraC treatment. To examine the methylation status of the critical STAT3 binding element, we used a bisulfite sequencing method which allows the determination of every methylated CpG site in any target sequence (Clark et al., 1994). As shown in Figure 3A, the CpG dinucleotide in the STAT3 binding site in the GFAP promoter was highly methylated in postmitotic neurons (column 1), while that in the AraC-untreated control cells, which are capable of expressing GFAP in response to LIF stimulation, was barely methylated (column 2). When astrocytes were enriched by incubation of neuroepithelial cells with LIF and BMP2 simultaneously for 4 days (Nakashima et al., 1999b, 1999c), a CpG site in the sequence was also barely methylated (column 3). We further examined a possible developmental change in the frequency of CpG methylation in the critical STAT3 binding site (−1518 to −1510) in the GFAP promoter in neuroepithelial cells. As shown in Figure 3B, methylation of this particular STAT3 binding site was very frequent in E11.5 neuroepithelial cells and less frequent in E14.5 neuroepithelial cells. When the latter cells were cultured for 4 days in vitro, methylation of this site became very low. In E11.5 neuroepithelial cells, over 50% of this site was still methylated after 4 days in culture. The primary data from the experiments in Figure 3 are summarized in Table 1.

RT-PCR analysis of mRNA from adult tissues such as



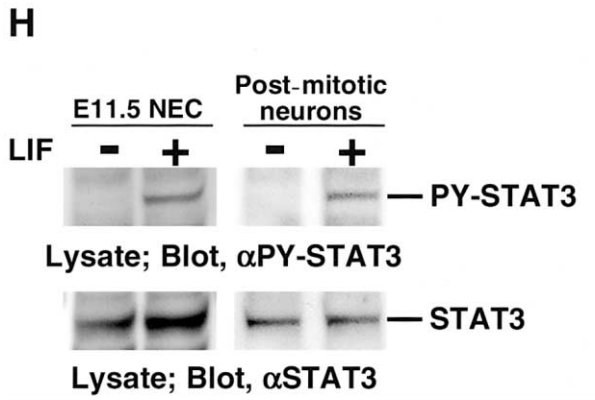


Figure 2. Requirement for STAT3 in LIF-Induced Astrocyte Differentiation and Activation of STAT3 in LIF-Nonresponsive E11.5 Neuroepithelial Cells and Postmitotic Neurons

(A) Neuroepithelial cells transfected with luciferase constructs containing the following GFAP promoter regions: an intact 2.5 kb promoter (GF1L), a 2.5 kb promoter with mutations in its STAT3 recognition sequence (−1518 to −1510) (GF1L-SBSPM), a minimal promoter region (GFMP) or a minimal promoter region with 8 repeats of the STAT3 binding sequence (SBS8GFMP). On the following day, cells were stimulated with LIF (80 ng/ml) for 8 hr.

(B–G) Neuroepithelial cells infected with recombinant adenoviruses engineered to express LacZ (B–D) or FLAG-tagged STAT3^{Y705F} (E–G) stimulated with LIF. After 4 days, cells were stained with antibodies against LacZ (B), FLAG (E), or GFAP (C and F). Superimposed views of (B) and (C) and of (E) and (F) are shown in (D) and (G), respectively. Bar = 50 μm.

(H) Cell lysates from E11.5 neuroepithelial cells (NEC) and postmitotic neurons after incubation with or without LIF (100 ng/ml) probed with antibodies against tyrosine-phosphorylated STAT3 or STAT3.

femoral muscle, heart, and liver revealed no expression of the GFAP gene (data not shown). We then examined the frequency of methylation in these tissues of the CpG dinucleotide in the STAT3 binding element (−1518 to −1510) critical for GFAP expression, and found that this element was almost completely methylated (Figure 3A, columns 4–6). These results further suggest a close correlation between GFAP expression and the methylation of the CpG site in the STAT3 binding element, which is applicable within and outside the nervous system.

Impairment of STAT3 Binding to the Methylated Recognition Sequence

In exerting transcriptional activation, it is essential for STAT3 to bind to its recognition sequence present in the target gene promoters. The foregoing results showed that the CpG dinucleotide in the STAT3 binding site was highly methylated in cells devoid of GFAP expression, suggesting that the methylation in the STAT3 binding element impedes accessibility of STAT3.

To substantiate this hypothesis, we conducted electrophoretic mobility shift assays (EMSA) with digoxigenin-labeled oligonucleotides containing the STAT3 binding site (−1518 to −1510) in the GFAP promoter (*GFS3) and its methylated form (*GFS3-Me). As a source of activated or control STAT3 protein, cell lysates were prepared from LIF-stimulated or nonstimulated COS7 cells which had been infected with recombinant adenovirus expressing STAT3. As shown in Figure 4A, *GFS3 formed a complex with LIF-stimulated, STAT3-containing lysates. This complex disappeared after the addition of a 100-fold excess of unlabeled probe (GFS3). In contrast, the addition of a 100-fold excess of methylated oligonucleotide (GFS3-Me) did not affect the complex formation. Furthermore, incubation with anti-STAT3 antibody led to retardation of the electrophoretic mobility of the complex, proving the presence of STAT3 protein in the complex. The methylated probe (*GFS3-Me) never formed a complex with STAT3 regardless of LIF stimulation. These results clearly demonstrate that STAT3 is

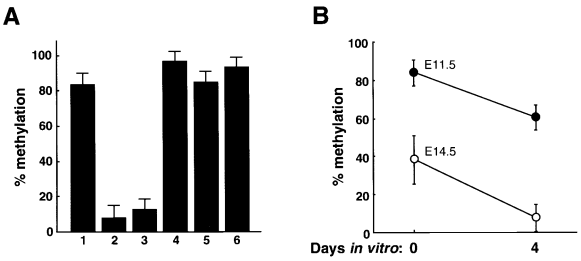


Figure 3. Cell Type-Specific and Developmental Stage-Dependent CpG Methylation in the STAT3 Binding Site in the GFAP Gene Promoter

(A) CpG methylation in the STAT3 recognition sequence (−1518 to −1510) of the GFAP promoter investigated by bisulfite sequencing in postmitotic neurons (column 1), proliferating neural precursors (column 2), and astrocytes (column 3), each prepared from E14.5 mouse telencephalon, and from adult femoral muscle (column 4), heart (column 5), and liver (column 6). PCRs were independently performed 3 times for each sample, then cloned. Frequencies of clones having methylation in the STAT3 recognition sequence are expressed as a percentage of the total number of clones sequenced (10 to 14 clones) for each independent PCR. The average methylation frequency in every cell type was calculated based on the frequency values derived from the three PCRs. Vertical bar represents SD.

(B) The STAT3 recognition sequence in the GFAP promoter investigated for its methylation status in neuroepithelial cells in freshly prepared E11.5 (closed circle) or E14.5 (open circle) mouse telencephalon or after 4 days in in vitro culture. Vertical bar represents SD.

incapable of binding to the methylated form of its recognition sequence.

To confirm whether this methylation-mediated interference with STAT3 binding takes place in vivo, we performed chromatin immunoprecipitation (ChIP) assays with postmitotic neurons or proliferating neural precursors which are incapable or capable, respectively, of expressing GFAP in response to LIF stimulation. It should be noted that even in the former cell type, STAT3 is activated (see Figure 2H). Immune complexes were prepared from the cell lysates using an anti-STAT3 antibody, and DNA fragments coprecipitated with STAT3 were examined by PCR for the presence of the GFAP promoter sequence. The data in Figure 4B (right-hand 4 lanes) provide clear evidence for an interaction between STAT3 and the relevant region of the GFAP promoter in LIF-stimulated neural precursors. In contrast, the interaction was hardly detectable in postmitotic neurons (Figure 4B, left-hand 4 lanes) which had the highly methylated STAT3 binding element in the GFAP promoter (see Figure 3A), even though STAT3 is activated in response to LIF (see Figure 2H). These results further suggest that STAT3 is unable to bind to the methylated form of its recognition sequence in the GFAP promoter both in vitro and in vivo.

Transcriptional Unresponsiveness of the GFAP Promoter with Methylated STAT3 Recognition Sequence

We and others have shown that the STAT3 binding sequence located between −1518 and −1510 is a prerequisite for GFAP gene expression, and we have demonstrated in this paper that methylation of this sequence interferes with STAT3 binding. We next attempted to examine whether methylation of the STAT3 binding element does indeed result in the inhibition of STAT3-mediated promoter activation. To this end, promoter assays were conducted with a reporter construct containing 8 repeats of the STAT3 binding element appended to the minimal promoter region of the GFAP gene (SBS8GFMP; see Figure 2A). Since it has been reported that various kinds of methylated CpG binding proteins exist in mammalian cells and confer nonspecific transcriptional repression to or below the detection limit (Bird and Wolffe, 1999), we employed *Drosophila melanogaster* cells in

Table 1. Methylation Frequency in the STAT3 Binding Site in the GFAP Promoter

PCR		Postmitotic Neurons	Proliferating Neural Precursors (E14.5 after 4 Days Culture)	Astrocytes	Femoral Muscle	Heart	Liver
#1	Methylated CpG	10	0	2	9	11	11
	n	11	10	10	10	12	11
	Percent	90.9%	0.0%	20.0%	90.0%	91.7%	100.0%
#2	Methylated CpG	8	1	1	11	8	9
	n	10	11	11	11	10	10
	Percent	80.0%	9.1%	9.1%	100.0%	80.0%	90.0%
#3	Methylated CpG	8	2	1	10	10	9
	n	10	14	13	10	12	10
	Percent	80.0%	14.3%	7.7%	100.0%	83.3%	90.0%

PCR		E11.5	E14.5	E11.5 after 4 Days Culture
#1	Methylated CpG	11	5	7
	n	13	10	13
	Percent	84.6%	50.0%	53.8%
#2	Methylated CpG	10	3	8
	n	13	12	13
	Percent	76.9%	25.0%	61.5%
#3	Methylated CpG	10	4	8
	n	11	10	12
	Percent	90.9%	40.0%	66.7%

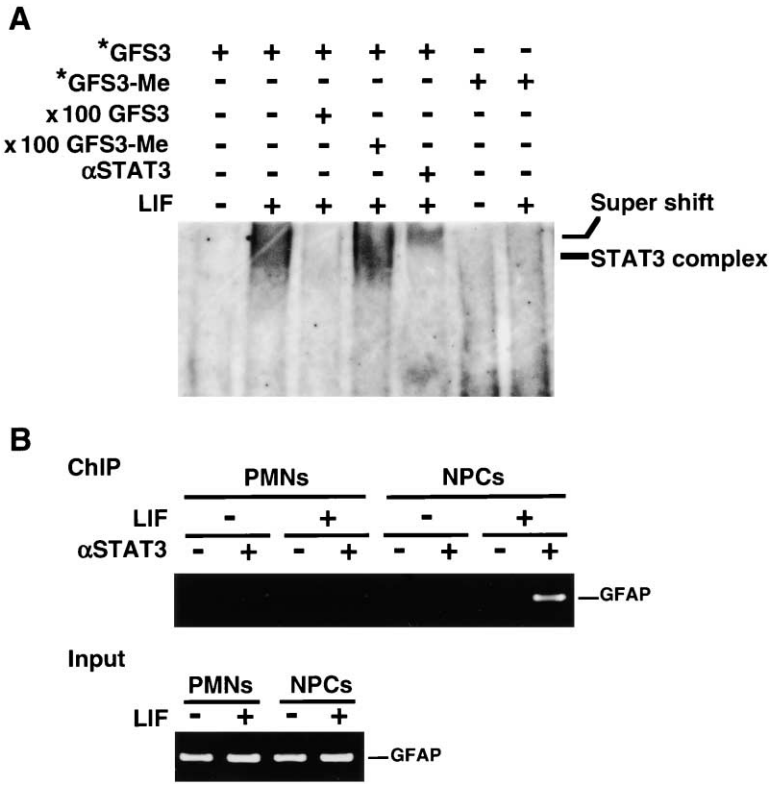


Figure 4. Impairment of STAT3 Binding to the Methylated Form of Its Target Sequence

(A) Electrophoretic mobility shift assay performed using a digoxigenin-labeled probe containing 3 tandem repeats of STAT3 recognition sequences (*GFS3) and its methylated version (*GFS3-Me). A 100-fold excess of nonlabeled GFS3 and GFS3-Me (X100 GFS3 and X100 GFS3-Me) were used as competitors. An antibody against STAT3 was used.

(B) Chromatin immunoprecipitation assay performed using formaldehyde-cross-linked, moderately sonicated chromatin from nonstimulated (−) or LIF-stimulated (+) postmitotic neurons (PMNs) and neural precursor cells (NPCs). The DNA fragments of the GFAP promoter associated with STAT3 were immunoprecipitated using an antibody against STAT3, and detected using PCR. The GFAP promoter fragment was detected in the anti-STAT3 immunoprecipitate obtained from LIF-stimulated neural precursor cells.

this study (SL2 cells) since these are considered to have few methyl binding proteins. We first examined whether STAT3 could be activated by joint expression with JAK1, a cytoplasmic tyrosine kinase functioning upstream of STAT3, in SL2 cells. Western blot analysis revealed that simultaneous expression of STAT3 and JAK1 resulted in activation of both proteins as assessed by their tyrosine-phosphorylation status (Figure 5A). The CpG-methylated form of the SBS8GFMP reporter was prepared by treatment with SssI in vitro. After the treatment with SssI, SBS8GFMP became resistant to HpaII digestion (Figure 5B), indicating that methylation was accomplished successfully. Methylated or unmethylated SBS8-GFMP were cotransfected into SL2 cells along with an empty vector or a set of JAK1 and STAT3 expression vectors. As shown in Figure 5C, methylation of SBS8GFMP significantly impeded STAT3-mediated transcriptional activation. Since the response of SBS8GFMP to STAT3 activation was fully dependent on the STAT3 binding sequence, TTCCGAGAA, within the construct (Figure 2A), it is conceivable that the reduced response of methylated SBS8GFMP to STAT3 activation can be attributed to the methylation in the sequence.

Demethylation Confers Responsiveness to Gliogenic Signal on E11.5 Neuroepithelial Cells

To verify the inhibitory role of methylation in GFAP expression, we incubated E11.5 neuroepithelial cells, which do not normally express GFAP in response to LIF (see Figure 1), with LIF in the presence or absence of a demethylating agent, 5-aza-2′-deoxycytidine (5-aza-CdR). As shown in Figure 6, GFAP-expressing cells appeared in response to LIF only in the culture containing 5-aza-CdR. This finding further confirms that unresponsiveness of E11.5 neuroepithelial cells to gliogenic signal is attributable to DNA methylation.

Discussion

As we and others previously demonstrated, the gp130-STAT3 signaling pathway is critical for astrocyte differentiation from neuroepithelial cells (Bonni et al., 1997; Nakashima et al., 1999a; Takizawa et al., 2001). However, it is obvious that this signaling pathway does not automatically force the cells to induce astrocytes. As shown in the present work, postmitotic neurons do not express the astrocytic marker GFAP even though STAT3 is activated. The same is true for neuroepithelial cells at a relatively early developmental stage, for instance on E11.5. In these cell types, the STAT3 binding element critical for GFAP expression is shown here to be methylated. The current work thus supports the important hypothesis that cell lineage specification in the developing brain is regulated by both cell-external cues and cell-intrinsic programs, where the former involves gp130 cytokines and the latter involves the methylation status of the promoter of the gene for astrocyte-specific protein GFAP in this case. It is worth noting that methylation of the STAT3 binding element abolishes the accessibility of STAT3 and concomitantly inhibits transcription activity. Thus, both external and internal cellular conditions appear to cooperate in determining lineage specification. In this sense, it appears likely that methylation of the STAT3 binding element in the GFAP gene promoter must become less frequent in E14.5 neuroepithelial cells from which astrocytes are readily differentiated in the 4-day

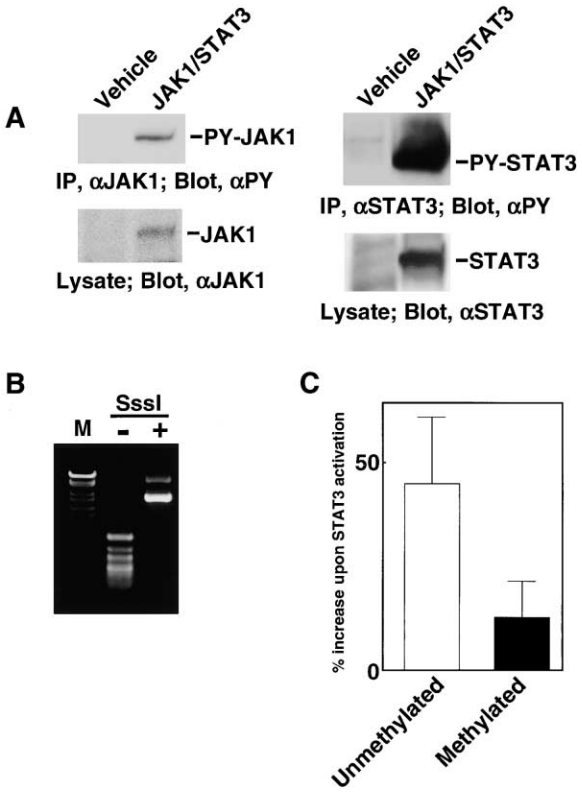


Figure 5. Methylation-Dependent Inhibition of STAT3-Induced Transcriptional Activation

(A) SL2 cells cotransfected with pAC5.1-JAK1 along with pAC5.1-STAT3. Cell lysates were subjected to immunoprecipitation with antibodies against JAK1 and STAT3, followed by immunoblotting with an anti-phosphotyrosine antibody. The presence of JAK1 and STAT3 were confirmed by blotting with either anti-JAK1 or anti-STAT3.

(B) Methylation of the reporter construct SBS8GFMP performed with SssI in vitro and confirmed by digesting with the methyl-sensitive enzyme, HpaII.

(C) Methylated or unmethylated SBS8GFMP cotransfected with either a combination of pAC5.1-JAK1 and pAC5.1-STAT3 or vehicle alone into SL2 cells and cell lysates analyzed for luciferase activity. Results are presented as the percentage increase in activity induced by JAK1-STAT3 coexpression in comparison with the control (vehicle transfection).

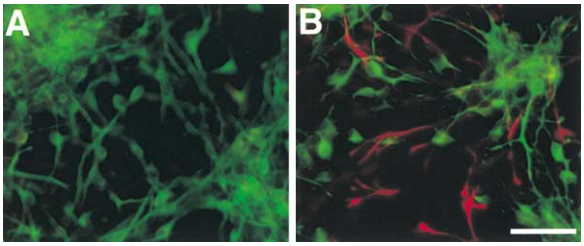


Figure 6. Precocious Acquisition of Responsiveness to Gliogenic Signal of E11.5 Neuroepithelial Cells by Forced Demethylation

(A and B) Neuroepithelial cells prepared from E11.5 mouse telencephalons cultured with LIF (80 ng/ml) for 4 days in the presence (B) or absence (A) of 5-aza-CdR (3 μM).

Cells were stained with antibodies against GFAP (red) or MAP2 (green). Bar = 50 μm.

stimulation with gp130-stimulating cytokines. It is interesting to note that E11.5 neuroepithelial cells became responsive to LIF-induced expression of GFAP, when the cells were cultured for 4 days in vitro prior to LIF stimulation (M.N. et al., unpublished data). These findings support the hypothesis that developmental changes in responsiveness to a certain external signal are determined by the methylation status in the promoter region of the signal's target gene(s). This is also strongly supported by the results that demethylation by 5-aza-CdR led to the acquisition of precocious responsiveness of E11.5 neuroepithelial cells to gliogenic signal (see Figure 6). This mechanism is of great importance in understanding cell lineage specification during the development of the brain where various kinds of cell-external cues (i.e., growth and differentiation factors) are present simultaneously. Taken together with the observation that the STAT3 binding site in the GFAP gene promoter is almost completely methylated in such tissues as muscle, heart, and liver, CpG methylation appears to function to avoid expression of genes at undesirable times and places. In conclusion, CpG methylation may be important not only in gene silencing but also in the timing of fate determination of neural precursors in the developing brain. The cell-fate switch of neural precursors may, in part, be attributable to the demethylation of methylated CpG sites. If it is the case, CpG methylation may occur simultaneously over whole genomic loci containing astrocyte-specific gene promoters in cells committed to becoming non-astrocytic cell types.

Our results demonstrate that methylation in the STAT3 recognition sequence abolishes binding of STAT3, leading to transcriptional inhibition of STAT3-induced GFAP gene expression. There is an alternative mechanism whereby methylation confers gene suppression, namely methyl-CpG binding protein-mediated gene silencing. MeCP2, for instance, is a member of a group of methylated-CpG binding proteins and is expressed at high levels in the postnatal brain (Coy et al., 1999; Lewis et al., 1992). It has been demonstrated that MeCP2 confers transcriptional repression via association with HDACs (Jones et al., 1998). While it remains unknown whether MeCP2 actually occupies the methylated STAT3 recognition sequence in the GFAP gene promoter in postmitotic neurons, we can say that, at least for the GFAP expression, reduction in the STAT3 binding affinity for its target sequence by CpG methylation is sufficient for silencing the GFAP gene. In humans, mutations in the MeCP2 gene cause Rett syndrome (RTT), a childhood neurological disorder (Dragich et al., 2000; Van den Veyver and Zoghbi, 2000). Mice deficient for MeCP2 show symptoms which resemble those of RTT patients and a decrease in the size of hippocampal neurons (Chen et al., 2001; Guy et al., 2001). These facts imply that MeCP2 plays important and nonredundant roles in the normal function of the nervous system.

As for the enzymes involved in the methylation of the CpG dinucleotide, there are two different types of DNA methyltransferase, among which Dnmt1 is a maintenance type of DNA methyltransferase (Lyko et al., 1999), and Dnmt3a and Dnmt3b are de novo DNA methyltransferases (Okano et al., 1999). These enzymes are considered to be required for prohibiting undesirable gene

expression or maintaining the stability of chromatin structures (Bestor, 2000). Analysis of conditional mutant mice lacking Dnmt1 in mitotic CNS precursor cells revealed that hypomethylated CNS neurons failed to exhibit appropriate function and were excluded at postnatal stages by as yet unknown mechanisms (Fan et al., 2001), implying the importance of CpG methylation for neurons. It is interesting to note that treatment with 5-aza-CdR led to induction of GFAP expression in mouse fibroblasts which do not normally express GFAP (Michalowsky and Jones, 1989). This supports the involvement of DNA methylation in cell-type-specific gene expression.

Besides lineage specification, DNA methylation has been shown to be involved in the regulation of tumor cell growth. It has been reported that a promoter region of the gene for p21, an inhibitor of cyclin-dependent kinases, contains a recognition sequence for STAT1, and that methylation in this sequence abolishes interferon γ-induced expression of p21, resulting in the failure of the growth arrest response in some tumor cell lines (Chen et al., 2000). Taken together with our current observations, methylation may be a pivotal event in determining the response of cells to the STAT family of proteins if their consensus binding sequences (TTNCN NNAA) in the target gene promoters contain CpG dinucleotides.

It has long been asked why astrocytes appear at a very late stage of brain development whereas neurons are differentiated from nearly the beginning of neuroectoderm formation. As we have shown in this study, it is of interest that E11.5 neuroepithelial cells possess a complete set of the gp130-STAT3 signaling machinery (see Figure 2H) but do not differentiate into astrocytes even in the presence of gp130-stimulating cytokines. More importantly, forced demethylation conferred precocious responsiveness to an astrocyte-inducing cue, LIF, upon the cells. Our present work sheds light on a mechanism by which developmental stage-dependent cellular differentiation occurs. Our conclusion is that STAT3 is a transcriptional factor sensitive to methylation in its target DNA sequence and that DNA methylation is important in the regulation of the gp130-STAT3 signal-mediated astrocyte-specific gene expression. Our results further suggest the mechanism underlying the cell type-specific and developmental stage-dependent responsiveness of cells to the STAT3-activation signal. To elucidate the mechanism of how methylation or demethylation of specific gene promoters is regulated during development will be an intriguing subject for further study, and will pave the way for better understanding of cell fate determination.

Experimental Procedures

Animals and Cell Preparation

Time-pregnant ICR mice were used to prepare neuroepithelial cells. Mice were treated according to the guidelines of Kumamoto University Center for Animal Resources and Development. Neuroepithelial cells were prepared from telencephalons of E11.5 or E14.5 mice and cultured as described previously (Nakashima et al., 1999b). Briefly, the telencephalons were triturated in Hank's balanced salt solution (HBSS) by mild pipetting with 1 ml pipet tip (Gilson). Dissociated cells were cultured for 4 days in N2-supplemented Dulbecco's Modified Eagle's Medium with F12 (GIBCO) containing 10 ng/ml basic FGF (R&D Systems) (N2/DMEM/F12/bFGF) on culture dishes which had been precoated with poly-L-ornithine (Sigma) and fibronectin (Life Technologies). For selection of postmitotic neurons, 1 μM AraC was added to the culture media.

Recombinant Adenovirus Construction and Infection

Recombinant adenoviruses were constructed as described previously (Saitoh et al., 1998). Infection of neuroepithelial cells with each recombinant adenovirus was performed at a multiplicity of infection (MOI) of 200 PFU/cell.

Immunofluorescent Staining

Cells cultured on chamber slides (Nunc) were washed with PBS, fixed in 4% paraformaldehyde in PBS, and stained with one of the following primary antibodies: anti-GFAP (Dako), anti-MAP2 (Sigma), anti-LacZ (Molecular Probes), or biotin-conjugated anti-FLAG (Sigma). The following secondary antibodies were used: FITC-conjugated goat anti-mouse IgG, rhodamine-conjugated donkey anti-rabbit, FITC-conjugated donkey anti-rabbit (Chemicon), and streptavidin-conjugated Texas Red (Vector). Nuclei were stained using bisbenzimide H33258 fluorochrome trihydrochloride (Nakaraitesque).

Luciferase Assay

Neuroepithelial cells cultured for 4 days on 10 cm dishes, as described above, were replated on 12-well plates (Nunc) and transfected the next day with the following reporter plasmids; GF1L, GF1L-SBSPM (Nakashima et al., 1999b), GFMP, or SBS8GFMP (see Figure 2A). Control transfections were made using sea pansy luciferase gene conjugated with human elongation factor 1α promoter (R-Luc) (Nakashima et al., 1999b). Transfections were performed using Trans-It LT1 (Pan Vera) according to the manufacturer's instructions. On the following day, the cells were stimulated with LIF (80 ng/ml) for 8 hr and then solubilized. For *Drosophila* cells, STAT3 and JAK1 cDNA were subcloned into a pAC5.1 vector optimized for expression in *Drosophila* cells (pAc5.1-STAT3 and pAc5.1-JAK1). Either SssI-methylated or unmethylated SBS8GFMP was cotransfected with pAcSp1 (Courey and Tjian, 1988) and a combination of pAc5.1-STAT3 and pAC5.1-JAK1 using a Cell Phect transfection kit (Amersham) according to the manufacturer's instructions and cultured for 24 hr. Luciferase activity was measured using the recommended procedures for the Pikkagene Dual Luciferase Assay System (Tokyo Ink Inc.) with the following modification: trypsin inhibitor (1 mg/ml, type III-0 from soy bean white; Sigma) was included in the cell lysis buffer. Sea pansy luciferase gene under a CMV promoter (CMV-Renilla) was used as an internal control. A micro Lumat LB96B luminometer (Wallac Berthold) was used for detection.

Immunoblotting

Cells were lysed using NP40 lysis buffer (0.5% NP40, 10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 3 mM pAPMSF [Wako Chemicals], 5 mg/ml aprotinine (Sigma), 2 mM sodium orthovanadate [Wako Chemicals], and 5 mM EDTA). Lysates were immunoprecipitated with anti-STAT3 (C-20; Santa Cruz) and anti-JAK1 (Santa Cruz) antibodies. Immunoprecipitates and lysates were subjected to SDS-PAGE and subsequent immunoblotting with antibodies against STAT3 (Transduction Laboratories), tyrosine-phosphorylated STAT3 (Cell Signaling), JAK1, or phospho-tyrosine (4G10; Upstate Biotechnology). Detection was performed using the ECL detection system (Amersham).

Bisulfite Sequencing

Sodium bisulfite treatment of genomic DNA was performed essentially as described previously (Clark et al., 1994). Briefly, 5 μg of genomic DNA was digested with SacI, denatured with 0.3 M NaOH at 37°C for 15 min, and incubated with 3.1 M sodium bisulfite and 0.5 mM hydroquinone at 55°C for 16 hr. The samples were purified using a desalting column (Promega) according to the manufacturer's instructions, and eluted in 50 μl of H_2O. Three molar NaOH (5.5 μl) was added and the samples were incubated at 37°C for 15 min. Samples were neutralized by the addition of 3 M ammonium acetate, ethanol precipitated, and dissolved in H_2O. The DNA fragment containing the STAT3 recognition sequence was amplified by PCR using the following set of primers: GFmS; 5′-GGGATTTATTAGGAGAATT

TTAGTAAGTAG-3′, GFmAS; 5′-TCTACCCATACTTAAACTTCTAA TATCTAC-3′. The PCR products were cloned into pT7Blue and 10 to 14 clones randomly picked from each of three independent PCRs were sequenced.

Electrophoretic Mobility Shift Assay

COS7 cells were infected with a recombinant adenovirus engineered to express STAT3 and cultured for 16 hr. After stimulation with LIF (80 ng/ml) for 15 min, cells were lysed in lysis buffer (0.1% NP-40, 10 mM HEPES [pH 7.8], 10 mM KCl, and 0.1 mM EDTA) containing protease and phosphatase inhibitors (3 mM pAPMSF, 5 mg/ml aprotinine, and 2 mM sodium orthovanadate). The sense (5′-GTAC TTCCGAGAAGGGTTCCGAGAAGGGTTCCGAGAA-3′) and antisense oligonucleotides with or without methylated CpG were synthesized separately and annealed together. For probes, the oligonucleotides were labeled at the 3′ end with digoxignenin (Roche) according to manufacturer's instructions. Binding reactions were performed in a total volume of 25 μl of the binding buffer (100 mM HEPES [pH 7.6], 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM DTT, 10% (w/v) Tween 20, and 150 mM KCl). Cell lysates were incubated for 20 min at room temperature with the labeled oligonucleotides in the presence or absence of unlabeled competitors. Supershift reactions were performed by incubating an antibody against STAT3 (C-20; Santa Cruz) with the cell lysates for 1 hr on ice prior to the addition of the labeled oligonucleotides. The samples were separated using native 8% polyacrylamide gels at 20 mA in 0.25× Tris-borate EDTA buffer. After electrophoresis, the gels were blotted onto nylon membranes for 30 min and baked for 30 min at 120°C. The membranes were probed with an alkaline phosphatase-conjugated antibody raised against digoxigenin (Roche) and detected by CDP Star according to the manufacturer's instructions (Roche).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation was performed as previously described (Clayton et al., 2000) with minor modifications. Cells were exposed to formaldehyde at a final concentration of 1% added directly to the tissue culture medium. Cells were centrifuged into a pellet after 10 min of formaldehyde exposure, lysed in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl [pH 8.1]) containing protease and phosphatase inhibitors (3 mM pAPMSF, 5 mg/ml aprotinine, 1 mg/ml pepstatin A [Wako Chemicals], and 2 mM sodium orthovanadate) and then incubated for 5 min on ice. Cell lysates were sonicated using a microtip until the DNA fragments were 600–1000 base pairs in length. Chromatin samples were diluted 1:10 with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], and 167 mM NaCl). One percent of the total volume was stored as input at −20°C until use. Immunoprecipitation was performed at 4°C overnight with 1 μg of an antibody against STAT3 (C-20) after samples were precleared with protein A Sepharose beads (Amersham) containing 5 μg of sonicated phage lambda DNA (TOYOBO). Immune complexes were collected by protein A Sepharose beads and washed with the following buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], and 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], and 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl [pH 8.1]), and TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 8.0]). Immune complexes were disrupted with elution buffer (1% SDS and 50 mM $NaHCO_3$) and the covalent links between immunoprecipitates and input chromatin were reversed by incubation with 300 mM NaCl at 65°C for 4 hr. DNA was further incubated with proteinase K, purified by phenol extraction, and ethanol precipitated. DNA pellets were dissolved in 30 μl of H_2O and used as a template for PCR with the following set of primers: GSS (5′-TAAGCT GAAGACCTGGCAGTG-3′) and GSAS (5′-TGCTGAATAGAGCCTTG TTCTC-3′).

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