Silencing of transgene transcription precedes methylation of promoter DNA and histone H3 lysine 9

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Transgenes stably integrated into cells or animals in many cases are silenced rapidly, probably under the influence of surrounding endogenous condensed chromatin. This gene silencing correlates with repressed chromatin structure marked by histone hypoacetylation, loss of methylation at H3 lysine 4, increase of histone H3 lysine 9 methylation as well as CpG DNA methylation at the promoter. However, the order and the timing of these modifications and their impact on transcription inactivation are less well understood. To determine the temporal order of these events, we examined a model system consisting of a transgenic cassette stably integrated in chicken erythroid cells. We found that histone H3 and H4 hypoacetylation and loss of methylation at H3 lysine 4 all occurred during the same window of time as transgene inactivation in both multi-copy and low-copy-number lines. These results indicate that these histone modifications were the primary events in gene silencing. We show that the kinetics of silencing exclude histone H3 K9 and promoter DNA methylation as the primary causative events in our transgene system.

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Introduction

Regulation of gene expression in eukaryotic organisms is a complex process that includes many levels of chromatin remodeling and modifications. The hierarchical order of events in the course of gene activation has been well studied (Cosma, 2002; Emerson, 2002); however, the changes in histone and DNA molecules that accompany transcription inactivation have been less thoroughly investigated even though several repressor complexes with chromatin remodeling activity have been described (Bird and Wolffe, 1999). It is well established that gene silencing correlates with DNA hypermethylation, and the addition of a methyl group to the cytosine base can influence transcription by preventing transcription factor binding and/or by forming silent chromatin structures (Bird and Wolffe, 1999; Jones and Baylin,

2002). Crosstalk between histone modifications and DNA methylation is thought to exist (Bird and Wolffe, 1999; Ben-Porath and Cedar, 2001; Bird, 2002; Richards and Elgin, 2002) and can lead to transcription repression, although it is not yet clear who initiates this ‘talk’, or when. In one possible pathway, DNA methylation is the first event that initiates a chain of events resulting in modification of the histone amino terminal tails and creates silenced chromatin. This model comes from the observation that unmethylated transgenes stably integrated into the genome become packaged with acetylated histones, while *in vitro* CpG-methylated transgenes become associated with deacetylated histones (Eden *et al.*, 1998; Schubeler *et al.*, 2000). These methyl-CpG-enriched regions may target methyl-CpG-binding proteins, which in turn recruit repressor complexes containing histone deacetylases (Bird and Wolffe, 1999) as well as histone methyltransferases (Fuks *et al.*, 2003). In a second proposed model, DNA methylation is a secondary event, induced by an already silenced chromatin (Bird, 2002). Recently it was found that in *Neurospora crassa* all DNA methylation is dependent on H3 lysine 9 (K9) methylation (Tamaru and Selker, 2001). Further chromatin immunoprecipitation experiments showed that trimethylated H3 K9, but not dimethylated H3 K9, marked chromatin regions for cytosine methylation in *N. crassa* (Tamaru *et al.*, 2003). Similarly in *Arabidopsis*, histone H3 K9 methylation is necessary for some of the CpNpG and asymmetric methylation (Jackson *et al.*, 2002). These observations suggest that DNA methylation acts downstream of H3 K9 methylation, at least in these two organisms. Does DNA methylation cause inactivation of transcription or is it a consequence of it? Which are the critical initiating events in gene repression and what is responsible for the maintenance of the silenced chromatin? Obviously the answers to these questions may depend on the gene system, but these are particularly important questions with respect to transgenes, since they concern their ability to maintain their pattern of expression after stable transfection into cells or animals. In many cases, such transgenes are silenced rapidly, probably under the influence of surrounding endogenous condensed chromatin in which they find themselves embedded. In this paper we focus on the silencing of such stably transfected genes. We show among other findings that the kinetics of silencing specifically exclude DNA methylation as the primary causative event.

Results

To determine the temporal order of epigenetic chromatin modifications and their impact on the process of transcription inactivation, we employed our previously studied model system of a transgenic cassette stably integrated in chicken erythroid cells (Pikaart *et al.*, 1998; Mutskov *et al.*, 2002). The gene encoding the Tac subunit of the interleukin 2 receptor

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(IL2R) driven by an erythroid-specific chicken β^A -globin promoter and the β/ϵ enhancer (Figure 3 bottom line) was integrated into the early erythroid 6C2 cell line. A hygromycin-resistant gene was cotransfected as a selectable marker for the transgene-expressing cells. When the hygromycin-resistant transformed clones were grown in the presence of hygromycin in the medium, all the cells expressed an antigenic IL2R cell surface marker (day 0—Figures 1A and 6A), due to the coordinated activity of the IL2R and hygromycin genes (Pikaart *et al.*, 1998). However, after removal of the drug, there was a gradual extinction of IL2R activity over a period of 10–80 days in culture (Figures 1A and 6A; Pikaart *et al.*, 1998). We followed the process of transgene silencing in a multicopy cell line carrying 13 copies of the IL2R reporter cassette. FACS analysis of the IL2R surface marker in these cells at day 0 (still in the presence of hygromycin) and at days 2, 4, 6, 8, 11, 13 and 19 after removal of hygromycin revealed an extinction of expression (Figure 1A). At day 11 almost all the cells were IL2R negative. We also performed quantitative (TaqMan) RT-PCR to detect the presence of IL2R transcripts during the time course of our experiment (Figure 1B). For each time point, the level of IL2R mRNA was compared to the level of folate receptor mRNA, which is constantly expressed in 6C2 cells (Prioleau *et al.*, 1999). This experiment confirmed the gradual extinction of IL2R transcription, which was abolished around days 10–13 (Figure 1B).

Figure 1 Extinction of IL2R transgene activity over an extended time in culture. (A) 6C2 cells carrying 13 copies of the IL2R reporter were grown for extended times without hygromycin selection. For IL2R cell surface activity, the cells were analyzed by flow cytometry at different days. The horizontal bars denote M1 and M2, which define the ranges of IL2R negative and positive cells, respectively. 6C2 cells untransfected with the IL2R construct were used to determine the IL2R negative cells zone on the histograms. (B) Quantitative RT-PCR of a 13-copy cell line at days 0, 3, 6, 9, 13, 18 and 20. Each point on the graph represents the amount of IL2R mRNA relative to the folate receptor mRNA per copy of the transgene. IL2R mRNA at day 0 is set to 100.

We previously demonstrated that transgene silencing in 6C2 chicken cells, due to chromosomal position effects of the integration site, is accompanied by changes in chromatin structure and DNA methylation (Pikaart *et al.*, 1998; Mutskov *et al.*, 2002). Hypermethylation of the promoter as well as the coding region of the transgene was observed at day 100; however, the critical CpG sites for silencing by DNA methylation were located over the promoter (Mutskov *et al.*, 2002). This finding suggested a link between transcription silencing and epigenetic modification, but it did not provide direct evidence as to whether DNA methylation caused IL2R inactivation or whether it was a consequence of it. For this reason we studied the kinetics of transition from the unmethylated, active state to the densely methylated and inactive state of the transgene by bisulfite genomic sequencing of genomic DNA, collected from the cells at different time points (days 0, 2, 4, 6, 12, 15, 19, 60 and 90) after release from selection (Figure 2). We amplified a fragment covering the main CpG cluster of the β^A -globin promoter, including the transcription start site and part of the IL2R cDNA (Figure 2B). In total, 20 clones from five individual PCR reactions were sequenced in each case to determine the level of methylation of individual CpG sites (numbered 1–21). The patterns of CpG methylation among the 20 clones were similar from clone to clone (data not shown). At day 0, the 13-copy cell line was hypomethylated at CpG dinucleotides 1–10 in the promoter region (Figure 2B), previously shown to be critical for gene expression (Mutskov *et al.*, 2002). This region contains binding sites for transcription factors that regulate the β^A -globin promoter in erythroid cells (Figure 2B; Emerson *et al.*, 1985; Gallarda *et al.*, 1989). The CpGs 19–21, which are downstream of the transcription start site, were partially methylated and they apparently did not interfere with transcription (Mutskov *et al.*, 2002).

Following removal of selection, we observed a gradual increase in DNA methylation levels over the entire region, including CpGs 1–10, which were methylated in up to 80% of sequenced clones at day 90. Importantly, however, during the time of transcription inactivation (between days 2 and 12) the patterns of promoter methylation were very similar, with quite low levels of modification (Figure 2A): only 5–25% of sequenced clones were methylated at sites 1–10 at day 12, even though transcription was abolished. Site 8, lying close to the TATA box, was an exception with a moderate level of methylation (45%). However, this level did not increase beyond 45% until after all expression had been extinguished. In contrast, significant DNA methylation (25–100%) over the promoter was observed at each CpG site at much later times—days 90 (Figure 2) and 100 (Mutskov *et al.*, 2002). Based on these observations it appears that promoter DNA methylation follows transgene inactivation and suggests a role in maintenance, not establishment, of gene silencing.

The fact that the transgene seemed to be subject to CpG methylation mainly in the inactive state implied that other mechanisms were likely involved in the initial steps of repressed chromatin formation and silencing of gene expression. To explore this, we employed chromatin immunoprecipitation (ChIP) assays to assess the kinetics of two major histone modifications associated with chromatin transcriptional states, acetylation of histones H3 and H4 and K9 dimethylation of histone H3, during transgene silencing. At different time points (days 0 and 1, 5, 7, 10, 13, 17, 19 and 74

Figure 2 Bisulfite genomic sequencing analysis of a multicopy cell line. (A) CpG methylation over the promoter and transcription start site proximal region of the IL2R transgene. Genomic DNA collected from the cells at different time points were bisulfite-treated, PCR-amplified and subcloned. In total, 20 individual clones were sequenced to give the methylation pattern of the 21 CpGs. The percentage of methylated clones for each CpG is plotted. (B) Distribution of CpG dinucleotides over the transgene. A primer set was designed to amplify the region corresponding to the main CpG cluster on the promoter, containing CpG dinucleotides 1–21. The nucleosome hypersensitive region corresponds to a part of the chicken β^A -globin promoter where transcription factors (rectangles and ovals) are known to bind.

after selection), cells were crosslinked with low concentrations of formaldehyde (up to 0.3% final concentration), which allowed shearing of chromatin into small-sized fragments for high resolution. We designed 10 primer sets and TaqMan probes (Figure 3, bottom line, primer sets 1–10) spanning the entire transgene including the promoter, the IL2R cDNA, the splice/polyadenylation signal element and the enhancer. A rapid loss of histone acetylation correlated with the extinction of IL2R activity (Figure 3). Histone H3 deacetylation in the promoter and the coding region appeared to occur perhaps slightly earlier than histone H4 deacetylation. During the same time points, a gradual increase in histone H3 dimethylation at lysine 9 was also observed. However, similar to the DNA cytosine methylation, significant dimethylation at H3 K9 occurred after the gene was repressed.

We built kinetic curves for all the studied processes—DNA methylation, histone H3 and H4 acetylation and H3 K9 methylation—with each curve representing an individual

neighborhood on the transgene, and compared these with the RT-PCR curve for transcription activity. Figure 4 shows a kinetic view for different regions within the IL2R construct, where the maximum values for the IL2R activity and for the histone and DNA modifications were taken as 100%. Histone deacetylation was coupled with gene inactivation and its kinetics were similar in the 10 studied regions (primer sets 1–10) across the IL2R transgene (Figure 4A–D, data not shown). Except for the region spanned by primer set 3, which showed the fastest kinetics of H3 K9 dimethylation (but still with a relatively low level of modification at day 10), the rest of the characterized regions had between 15 and 40% of the maximally modified H3 K9 nucleosomes at day 10. This modification attained its maximum value long after the gene had been inactivated (days 19 and 74, Figures 3 and 4A–D).

The kinetic curves show that CpG methylation of the promoter region also occurs later than gene inactivation (Figure 4A–D, data not shown). CpG sites 1, 2 and 6 are presented in Figure 4A, B and C, respectively. Even CpG site

Figure 3 ChIP analysis of histone modifications in different regions of the IL2R transgene in a multicopy cell line. Cells carrying the transgene were fixed with formaldehyde at different time points and immunoprecipitated with antibodies against acetylated histones H3 and H4, dimethylated histone H3 K9 and nonimmune IgG. Primer sets 1–10 with TaqMan probes spanning the transgene were used to amplify the bound and input DNA. In parallel, these DNA samples were amplified with primers specific for the chicken β -globin locus as an internal control (primer sets #5.613 or #10.35, described in Litt *et al.*, 2001b). The values from the ChIPs were corrected by subtraction of the nonspecific signal derived from the normal rabbit IgG ChIP. We compared the relative abundance of transgene sequences to the chicken internal control sequences in each bound fraction versus the input fraction. The presented diagrams show the fold differences of the analyzed proteins, where the highest relative-difference data point set is equal to 1.0. A map of the IL2R transgene is presented with the positions of the different primer sets used. Positions of the various transcription-factor-binding sites over the promoter are shown in Figure 2.

1, the fastest to be methylated in the entire promoter, became methylated only after inactivation of expression was nearly complete (Figure 4A). Collectively, these data suggest that the primary mechanism of inactivation does not involve methylation of the promoter DNA or of histone H3 K9, since these events follow transgene repression. However, the high level of DNA methylation of some CpGs in the gene body is not changed during the time of gene inactivation or of histone deacetylation/methylation (Figure 4D, CpG site 21), consistent with the previous observation that gene body DNA methylation does not interfere with gene activity (Mutskov *et al.*, 2002).

There has been considerable interest recently in the association of methylation at lysine 4 of histone H3 with transcriptionally active chromatin (Litt *et al.*, 2001a; Santos-Rosa *et al.*, 2002), and particularly in the difference between the roles of the dimethylated and trimethylated states (see Discussion). We therefore repeated our kinetic ChIP analysis making use of antibodies specific for these two states. As shown in Figure 5A–C (and data not shown), the earliest events associated with the disappearance of the IL2R transcript once again involve histone modification at all the sites in the promoter and gene body. Both the di- and trimethylated

forms of H3 K4 disappear quite early, together with K9 acetylation. There is in fact some indication that loss of the dimethyl H3 K4 modification may occur slightly earlier than the other modifications (Figure 5A–C, data not shown), but the data do not permit a strong conclusion to be drawn.

The presence of multiple homologous copies of transgenes within an array may result in repeat-induced silencing, the mechanism of which is still unknown (Dorer and Henikoff, 1997). Copy number reduction in transgenic mouse lines caused a marked increase in expression of the transgenes and less compaction of the chromatin at the transgene locus (Garrick *et al.*, 1998). We were interested in knowing if a low-copy-number transgenic cell line would have the same kinetics of chromatin modifications during inactivation as already seen for the high-copy line. A single or a low copy number of IL2R reporters stably integrated in erythroid 6C2 cells were subjected to position effect silencing (Pikaart *et al.*, 1998; data not shown). However, most of these lines (but not all of them) lost expression at a slower rate as compared to the cell lines carrying a high copy number (data not shown). We performed kinetic experiments with a two-copy transgenic line, which had a gradual extinction of activity in culture (Figures 4E and 6A). At days 28–29, over 95 % of

the cells were IL2R inactive (Figure 6A) and, similar to the 13-copy line, at this time point the promoter DNA methylation was relatively low (Figures 4E and 6B,C). We determined the extent of CpG methylation for the low-copy line by *HpaII* digestion of four sites in the transgene promoter (Figure 6B), as well as by bisulfite analysis (Fig 6C). We obtained similar patterns of low-level methyl-CpG-dependent digestion (indi-

cating no increased CpG methylation at the *HpaII* sites) during days 1–29, the time during which transgene inactivation occurred in these lines (Figure 6B). Significant increases were observed only at days 100 and later. This result was confirmed by bisulfite genomic sequencing analysis (Figure 6C). Finally, we compared the kinetics of histone deacetylation, histone H3 K9 dimethylation and DNA methy-

Figure 5 Combined kinetics of disappearance of mRNA, loss of histone H3 acetylation and loss of histone K4 di- and trimethylation in the multicopy cell line. The relative times of occurrence of histone modifications when compared to loss of gene expression are similar to those observed in (Figure 4A–D). However, there are some differences in the time in culture at which observable silencing commences, possibly reflecting differences in the time of incubation before hygromycin selection is removed. (A) Combined kinetics for histone modification at promoter region 1 (primer set 1 in Figure 3, bottom line). (B) Kinetic curves for histone modification in promoter region 3 (see Figure 3, bottom line). (C) Combined data from the gene body at region 6 (see Figure 3, bottom line).

lation with gene activity (Figure 4E). The relationship between these chromatin modifications and gene repression for the two-copy IL2R cell line was similar to that already seen in the multicopy line (compare Figure 4A–C with Figure 4E).

Discussion

The four best-characterized hallmarks of repressed chromatin, histone H3 and H4 hypoacetylation, histone H3 K9

methylation and cytosine methylation clearly are closely linked (Bird and Wolffe, 1999; Ben-Porath and Cedar, 2001; Bird, 2002; Richards and Elgin, 2002), but their role in the dynamic transitions between transcriptionally permissive and transcriptionally silent chromatin remains to be defined. In this study we performed experiments to monitor the kinetics of the chromatin modifications that accompany the gradual silencing of a transgene integrated in chicken erythroid cells. The observed reduction in transcript abundance with time is not likely to reflect an alteration in the stability of

Figure 4 Kinetics of transgene inactivation, histone modifications and DNA methylation for multicopy (A–D) and low-copy-number (E) number cell lines. The kinetic curve for the transgene activity in the timing experiment is built based on the level of IL2R mRNA at different days (pink color line, mRNA). The kinetics of changes in histone H3 acetylation, H4 acetylation and H3 K9 dimethylation are made using the ChIP data, and are indicated as H3Ac (green color line), H4Ac (red color line), and H3Me (blue color line), respectively. The kinetics of methylation of an individual CpG site on the transgene accompanying the extinction of IL2R expression are presented as MeCpG (orange color line). The maximum values of mRNA level, histone H3 and H4 acetylation, histone K9 H3 methylation and individual CpG methylation are set to 100. Multicopy cell line: (A) Combined kinetics for histone modification at promoter region 3 (primer set #3 in Figure 3, bottom line) and methylation of CpG site 1 lying in the same region. (B) Combined data from the same region 3 but with the next CpG site: CpG site 2. (C) Kinetics for another region from the transgene promoter: primer set #4 (see Figure 3, bottom line) and CpG site 6. (D) Kinetics of DNA methylation of CpG site 21 from the gene body and the histone modifications kinetics at this region #6 (see Figure 3, bottom line). Low-copy-number cell line: (E) Kinetic curves for IL2R activity and histone modification in the promoter region #3. Changes in the DNA methylation state of the transgene promoter over time were determined by Southern blotting in Figure 5. Phosphor Imager analysis was performed to quantitate the intensity of the three bands on the blot for each time point. The band ‘a’ intensity is compared to the sum of the intensities of bands ‘a’, ‘b’ and ‘c’.

A

B

Bisulfite genomic sequencing analysis

C

Figure 6 Extinction of IL2R transgene activity and accompanying DNA methylation of a low-copy-number cell line. **(A)** FACS analysis of IL2R activity at day 0 (in the presence of hygromycin) and at days 8, 16, 23, 28 and 100 after removal of hygromycin. **(B)** Southern blots at different days in culture to determine the extent of DNA methylation at *HpaII* restriction sites in the transgene. The top diagram shows the positions of recognition sites for the methylation-sensitive enzyme *HpaII* within the IL2R transgene. Horizontal black lines with asterisks at the ends indicate the three products expected from complete digestion with the *HpaII* enzyme. The sites protected from digestion due to CpG methylation are indicated as M. The bottom panel shows DNA isolated at different days, digested with the *XbaI/HpaII* restriction enzymes and analyzed by Southern blotting after separation on an agarose gel. The membranes were hybridized with a *BamHI-XbaI* probe from the IL2R cDNA, indicated in the top diagram. **(C)** Bisulfite genomic sequencing analysis of a low-copy-number cell line at days 1, 11, 21, 29, 100 and 160. In total, 20 individual clones were sequenced to give the methylation pattern of the 21 CpGs. The percentage of methylated clones for each individual CpG is plotted.

the mRNA in transfected cells. As reported in earlier work (Pikaart *et al*, 1998), some transgenic 6C2 cell lines, carrying the identical reporter used here, maintain expression for long

periods of time after the removal of hygromycin selection marker, presumably because of effects of the integration site. Furthermore, these constructs surrounded by insulators ex-

Figure 7 Schematic representation of the sequence of events leading to silencing of a transgene stably integrated in the genome. Initially, the transgene is expressed and is not affected by the surrounding heterochromatin. The histones over the gene are acetylated (Ac) by HAT and the DNA is not methylated at the promoter region (open circles). After a period of propagation in culture, histone deacetylase activity (HDAC) is recruited to the transgene and the loss of histone acetylation is accompanied by transcription inactivation. Histone H3 that is di- or trimethylated at lysine 4 is also lost at this time. At the next step histone H3 K9 and DNA methyltransferases (HMT and DNMT) methylate their substrates (M for the histones and filled circles for the CpGs) in the already inactive transgene and form a more stable silenced state. Propagation of the heterochromatic state by heterochromatin protein 1 (HP1) and the binding of methyl-CpG-binding proteins (MBD) may ‘lock’ the silenced transgene.

press at high and constant levels for long periods of time (Pikaart *et al*, 1998).

Hypermethylation of promoter CpG islands is involved in the epigenetic silencing of genes on the inactivated X-chromosome, imprinted genes, tumor suppressor genes and exogenous integrated genes (Bird and Wolffe, 1999; Jones and Baylin, 2002). However, most of these observations were made on already repressed genes, leaving open the question of whether cytosine methylation is always a primary agent of

gene silencing or is in some cases a secondary modification affecting only the genes that have already been inactivated. The relationship of CpG methylation to gene silencing is an issue that has been raised by many investigators. In the case of the transgene silencing mechanism we describe here, the observation that promoter methylation occurs later than transcription inactivation implies that this epigenetic mark is involved in maintaining the inactive state of the chromatin rather than initiating it.

Other inactivation pathways

Under other circumstances, for transgenes in which the DNA had been methylated *in vitro* before transfection, methylation has been shown to serve an initiating function in the formation of repressed chromatin, leading to H3/H4 hypoacetylation (Eden *et al.*, 1998; Schubeler *et al.*, 2000) and H3 K9 methylation (Santoro *et al.*, 2002) after their integration into the genome. Both methylation-dependent and -independent mechanisms of repression have been observed in the case of retroviral expression in embryonic stem cells (Cherry *et al.*, 2000; Pannell *et al.*, 2000). In another example where gene silencing is independent of DNA methylation, it has been found that the tumor suppressor gene p16^{INK4a} can be silenced in cell lines carrying a double knockout of DNMT1 and DNMT3b (DNMT: DNA methyltransferase). These mutations reduce genomic DNA methylation by >95%, and result in demethylation and initial loss of silencing of the gene (Rhee *et al.*, 2002). However, the p16^{INK4a} gene is re-silenced with continued passage of the cells despite the absence of DNA methylation (Bachman *et al.*, 2003).

The situation with the genes on the inactivated X-chromosome is less clear. Early kinetic studies showed that the hypoxanthine phosphoribosyltransferase (Hprt) gene is silent on the inactive X-chromosome before DNA methylation occurs in intron 1 sequences. This was determined by digestion with cytosine methylation-sensitive restriction endonucleases (Lock *et al.*, 1987). However, the kinetic analyses of the CpG island in the same Hprt gene using the bisulfite genomic sequencing method revealed that methylation occurred at the initiation of X-chromosome inactivation (Park and Chapman, 1997).

Histone H3 methylation

Methylation of histone H3 K9 has so far been implicated in heterochromatin repression, promoter regulation and propagation of repressed chromatin (Litt *et al.*, 2001a; Nielsen *et al.*, 2001; Fahrner *et al.*, 2002; Nguyen *et al.*, 2002; Sacconi and Natoli, 2002; Kondo *et al.*, 2003). Our observation that histone H3 K9 dimethylation occurs over the IL2R reporter (Figures 3 and 4) also demonstrates that histone methylation is associated with silencing of the stably integrated transgenic constructs in eukaryotic cells.

This raises the question of whether DNA methylation in mammalian cells is dependent on the activity of histone methyltransferases (HMTs), as has been shown in *Neurospora* and *Arabidopsis* (Tamaru and Selker, 2001; Jackson *et al.*, 2002). It is known that DNMT3 proteins colocalize with HP1 (which binds to methylated histones) at the heterochromatic loci in embryonic stem cells (Bachman *et al.*, 2001); however, a direct site-specific role for histone methylation in the recruitment of DNMTs has not been established. The kinetic data presented here do not allow us to determine whether histone methylation over the promoter precedes DNA methylation. Both occur, within our limits of resolution, at about the same time. We note however that the coding region DNA of the IL2R transgene is largely methylated at early times despite the fact that H3 K9 dimethylation levels are low over the same region (Figure 4D; Mutskov *et al.*, 2002). This is connected to the fact that the IL2R transgene is still active at that point; as shown earlier (Mutskov *et al.*, 2002), there is no correlation between tran-

scriptional activity and DNA methylation over the coding region.

It appears from our data that dimethylation of H3 K9 lies downstream of histone deacetylation and transgene inactivation. This is not surprising because H3K9 can be either acetylated or methylated and, in the process of inactivation, deacetylation must happen first. Our observations of the timing of modifications over the gene promoter are not inconsistent with some recent data on the timing of X-chromosome inactivation (Heard *et al.*, 2001). The chromosome-wide enrichment of K9 H3 dimethylation is thought to be an early event, and marks a 'hotspot' important for the initial steps in the X-inactivation process. However, a ChIP experiment showed that the promoter regions of the X-inactivated genes MeCP2 and G6pd were enriched in dimethyl-K9 H3 later (by day 5 of differentiation) (Heard *et al.*, 2001), indicating a difference between chromosome-wide compared to promoter-specific methylation on the X-chromosome.

One of the goals of this investigation was to determine whether an increase in H3 K9 methylation, which is usually associated with silencing, preceded or followed inactivation of the transgene. It is possible that H3 K9 methylation is inhibited by methylation of lysine 4 at the same molecule. This interplay has been shown by *in vitro* experiments to be specific for Suv39h1 histone methyltransferase (Wang *et al.*, 2001; Nishioka *et al.*, 2002), but not for other H3 K9 methyltransferases: G9a (Nishioka *et al.*, 2002) and SETDB1 (Schultz *et al.*, 2002). However, we have also examined in separate experiments the parallel changes in H3 K4 methylation. This modification has been associated with transcriptionally active regulatory regions (Litt *et al.*, 2001a) and coding regions (Litt *et al.*, 2001a; Bernstein *et al.*, 2002). As shown in Figure 5, loss of the dimethylated and trimethylated K4 modifications occurs early in the inactivation process. This is certainly consistent with the fact (but does not prove) that K4 must be demethylated before K9 is methylated. The dimethylated and trimethylated K4 modifications are lost at similar rates over both the promoter and coding region of the IL2R reporter (Figure 5A–C, data not shown).

Histone modification and gene silencing

The early loss of methyl groups from H3 K4, and of acetyl groups from lysines on both H3 and H4 suggest that these modifications may play an important role in switching off gene transcription. It is important to emphasize that our data are consistent with such a role, but do not prove it. Our earlier results (Mutskov *et al.*, 2002) showed that silencing of the transgenic cell lines carrying the same reporter used here could be inhibited by growing the cells constantly in the presence of Trichostatin A, which blocks histone deacetylation. In contrast, the growth of cells in 5-aza-cytidine, an inhibitor of DNA methylation, did not affect the rate of inactivation, suggesting that silencing of the transgene was not controlled by this modification (Mutskov *et al.*, 2002). Thus, maintenance of acetylation is at least sufficient to prevent inactivation. It has been established that specific histone acetylation patterns mediate regulatory factor interactions critical for gene activity (Cosma, 2002; Emerson, 2002; Turner, 2002). The constant recruitment of proteins that carry histone acetyltransferase (HAT) activity and maintain hyperacetylated histones can help prevent switching to

the inactive state. Insulator elements such as the HS4 chicken β -globin insulator also help maintain a high level of histone acetylation over the protected region (Pikaart *et al*, 1998; Mutskov *et al*, 2002). The promoter of the gene adjacent to the insulator is more accessible to transcription factors, and the binding of these factors in turn protects the promoter DNA from methylation (Mutskov *et al*, 2002). Immunity to DNA methylation caused by active chromatin was also described for locus control regions (Santoso *et al*, 2000) and enhancer elements (Schubeler *et al*, 2000; Mutskov *et al*, 2002), which are tissue-specific centers for high levels of histone acetylation.

Our parallel kinetic studies of the changes in transgene expression, histone acetylation and methylation, and DNA methylation suggest a sequence of events in which loss of histone acetylation and H3 K4 di- and trimethylation are early steps in the sequence of events leading to transgene silencing (Figure 7, step 1). At this stage the gene is repressed, but still reversibly, because transcription can be partially reactivated by inhibition of histone deacetylases (TSA) (Pikaart *et al*, 1998). Methylation of histone H3 K9 and of CpG sites on promoter DNA are later events (Figure 7, step 2). These two chromatin modifications may target HP1 and MBD-protein repressor complexes that could 'lock' this stable silenced chromatin state (Figure 7, step 3); in any case, the presence of promoter CpG methylation almost certainly implies inability to return to an active state. Moreover, histone and DNA methylation marks may signal to one another to ensure propagation of this more compact form of chromatin. We previously observed binding of MBD proteins to the silenced IL2R transgene at late stages (Mutskov *et al*, 2002), which could reinforce the histone modification patterns via recruitment of HDACs (Bird and Wolffe, 1999) and H3 K9 histone methylase activity (Fuks *et al*, 2003).

Although our specific results may serve as a more general model for the silencing of transgenes stably integrated into a cell or animal, we do not believe that they necessarily provide a universal mechanism for the regulation of endogenous genes, in which the order of events for gene silencing is likely to be under much stricter control. For example, the endogenous chicken embryonic ρ -globin gene remains associated with hyperacetylated histones at day 15 of differentiation, even though this gene starts to be inactivated after day 5 and transcription is barely detected at day 15 (Hebbes *et al*, 1992; Litt *et al*, 2001b). Recent results have indicated that transcription activation in eukaryotes requires the recruitment of chromatin remodeling and histone modification factors, but the order and the recruitment timing are gene-specific events (Cosma, 2002; Emerson, 2002). Our results show however that promoter DNA methylation and histone H3 K9 dimethylation are not early events in the silencing of a transgene. Extinction of expression is instead correlated with loss of histone acetylation and of H3 K4 methylation. It seems reasonable to focus future studies on one or both of these early events as primary causes of silencing.

Materials and methods

Stable transfection in cell culture and FACS analysis

The IL2R reporter construct under the control of the chicken β^A -globin promoter and β/ϵ enhancer has been described previously (Pikaart *et al*, 1998). 6C2 is a CFU-E stage erythroid precursor cell

line, obtained by transformation of chicken bone marrow with a wild-type avian erythroblastosis virus (AEV). 6C2 cells were grown as described (Boyes and Felsenfeld, 1996) and stably transfected with the reporters in the presence of DNA fragments encoding the hygromycin-resistant gene derived from the pREP 7 plasmid (Invitrogen), by electroporation (Boyes and Felsenfeld, 1996), or with effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Individual hygromycin-resistant colonies were picked after 2–3 weeks of culture in α -MEM plus 2% methocel and 2000 U/ml hygromycin (Calbiochem), and expanded in hygromycin-containing medium (1250 U/ml) for a further 6 days. IL2R expression was monitored by FACS analysis as described (Pikaart *et al*, 1998) using a FACSCalibur immunocytometer (Becton Dickinson). Cells were maintained in log-phase growth for all experiments.

Determination of transgene copy number in stably transfected cell lines

Genomic DNA from the stably transfected 6C2 cell lines was isolated by a standard procedure (Sambrook *et al*, 1989) and series of two-fold dilutions were made. Equal amounts of DNA from each point of the double dilutions were analyzed by real-time PCR, in parallel with two different primer sets with a very similar efficiency of amplification. The first set of primers, including a TaqMan probe, was specific for the IL2R transgene (primer set IL2R2800 described in Mutskov *et al*, 2002) and the second set amplified the endogenous folate receptor gene (primer set FolateExon 4, described in Mutskov *et al*, 2002). The number of integrated transgenes was estimated by comparison of the IL2R signal to the signal from one copy of the endogenous gene (half of the total folate receptor gene signal). As a control, the same experiment was performed with genomic DNA isolated from nontransfected 6C2 cells.

Quantitative TaqMan RT-PCR assay

Total RNA from transfected 6C2 cells was isolated at different time points (see the Results section) and reverse transcribed as described previously (Mutskov *et al*, 2002). A series of two-fold dilutions of the cDNA products were made, and equal amounts of DNA were amplified by real-time PCR using specific primers and TaqMan probes for the IL2R transgene and the folate receptor gene as described (Mutskov *et al*, 2002).

Southern blot hybridization

Isolation of high molecular weight genomic DNA was performed by a standard method (Sambrook *et al*, 1989). In total, 10 μ g of each DNA sample was subjected to double digestion with the restriction endonucleases *Xba*I and the DNA methylation-sensitive *Hpa*II. The digested DNA was resolved on an agarose gel, blotted on to Hybond-N⁺ nylon membrane (Amersham) and hybridized with an IL2R probe in QuikHyb solution (Stratagene) according to the manufacturer's protocol.

Bisulfite genomic sequencing

Bisulfite conversion of DNA was carried out by the method developed by Clark *et al* (1994) with minor modifications described previously (Mutskov *et al*, 2002). Each bisulfite-modified DNA sample was subjected to PCR reactions using primers specific for the bisulfite-converted sequence of the IL2R transgene (primer set C1–C2, described in Mutskov *et al*, 2002). Amplification, PCR product purification and subcloning were carried out as described (Mutskov *et al*, 2002). Individual clones were sequenced using M13 reverse or M13 forward primers and the BigDye terminator cycle sequencing kit (PE Applied Biosystems) and applied to an ABI PRISM 310 DNA sequencer (Perkin Elmer).

Formaldehyde crosslinking and ChIP

Formaldehyde crosslinking *in vivo*, sonication of the chromatin and chromatin immunoprecipitation assays were performed as described previously (Mutskov *et al*, 2002). At different time points of our experiment (see the Results section), 6C2 cells stably transfected with the IL2R transgene were fixed with 0.3% of formaldehyde at room temperature for 8 min, followed by incubation at 4°C for another 30 min. After isolation of nuclei, sonication and lysis with 1% SDS, the soluble chromatin was pre-cleared with salmon sperm DNA/protein A agarose (Upstate Biotechnology) before the immunoprecipitation procedure. Different antibodies

purchased from Upstate Biotechnology were used for ChIP: acetylated histone H3 antibody, acetylated histone H4 antibody, dimethyl histone H3 (K9) antibody, dimethyl histone H3 (K4) antibody, trimethyl histone H3 (K4) antibody as well as normal rabbit IgG in the control experiments.

Quantitative real-time PCR

DNA samples from input (In) and antibody-bound (IP) chromatin were analyzed by real-time PCR using the TaqMan Universal PCR Master Mix (PE Applied Biosystems) and an ABI Prism 7700 sequence detector according to the manufacturer's protocols. In total, 10 primers and TaqMan probes were selected from the IL2R transgene. Their sequences have been described previously (Mutskov *et al.*, 2002): primer set #1 as IL2R1060, primer set #2 as IL2R1420, primer set #3 as IL2R1900, primer set #4 as IL2R2020, primer set #5 as IL2R2140, primer set #6 as IL2R2560, primer set #7 as IL2R2800, primer set #8 as IL2R3160, primer set #9 as IL2R3640, and primer set #10 as IL2R3880. Individual PCRs were carried out in triplicate to control for PCR variation and the C_t values were collected. Data quantification was performed by applying the comparative C_t method, as described by the manufacturer's protocols (PE Applied Biosystems), giving the fold difference of a target sequence (t) in the IP fraction versus a fixed amount of In DNA as a standard, where

$$IP/In = 2^{-\Delta\Delta C_t} = 2^{-(C_t(IP) - C_t(In))}.$$

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For each primer set, these fold difference values were corrected by subtraction of the nonspecific signal derived from the nonimmune rabbit IgG ChIP (t_0):

$$(IP/In)^t - (IP/In)^{t_0}.$$

In parallel, DNA samples were amplified with primers specific for the chicken β -globin locus as an internal control (c) (primer sets #5.613 or #10.35, described in Litt *et al.*, 2001b). Finally, we normalized the relative abundance of transgene sequences to the chicken internal control sequence for each individual chromatin immunoprecipitation reaction using the following formula:

$$[(IP/In)^t - (IP/In)^{t_0}] / [(IP/In)^c - (IP/In)^{c_0}].$$

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