Histone Modifications are Associated with the Persistence or Silencing of Vector-mediated Transgene Expression *In Vivo*

Efren Riu[1,2], Zhi-Ying Chen[1,2], Hui Xu[1,2], Chen-Yi He[1,2] and Mark A Kay[1,2]

[1]*Department of Pediatrics, Stanford University School of Medicine, Stanford, California, USA;* [2]*Department of Genetics, Stanford University School of Medicine, Stanford, California, USA*

One of the major obstacles to success in non-viral gene therapy is transcriptional silencing of the DNA vector. The mechanisms underlying gene silencing/repression in mammalian cells are complex and remain unclear. Because changes in chromatin structure and, in particular, histone modifications are involved in transcriptional regulation of endogenous genes, we hypothesized that changes in the pattern of histone modifications were related to the observed transcriptional silencing of exogenous DNA vectors. We used antibodies against specific modified histones to perform chromatin immunoprecipitation (ChIP) analyses on liver lysates from mice transfected with two types of plasmids: (i) DNA minicircles (MCs) devoid of bacterial plasmid backbone DNA, which showed marked persistence of transgene expression, and (ii) their parental plasmids, which were silenced over time. Silencing of the transgene from the parental vectors was accompanied by an increase in heterochromatin-associated histone modifications and a decrease in modifications typically associated with euchromatin. Conversely, the pattern of histone modifications on the MC DNA was consistent with euchromatin. Our data indicates that (i) episomal vectors undergo chromatinization *in vivo,* and (ii) both persistence and silencing of transgene expression are associated with specific histone modifications.

Received 13 November 2006; accepted 20 March 2007; published online 24 April 2007. doi:10.1038/sj.mt.6300177

INTRODUCTION

Gene transfer as a therapeutic strategy for the treatment of diverse human diseases requires the ability to achieve persistent and efficacious levels of gene expression *in vivo*. Traditionally, most gene transfer protocols in animals and humans have been performed with viral-based vectors but the use of non-viral based vectors is an alternative that is gaining in popularity. In addition to having low toxicity these vectors are stable and autonomous *in vivo*, forming episomes without integration into the host genome.[1,2] One obstacle that has been encountered with episomal vectors has been silencing of the ectopic gene in quiescent tissues.[1,2] It is believed that if this limitation can be circumvented, gene transfer using non-viral episomal vectors will have tremendous therapeutic potential.

In eukaryotes, genomic DNA is tightly associated with histones and other nuclear proteins to form chromatin.[3] Depending on the level of compaction or state of the chromatin, the DNA is either accessible or inaccessible to *trans*-acting factors that carry out fundamental nuclear processes such as gene transcription, repair, or silencing.[4,5] A key molecular mechanism for regulating chromatin accessibility is achieved by dynamic post-translational covalent modification of the N-terminal tails of histone proteins. Examples of such modifications include acetylation, methylation, phosphorylation, and ubiquitination.[4,5] Certain combinations of these modifications have a profound impact on transcriptional regulation.[5] The recognition of the dynamic interplay between such histone modifications led to the "histone code" hypothesis. The hypothesis is that specific patterns of histone modifications are recognized by different DNA and chromatin effector molecules, and that these activities in turn initiate downstream pathways such as gene silencing.[6] In this way, molecular structures on chromatin are translated by the histone code into distinct functional outcomes. Specifically, epigenetic hallmarks of silenced or heterochromatic DNA include hypoacetylation of histone tails, histone H3 methylation at lysine residue 9 (H3K9me), and recruitment of heterochromatin protein 1-α (HP1α).[4,5] In contrast, open or active chromatin is associated with acetylated histones as well as methylation at the lysine 4 residue of histone H3 (H3K4).[4,5] Recent data have shown that while di-methylated H3K4 (H3K4me2) is found distributed throughout the entire gene, tri-methylated lysine 4 (H3K4me3) and acetylated K9 + K14 of histone H3 are both highly localized to the 5′ regions of transcriptionally active human genes, thereby suggesting that mammalian promoters have chromatin configurations similar to those described in yeast.[7,8] In addition, when histone 3 phosphorylation on serine 10 and acetylation on lysine 9/14 are present, H3K9 methylation cannot occur and HP1α is not recruited, thereby suggesting that phosphoacetylation of histone H3 could be a general mechanism allowing the cell to overcome

Correspondence: *Mark A. Kay, Departments of Pediatrics, Stanford University School of Medicine, 300 Pasteur Drive, Rm G305, Stanford, California 94305-5208, USA. E-mail: markay@stanford.edu*

HP1α-mediated transcriptional repression.[9,10] While a great deal of progress has recently been made in understanding gene silencing mechanisms in the context of chromatin structure on genomic DNA, there is virtually no information regarding the nature of chromatin structure on exogenously added episomal DNA in the tissues of whole animals, and its impact on gene transfer.

In previous work, through several *in vitro* and *in vivo* alterations of DNA components in both the expression cassette and the bacterial backbone, we and others have demonstrated that sequences present in episomal vectors that originate from bacteria, and that are linked covalently to the expression cassette, play a critical role in transcriptional silencing of transgenes *in vivo*.[11–16]

Nonetheless, in order to overcome silencing, we have developed vectors devoid of bacterial DNA sequences, named minicircles (MCs), that display more robust and stable gene expression *in vivo* compared with the parental plasmid vector.[15,17] On the basis of these findings, we hypothesize that the chromatinization in these non-viral vectors will affect the pattern of expression and will have an impact on gene transfer/therapy by packaging the episomal DNAs into a different euchromatic/heterochromatic state.

Thus, in this report we aim to identify important chromatin modifications in an attempt to begin to explain the mechanisms involved in regulating transgene silencing *in vivo*, and to unravel the molecular mechanisms by which non-viral gene transfer vectors are silenced. In the long term, this work will be helpful in developing new tools and strategies to achieve persistent gene expression in a clinical setting.

RESULTS

Histone modification patterns in the pBSRSVhAAT plasmid-MC system *in vivo*

We hypothesized that differences in chromatin structure/histone modification between the different DNA molecules might influence the silencing/activation of episomal vectors *in vivo*. In order to study this, we evaluated the *in vivo* chromatinization of episomal liver DNA from tissue lysates after administering the RSVhAAT plasmid (P) and MC system into mice (**Figure 1a**). As we have previously reported, after *in vivo* administration of these two DNA molecules, expression from the parental vector was silenced over a period of a few weeks (**Figure 1b**). In contrast, the MC RSVhAAT, which lacks bacterial plasmid backbone DNA, displayed a 100-fold higher level of transgene expression compared to the parental plasmid (**Figure 1b**). In line with what we found earlier,[15,16] no differences in the vector DNA copy number in the livers of these mice were found, thereby excluding the possibility that the differences in transgene expression observed between the experimental groups result from different amounts of vector DNA (P = 7.8 ± 1 and MC = 8.1 ± 2 human α-1-antitrypsin (hAAT)/glyceraldehyde-3-phosphate dehydrogenase relative copies). With this system in hand, we further aimed to determine whether histones associate with episomal vectors *in vivo,* and whether the presence of histone modifications that are predicted to accompany inaccessible chromatin structure might lead to gene silencing of these exogenous vectors *in vivo*. To this end, we used a battery of chromatin immunoprecipitation (ChIP) experiments to map the pattern of histone modifications in our plasmid and MC systems.

In order to determine whether the development of the histone association on episomal DNA occurs over time and is parallel to the transgene silencing/activation, we analyzed the level of euchromatin and heterochromatin markers on the DNA molecules in mice injected with the RSVhAAT plasmid and MC system, at early and late time points after DNA administration. On day 1 after the injection, we did not detect significant differences between the two DNA species isolated from mice for any of the chromatin markers analyzed (**Figure 1c–f**). This possibly indicates that part of the exogenous DNA was not forming fully appropriate native chromatin, or that some of the DNA was not in the appropriate nuclear compartment required for transcription. Similar results were observed 7 days after the DNA administration when ChIP was performed for detection of several histone modifications, such as H3Ac and H4Ac (data not shown).

In contrast, 35 days after the DNA administration, mice injected with the parental plasmid RSVhAAT showed an enrichment of approximately twofold to fivefold when compared with the MC injected mice, for most of the heterochromatin markers analyzed, namely, di- and tri-methylation in lysine 9 on histone 3 (H3K9me2 and H3K9me3, respectively), di- and tri-methylation on lysine 20 of histone 4 (H4K20me2 and H4K20me3, respectively), histone deacetylase 2 (HDAC2), HP1α and the H3K9 methylase SUV39H1, the human ortholog of *Drosophila* gene [*Su(var)3-9*] associated with the promoter and complementary DNA (cDNA) coding region (**Figure 1c** and **e**). Low levels of heterochromatin markers were also found in the MC samples, possibly due to the presence of some parental plasmid contaminants in the MC preparation. All these markers have been linked to chromatin modification and transcriptional repression (see ref. 5). These results were consistent with the episomal transgene silencing *in vivo* expression data observed in these mice.

Conversely, in similar experiments using MC DNA there was a twofold to sixfold increase compared with the parental plasmid DNA, in euchromatin histone modification markers:acetylation in histones 3 and 4 (H3Ac and H4Ac, respectively), di- and tri-methylation in lysine 4 on histone 3 (H3K4me2 and H3K4me3, respectively), di-methylation on lysine 79 of histone 3 (H3K79me2) and acetylation of lysine 9 and phosphorylation in serine 10 on histone 3 (H3K9Ac + S10P), (**Figure 1d** and **f**). These markers have been linked to euchromatin domains and transcriptional activation (see ref. 5). These results therefore suggest that persistent and sustained transgene expression is associated with an open chromatin status.

ChIP real-time polymerase chain reaction analysis in EF-1-α-hAAT plasmid/MC system injected mice

In order to establish that these results were not due only to a particular structure of the RSVhAAT transgene, we performed ChIP polymerase chain reaction (PCR) analysis on liver lysates using another plasmid/MC system. We injected into mice the elongation factor 1-α (EF1-α) promoter[18] driving hAAT expression in the plasmid/MC system (**Figure 2a**). As in the previous plasmid/MC system, hAAT expression from the parental vector pBSEF-1hAAT (P) was silenced over a period of a few weeks. However,

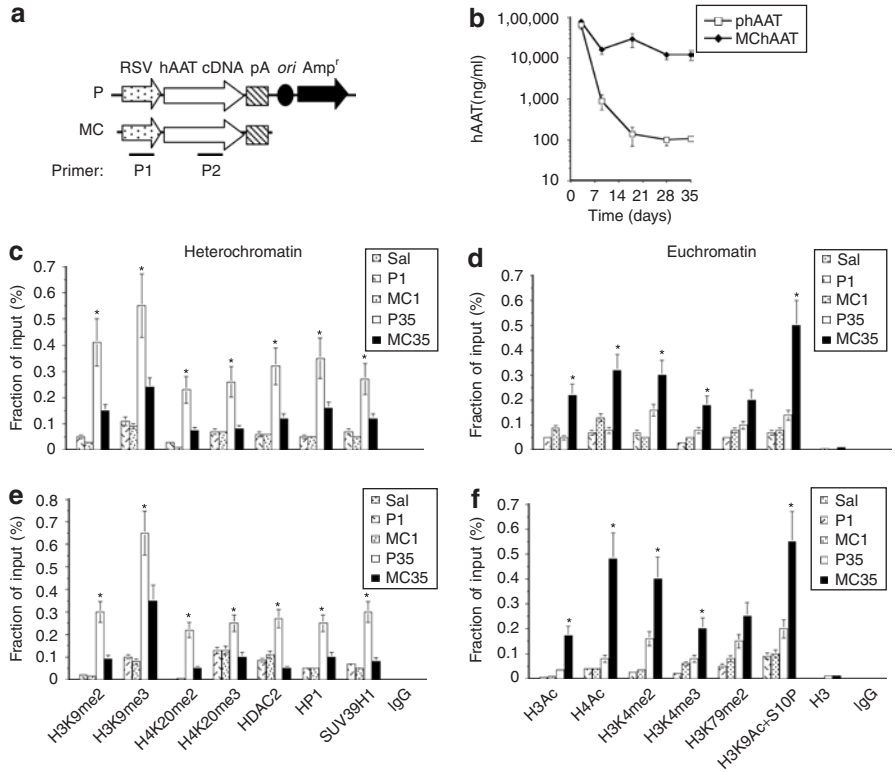

Figure 1 Chromatin immunoprecipitation (ChIP) analysis on parental plasmid (P) and MC RSV/hAAT transgene (MC). (**a**) Scheme of the RSV/hAAT plasmid (P) and MC system used for injection into mice. The location of the two pair of oligos (P1 and P2) used for quantification of ChIP data using real-time quantitative polymerase chain reaction (qPCR), as described in Materials and Methods (*ori,* plasmid origin of replication; Amp^r, ampicillin resistance gene; pA, bovine growth hormone polyadenylation signal) are shown. (**b**) Serum hAAT levels in mice injected with 20 µg of the pBSRSV/hAAT plasmid (phAAT) and 8 µg of the equivalent minicircle (MChAAT). (**c–f**) ChIP analysis using real-time qPCR of heterochromatin and euchromatin markers (see text for abbreviations), from saline control mice (Sal), plasmid (P) and minicircle RSVhAAT (MC) liver lysates on days 1 and 35 after the injection. (**c**) qPCR of heterochromatin markers using Primer P1 (RSV promoter); (**d**) qPCR of euchromatin markers using Primer P1 (RSV promoter); (**e**) qPCR of heterochromatin markers using Primer P2 (hAAT complementary DNA (cDNA)); (**f**) qPCR of euchromatin markers using Primer P2 (hAAT cDNA). The data represent the mean ± SEM of three independent experiments. An "*" indicates $P < 0.05$ between the P35 and MC35 groups.

the MC EF1-α hAAT vector displayed an approximately sixfold higher transgene expression when compared with the parental vector (**Figure 2b**). As before, no differences were found between the experimental groups in the vector DNA copy number in the livers (P = 5.8 ± 1 and MC = 4.6 ± 1 hAAT/glyceraldehyde-3-phosphate dehydrogenase relative copies).

After performing the ChIP, the EF1-α promoter and hAAT coding sequences were quantified by real-time PCR using specific primers (depicted in **Figure 2a**). As in the previous plasmid/MC system, we could not detect significant differences between the two DNA species in injected mice for any of the euchromatin/heterochromatin markers analyzed 1 day after the injection (**Figure 2c–f**). However, 35 days after administration of the DNA, mice injected with the parental plasmid EF1-α/hAAT showed an increase (approximately 1.5- to 3-fold) in the heterochromatic histone markers analyzed (H3K9me2, H3K9me3, H4K20me2 and HP1α) when compared with the MC injected mice, both in the promoter and the cDNA region of the DNA molecule (**Figure 2c** and **e**). These results were also concordant with the observed episomal transgene silencing *in vivo*.

In contrast, higher amounts (1.5- to 4-fold) of euchromatin histone modification markers were observed on the MC EF1-α hAAT DNA (H3Ac, H4Ac, and H3K4me2), as compared to its parental plasmid (**Figure 2d** and **f**). All these results suggest that persistent and sustained expression of a transgene correlates with an open chromatin state.

Chromatin profiling of persistent plasmid and MC DNA system *in vivo*

For the purpose of further study, and in order to characterize the effects of chromatin modification on exogenous plasmid DNA and its correlation with gene transcription, we injected into mice a third plasmid and MC system, based on the human cellular Ubiquitin C promoter (UBI)/hAAT transgene expression cassette (**Figure 3a**). In contrast to the previous two plasmid/MC models, in which the plasmid DNA was silenced a few weeks after administration, the Ubiquitin C promoter is a strong ubiquitous mammalian promoter that has been shown to maintain persistent transgene expression.[19] In fact, of the many expression cassettes tested to date,[18] this is the only one we have observed that was not transcriptionally silenced over time. As shown in **Figure 3b**, both plasmid (P) and MC UBI/hAAT showed very high and persistent levels of serum hAAT over 35 days, which was the duration of the experiment. As observed in the previous

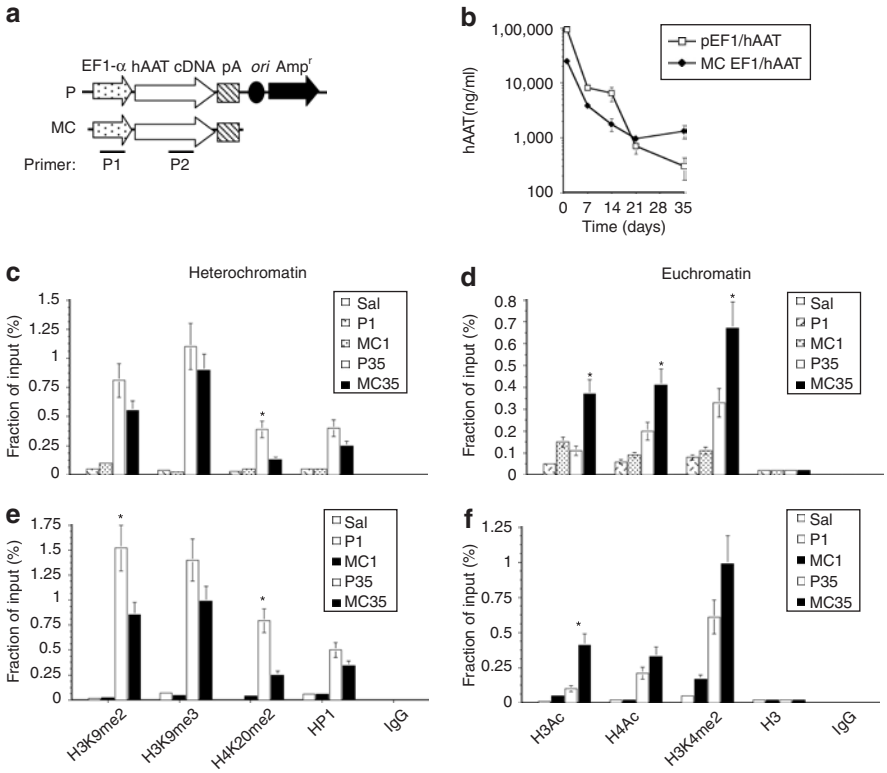

Figure 2 Chromatin immunoprecipitation (ChIP) analysis on parental plasmid (P) and MC EF1-α/hAAT transgene (MC). (**a**) Scheme of the EF1-α/hAAT plasmid (P) and MC system used for injection into mice. The location of the two pairs of oligos (P1 and P2) used for quantification of ChIP data using real-time quantitative polymerase chain reaction (qPCR), as described in Materials and Methods (*ori*, plasmid origin of replication; Amp^r, ampicillin resistance gene; pA, bovine growth hormone polyadenylation signal) are shown. (**b**) Serum hAAT levels in mice injected with 20 µg of the pBSEF1α/hAAT plasmid (pEF1/hAAT) and 8 µg of the equivalent minicircle (MC EF1/hAAT). (**c–f**) ChIP analysis using real-time qPCR of heterochromatin and euchromatin markers (see text for abbreviations), from saline control mice (Sal), plasmid (P), and minicircle EF1-α/hAAT (MC) liver lysates on days 1 and 35 after the injection. (**c**) qPCR of heterochromatin markers using Primer P1 (EF1-α promoter); (**d**) qPCR of euchromatin markers using Primer P1 (EF1-α promoter); (**e**) qPCR of heterochromatin markers using Primer P2 (hAAT complementary DNA (cDNA)); (**f**) qPCR of euchromatin markers using Primer P2 (hAAT cDNA). The data represent the mean ± SEM of three independent experiments. An "*" indicates $P < 0.05$ between the P35 and MC35 groups.

plasmid and MC systems, no significant differences were found between the experimental groups in the vector DNA copy number in the mice livers (P = 12 ± 3 and MC = 7 ± 2 hAAT/glyceraldehyde-3-phosphate dehydrogenase relative copies).

Since both parental plasmid and MC showed persistent expression we hypothesized that, if chromatin modifications underlie gene transcription, euchromatin markers should be predominantly detected in both DNA molecules. As in the earlier plasmid/MC models, 1 day after the DNA administration no differences were observed between the two DNA species in injected mice in any of the euchromatin/heterochromatin markers that were analyzed (**Figure 3c–f**). Not surprisingly, low levels of heterochromatin histone modification markers involved in gene repression were observed in these DNAs (**Figure 3c** and **e**) 35 days after DNA injection while very high levels of euchromatin markers were observed on both plasmid and MC DNAs [H3Ac, H4Ac, H3K4me2, H3K4me3, di- tri-methylation in lysine 36 on histone 3 (H3K36me2 and H3K36me3), and H3K79me2] in both the promoter and cDNA regions of the DNA molecule (**Figure 3d** and **f**). All these results also indicate that persistent transgene expression correlates with an open/active chromatin state.

Correlation between histone modifications and gene expression in the different plasmid/MC DNA systems

In order to be able to make direct comparisons among the chromatin status of the three plasmid/MC vector genomes in the injected mice, we determined the ratio of DNA associated with H3K9me2 to that with H3K4me2 in these samples.[20] H3K9me2 and H3K4me2 have been reported to localize in heterochromatin and euchromatin domains, respectively.[5] The ratio was calculated with each primer pair (**Figure 4a**) with samples obtained 1 day and 35 days after the injection. We predicted that the higher the ratio, the greater would be the degree of transcriptional inactivation. In agreement with the expression data, the silenced parental plasmid DNAs (the Rous sarcoma virus (RSV) and EF1-α cassettes) showed a markedly increased H3K9me2 to H3K4me2 ratio when compared with the corresponding MCs using the promoter (**Figure 4b**, left panel) or hAAT cDNA as a probe (**Figure 4b**, right panel). In contrast, when analyzing the MC samples, lower ratios of H3K9me2 to H3K4me2 were observed in comparison with the parental plasmid injected mice, thereby indicating that MC associates preferentially with transcriptionally active H3K4me2. Thus, taken altogether, our results suggest that silencing of the transgene from the parental

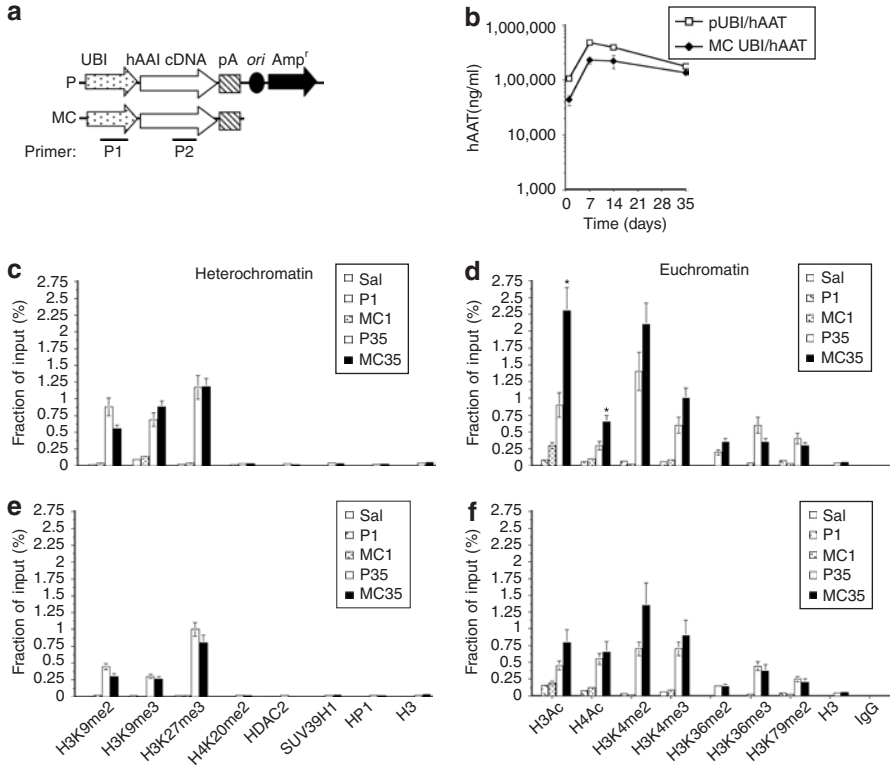

Figure 3 Chromatin immunoprecipitation (ChIP) analysis on parental plasmid (P) and MC UBI/hAAT transgene (MC). (**a**) Scheme of the UBI/hAAT plasmid (P) and MC system used for injection into mice. The location of the two pairs of oligos (P1 and P2) used for quantification of ChIP data using real-time quantitative polymerase chain reaction (qPCR), as described in Materials and Methods (*ori*, plasmid origin of replication; Amp^r, ampicillin resistance gene; pA, bovine growth hormone polyadenylation signal) are shown. (**b**) Serum hAAT production in mice injected with 20 µg of the pUbiquitin/hAAT plasmid (pUBI/hAAT) and 10 µg of the equivalent minicircle (MC UBI/hAAT). (**c–f**) ChIP analysis using real-time qPCR of heterochromatin and euchromatin markers (see text for abbreviations), from saline control mice (Sal), plasmid (P) and minicircle UBI/hAAT (MC) liver lysates on days 1 and 35 after the injection. (**c**) qPCR of heterochromatin markers using Primer P1 (UBI promoter); (**d**) qPCR of euchromatin markers using Primer P1 (UBI promoter); (**e**) qPCR of heterochromatin markers using Primer P2 (hAAT complementary DNA (cDNA)); (**f**) qPCR of euchromatin markers using Primer P2 (hAAT cDNA). The data represent the mean ± SEM of three independent experiments. An "*" indicates $P < 0.05$ between the P35 and MC35 groups.

vector was accompanied by an increase in heterochromatin-associated histone modifications and a decrease in modifications typically associated with euchromatin. Conversely, the pattern of histone modifications on the MC DNA was consistent with euchromatin.

DISCUSSION

In this study we established that episomal vectors undergo chromatinization after transfection of mouse liver. Interestingly, our data strongly support the proposition that successful expression of the transgene is critically dependent on the type of chromatin structure that forms on the exogenous DNA, in a manner similar to that observed in the mammalian genome. Thus, silencing of the transgene from the parental plasmid vector is accompanied by an increase in heterochromatin-associated histone modifications (H3K9 and H4K20 methylation) and in proteins found in heterochromatin domains (HDAC2, HP1α and SUV39H1), as well as a decrease in modifications typically associated with euchromatin. Further, the pattern of histone modifications on transcriptionally active plasmid and MC DNAs devoid of any bacterial plasmid DNA sequence, is consistent with euchromatin, because increased euchromatin-associated histone modifications (H3/H4 acetylation and H3K4/36/79 methylation) were found at high concentrations. The results from these studies are consistent with the hypothesis that histone modifications underlie gene regulation on exogenous DNA and are responsible for the observed differences in transcriptional silencing/activation of plasmid/MC DNAs *in vivo*. These results may explain many of our other observations, including the requirement for a covalent connection between the bacterial DNA and the expression cassette for the silencing to occur.[18,21] The mechanism of silencing appears to be primarily at the transcriptional level. This is evident from our preliminary real-time reverse transcriptase (RT) studies (not shown), that indicate large differences in the messenger RNA levels correlating with the differences in transgene product observed in MC versus parental plasmid delivery into the liver. However, we cannot, at this point, formally exclude the possibility that some other mechanism may contribute to transgene silencing.

In mammalian cells, transcriptional gene silencing is most often related to hypermethylation of the promoter region and a closed chromatin conformation associated with specific histone modifications.[4,6,22,23] H3K9me and H4K20me have been associated with silencing of euchromatic genes and are typical

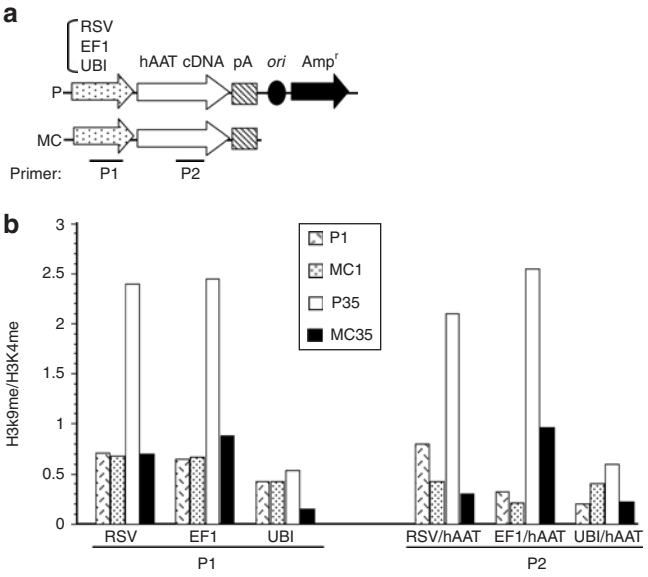

Figure 4 Time course modifications in chromatin on two regions of the three plasmid/minicircle (MC) systems injected into mice. (**a**) Scheme of the three plasmid (P)/MC models and the approximate locations of the two pairs of oligos (P1 and P2) used for quantification of chromatin immunoprecipitation data using real-time quantitative polymerase chain reaction (qPCR), as described in Materials and Methods. (**b**) Ratio of DNA associated with H3K9me2 to that associated with H3K4me2 was calculated for the promoter (left) and the hAAT complementary DNA (cDNA) (right) regions on plasmid (P) and MC on days 1 and 35 after the injection. The higher the ratio, the lower the level of transcription. EF-1, elongation factor 1; RSV, Rous sarcoma virus; UBI, ubiquitin C; Amp^r, ampicillin resistant.

of heterochromatin regions.[5,22,24] In addition, H3K9me and histone deacetylation by HDAC2 can function independent of CpG methylation to initiate the formation of heterochromatin by recruiting other non-histone proteins, primarily the complexed SUV39H1 and HP1α.[5,25–27] For instance, HDACs, SUV39H1, the enzyme that methylates H3K9 residues[28,29] and HP1α are tightly associated, and are found mostly in heterochromatin regions.[30,31] Indeed, the SUV39H1 and HP1α complex stabilizes and propagates the H3K9me epitope to adjacent nucleosomes, causing the spreading of heterochromatin and resulting in gene silencing.[32–34] Similarly, increased levels of heterochromatin markers such as H3K9me and H4K20me, as well as HDAC2, SUV39H1 and HP1α, were found in our silenced plasmid systems. Therefore, on the basis of our findings and on those from previous studies on heterochromatin formation in mammalian and non-mammalian systems, we hypothesize that silencing of exogenous plasmid DNA *in vivo* may follow a similar pattern to that observed in other systems. Histones newly deposited after DNA administration become acetylated, and modifications may continue to occur at any time by a mechanism which is as yet unknown.[35] On the basis of our data, we predict that in parental plasmid DNA, deposited histones become deacetylated in H3K9 residues by HDAC2. Because of the association of SUV39H1 and HP1α with the HDAC complex, the H3K9 deacetylated residues become methylated by SUV39H1. In conjunction with HP1α, heterochromatin is further propagated to adjacent plasmid DNA regions in a manner similar to the one described earlier for transcriptional silencing in mammalian cells.[32–34] Therefore, inactive forms of chromatin might originate at the bacterial sequences and then spread through the plasmid molecule, resulting in gene repression/silencing over time in mammalian cells *in vivo*.

Conversely, and consistent with this hypothesis, MC DNA expression remains stable and persistent, maintaining the euchromatin state (denoted by increased H3 and H4 acetylation and increased levels of H3K4-36-79me). This could be because the absence of bacterial sequences might prevent the heterochromatin machinery from associating with them. Interestingly, our MC DNAs show similar histone modification patterns to those observed in DNA insulators.[36]

An intriguing observation we made is that an intact plasmid DNA expressing the same hAAT transgene under the control of the Ubiquitin promoter C was not silenced over time, and had a euchromatin profile similar to the MC DNA. A likely explanation could be that this promoter contains several recognition sites associated with the transcriptional activator Sp1 and other transcription factors that serve as dominant chromatin marks.[19] It has been shown that Sp1 is associated with histone acetyl transferases[37] which, in turn, are able to maintain the original euchromatin mark.

The mechanism/s that trigger the chromatin remodeling/structure machinery (HDAC2, etc.) to turn euchromatin (active) into a heterochromatin (repressed/silenced) state are not known. There are several factors that could contribute to triggering the gene silencing response observed in the exogenous DNA. These include nucleosome positioning/phasing;[38] RNAi-mediated gene silencing;[39,40] nuclear positioning;[21,32] and/or CpG methylation.[41] In addition, it has been shown that characteristic prokaryotic sequences (*i.e.*, GC/AT ratios) might be rapidly sensed by unknown host defense molecular mechanisms, and that this may facilitate discrimination between exogenous and endogenous DNA by associating inactive forms of histone molecules on the plasmid DNA.[20,42] Moreover, we cannot rule out the roles played by other factors in the gene silencing mechanism; for instance, binding of histones to episomal or exogenously delivered DNAs may not be able to form a fully appropriate native chromatin. Further experiments will be required in order to address these questions.

In summary, we have identified and characterized the histone modifications on exogenously administered episomal plasmid and MC DNAs *in vivo*. Understanding the mechanism/s by which changes in chromatin remodeling/structure are involved in transcriptional gene silencing is a fundamental prerequisite to unraveling the mechanism of transcriptional regulation in mammalian systems. Ultimately, this will lead to future research efforts aimed at increasing the persistence of transgene expression *in vivo* through regulation of this pathway.

MATERIALS AND METHODS

Vector construction, DNA preparation and antibodies. Construction of the plasmids and corresponding MCs pBShAAT, a pBluescript KS+ (Stratagene, La Jolla, CA) containing the hAAT cDNA under the control of the Rous sarcoma virus (RSV) long terminal repeat promoter, and pBSEFhAAT, a pBluescript KS+ containing the hAAT cDNA under the

control of the EF1-α promoter has been previously described.[18] To prepare the pBSUBIhAAT plasmid, containing the hAAT cDNA under the control of the Ubiquitin C promoter (UBI), we first generated an intermediate plasmid (pKBHhAAT) by replacing the *Kpn*I–*Hin*dIII fragment encoding the RSV promoter in pBShAAT with a PCR primer embedding a *Bgl*II site. The final pBSUBIhAAT plasmid was constructed by inserting the *Bgl*II–*Hin*dIII fragment of pUB6/V5-His (Invitrogen, Carlsbad, CA), encoding the UBI promoter into the *Bgl*II–*Hin*dIII site into the pKBH-hAAT plasmid. To further generate the MC UBIhAAT, the *Xho*I fragment from the pBSUBIhAAT plasmid containing the whole transgene cassette was inserted into the *Xho*I site of the pBADφC31, resulting in the intermediate MC precursor pBADφC31.UBI. Preparation and purification of MC DNA was performed as described previously.[15,17] In brief, MCs were generated in bacteria from a parental plasmid containing a φC31 integrase gene, the I-*Sce*I endonuclease and its target site, and the different hAAT expression cassettes with *att*B and *att*P sites. After the *att*B and *att*P recombination and subsequent linearization and degradation of the plasmid bacterial backbone in bacteria, the MCs were then isolated by routine plasmid purification procedures.[15,17] After the purification, more than 85% of the product was the expected supercoiled MC DNA (data not shown).

Plasmid DNA was amplified in DH10B (Gibco BRL, Gaithersburg, MD) cells and purified by using Qiagen (Valencia, CA) endotoxin-free kits. All plasmid preparations were filtered through a 0.22-μm syringe filter unit and were dialyzed against TE for 24 hours before *in vivo* administration.

H3Ac, H4Ac, H3K9Ac + S10P, H3K36me2, H4K20me2, HP1α, SUV39H1 and H3 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). H3K4me2 (ab7766), H3K4me3 (ab8580), H3K36me3 (ab9050), H3K79me2 (ab3594), H3K9me2 (ab7312), H3K9me3 (ab8898), H4K20me3 (ab9053), and HDAC2 (ab7029) antibodies were purchased from Abcam (Cambridge, MA). Non-specific rabbit immunoglobulin G antibody was purchased from Rockland (Gilbertsville, PA).

Animal studies. We obtained 6- to 8-week-old C57BL/6 mice from Jackson Laboratory (Bar Harbor, ME). We delivered 20 μg of parental plasmids and their corresponding equimolar amount of MC to mouse livers by using a hydrodynamics-based technique. The amount of plasmids and MCs was determined based on the molecular weight of the DNA molecule, such that the mice would receive the same number of molecules in comparable groups. This hydrodynamics-based technique was used because it is the most efficient way to deliver DNA into hepatocytes of experimental animals *in vivo*.[2,43] We collected mouse blood periodically using a retro-orbital procedure and determined serum hAAT by enzyme-linked immunosorbent assay (ELISA) as described earlier.[15] The National Institutes of Health and Stanford University Animal Care Guidelines were followed at all times.

Mouse liver plasmid ChIP analysis. We developed a protocol to immunoprecipitate plasmid DNA from liver lysates. The protocol was an adaptation from[44,45] with modifications. Briefly, mice were sacrificed by CO_2 asphyxiation 1 day or 5 weeks after the plasmid and MC DNA injection, and livers were dissected and Dounce homogenized in 10 ml of Dulbecco's modified Eagle's medium supplemented with 10 mmol/l sodium butyrate and protease inhibitors (Roche Molecular Diagnostics, Pleasanton, CA), followed by addition of 1% formaldehyde (Fisher Scientific, Pittsburgh, PA). Fixation proceeded for 15 minutes at room temperature. The reactions were quenched by addition of 125 mmol/l glycine. After centrifugation (2,000 rpm for 5 minutes), samples were washed two times with phosphate-buffered saline (supplemented with 10 mmol/l sodium butyrate and protease inhibitors), resuspended in nuclei lysis buffer (50 mmol/l Tris-Cl, pH 8.0, 10 mmol/l EDTA, 1% sodium dodecyl sulfate, protease inhibitors), incubated 30 minutes on ice and sonicated with a Fisher Scientific sonifier mounted with a microtip for four 20-second pulses (output, 20%). Cross-linked chromatin was purified on a CsCl step gradient (1 ml of 1.75 g/ml, 1 ml of 1.5 g/ml, and 1 ml of 1.35 g/ml CsCl in 0.5% *N*-lauroylsarcosine and 1 mmol/l EDTA, pH 8) in a Beckman SW55 rotor for 19 hours at 31,000 rpm at 20 °C. Peak DNA-containing fractions, identified by agarose gel electrophoresis and ethidium bromide staining, were pooled, and then dialyzed overnight against Tris-EDTA (10 mmol/l Tris, pH 8, and 1 mmol/l EDTA). The purified, cross-linked chromatin was aliquoted and stored at 80 °C. Chromatin pre-clearing was performed by adding 50 μl of Protein A magnetic microbeads (Miltenyi Biotec, Auburn, CA) per ml of chromatin, and then incubating on a rotator at 4 °C for 45 minutes. The suspension was passed over a magnetic cell sorter (MACS) μcolumn attached to a magnet and the flow-through was collected. Ten micrograms of pre-cleared chromatin was incubated in IP buffer (0.01% sodium dodecyl sulfate, 1.1% Triton X-100, 1.2 mmol/l EDTA, 16.7 mmol/l Tris-Cl, pH 8.0, 167 mmol/l NaCl) overnight at 4 °C, rotating with 5 μg of the specific antibodies, as well as with no antibody and with non-specific antibody (immunoglobulin G) as negative controls. After the incubation, 50 μl of Protein A magnetic microbeads were added to each sample and incubated on a rotator for 1 hour at 4 °C. The samples were applied to individual magnetic cell sorter columns and only the flow-through for the "no antibody" control in a microfuge tube was collected as the "total input". After washing the column five times with 500 μl IP wash buffer (100 mmol/l Tris-Cl, pH 9.0, 500 mmol/l LiCl, 1% NP-40, 1% deoxycholic acid), samples were eluted with 300 μl of elution buffer (100 mmol/l $NaHCO_3$, 1% sodium dodecyl sulfate). One microliter RNase A (10 mg/ml) and 5 mol/l NaCl, to a final concentration of 0.3 mol/l, were added and samples reversed cross-linked by incubating at 65 °C for 6–8 hours. To analyze the DNA, the material was treated with proteinase K, extracted with phenol:chloroform, and ethanol-precipitated in the presence of 20 μg of glycogen as carrier. The final immunoprecipitated DNA products were resuspended in 50 μl of 10 mmol/l Tris-Cl, pH 8.0, for PCR analysis. The "total input" material was diluted 1:100 prior to PCR analysis. The amount of immunoprecipitated DNA was quantified by real-time PCR on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR green PCR kit (Applied Biosystems, Foster City, CA) and was calculated according to the following equation: $([DNA_{\text{specific IP}}] - [DNA_{\text{non antibodyIP}}])/[DNA_{\text{input}}]$ where $[DNA_{\text{specific IP}}]$ is the amount of DNA immunoprecipitated using the antibody of interest, $[DNA_{\text{non antibodyIP}}]$ is the amount of DNA immunoprecipitated by the "no antibody" control condition and DNA_{input} was the "total input" as described earlier, used for normalizing a sample to the amount of chromatin added to each ChIP. Results were presented as a fraction (%) of input DNA. Plasmid and MC ChIP samples from livers of injected mice were performed in duplicate. All data shown are the averages of three independent experiments. All the amplified products were electrophoresed and confirmed to have no non-specific PCR products, such as primer dimers (data not shown). Detailed conditions and primer sequences are available upon request. Unpaired *t*-tests were used to analyze differences within experimental groups. $P < 0.05$ was considered statistically significant.

Determination of transgene copy number. The copy numbers were determined by quantitative PCR analysis on 5 μl of the "total input" DNA for each of the different experimental conditions described earlier, by using oligonucleotides complementary to hAAT cDNA and, to normalize each sample, mouse glyceraldehyde-3-phosphate dehydrogenase sequences were used. Reactions were analyzed using the ABI PRISM 7700 Sequence Detection System with a SYBR green PCR kit. Quantitative values were obtained from the threshold cycle (C_t) number that indicated exponential amplification of the PCR product.

ACKNOWLEDGMENTS

We thank Kenneth Zaret (Fox Chase Cancer Center) and Linda Boxer (Stanford University) for providing helpful advice on the ChIP procedure. This work has been supported by NIH-NHLBI 64274 (M.A.K.).

REFERENCES

1. Niidome, T and Huang, L (2002). Gene therapy progress and prospects: nonviral vectors. *Gene Ther* **9**: 1647–1652.

2. Herweijer, H and Wolff, JA (2003). Progress and prospects: naked DNA gene transfer and therapy. *Gene Ther* **10**: 453–458.
3. Kass, SU, Landsberger, N and Wolffe, AP (1997). DNA methylation directs a time-dependent repression of transcription initiation. *Curr Biol* **7**: 157–165.
4. Grewal, SI and Moazed, D (2003). Heterochromatin and epigenetic control of gene expression. *Science* **301**: 798–802.
5. Margueron, R, Trojer, P and Reinberg, D (2005). The key to development: interpreting the histone code? *Curr Opin Genet Dev* **15**: 163–176.
6. Rice, JC and Allis, CD (2001). Code of silence. *Nature* **414**: 258–261.
7. Mateescu, B, England, P, Halgand, F, Yaniv, M and Muchardt, C (2004). Tethering of HP1 proteins to chromatin is relieved by phosphoacetylation of histone H3. *EMBO Rep* **5**: 490–496.
8. Liang, G, Lin, JC, Wei, V, Yoo, C, Cheng, JC, Nguyen, CT *et al.* (2004). Distinct localization of histone H3 acetylation and H3-K4 methylation to the transcription start sites in the human genome. *Proc Natl Acad Sci USA* **101**: 7357–7362.
9. Nowak, SJ and Corces, VG (2000). Phosphorylation of histone H3 correlates with transcriptionally active loci. *Genes Dev* **14**: 3003–3013.
10. Nowak, SJ and Corces, VG (2004). Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. *Trends Genet* **20**: 214–220.
11. Felgner, PL and Liang, X (1999). Debugging expression screening. *Nat Biotechnol* **17**: 329–330.
12. Sykes, KF and Johnston, SA (1999). Linear expression elements: a rapid, *in vivo*, method to screen for gene functions. *Nat Biotechnol* **17**: 355–359.
13. Hofman, CR, Dileo, JP, Li, Z, Li, S and Huang, L (2001). Efficient *in vivo* gene transfer by PCR amplified fragment with reduced inflammatory activity. *Gene Ther* **8**: 71–74.
14. Chen, ZY, Yant, SR, He, CY, Meuse, L, Shen, S and Kay, MA (2001). Linear DNAs concatemerize *in vivo* and result in sustained transgene expression in mouse liver. *Mol Ther* **3**: 403–410.
15. Chen, ZY, He, CY, Ehrhardt, A and Kay, MA (2003). Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression *in vivo*. *Mol Ther* **8**: 495–500.
16. Riu, E, Grimm, D, Huang, Z and Kay, MA (2005). Increased maintenance and persistence of transgenes by excision of expression cassettes from plasmid sequences *in vivo*. *Hum Gene Ther* **16**: 558–570.
17. Chen, ZY, He, CY and Kay, MA (2005). Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression *in vivo*. *Hum Gene Ther* **16**: 126–131.
18. Chen, ZY, He, CY, Meuse, L and Kay, MA (2004). Silencing of episomal transgene expression by plasmid bacterial DNA elements *in vivo*. *Gene Ther* **11**: 856–864.
19. Schorpp, M, Jager, R, Schellander, K, Schenkel, J, Wagner, EF, Weiher, H *et al.* (1996). The human ubiquitin C promoter directs high ubiquitous expression of transgenes in mice. *Nucleic Acids Res* **24**: 1787–1788.
20. Suzuki, M, Kasai, K and Saeki, Y (2006). Plasmid DNA sequences present in conventional herpes simplex virus amplicon vectors cause rapid transgene silencing by forming inactive chromatin. *J Virol* **80**: 3293–3300.
21. Bishop, CL, Ramalho, M, Nadkarni, N, May Kong, W, Higgins, CF and Krauzewicz, N (2006). Role for centromeric heterochromatin and PML nuclear bodies in the cellular response to foreign DNA. *Mol Cell Biol* **26**: 2583–2594.
22. Richards, EJ and Elgin, SC (2002). Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell* **108**: 489–500.
23. Rice, JC, Briggs, SD, Ueberheide, B, Barber, CM, Shabanowitz, J, Hunt, DF *et al.* (2003). Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol Cell* **12**: 1591–1598.
24. Nishioka, K, Rice, JC, Sarma, K, Erdjument-Bromage, H, Werner, J, Wang, Y *et al.* (2002). PR-Set7 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and is associated with silent chromatin. *Mol Cell* **9**: 1201–1213.
25. Nielsen, AL, Oulad-Abdelghani, M, Ortiz, JA, Remboutsika, E, Chambon, P and Losson, R (2001). Heterochromatin formation in mammalian cells: interaction between histones and HP1 proteins. *Mol Cell* **7**: 729–739.
26. Hwang, KK, Eissenberg, JC and Worman, HJ (2001). Transcriptional repression of euchromatic genes by Drosophila heterochromatin protein 1 and histone modifiers. *Proc Natl Acad Sci USA* **98**: 11423–11427.
27. Lachner, M, O'Carroll, D, Rea, S, Mechtler, K and Jenuwein, T (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**: 116–120.
28. Vaute, O, Nicolas, E, Vandel, L and Trouche, D (2002). Functional and physical interaction between the histone methyl transferase Suv39H1 and histone deacetylases. *Nucleic Acids Res* **30**: 475–481.
29. Rea, S, Eisenhaber, F, O'Carroll, D, Strahl, BD, Sun, ZW, Schmid, M *et al.* (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**: 593–599.
30. Ayyanathan, K, Lechner, MS, Bell, P, Maul, GG, Schultz, DC, Yamada, Y *et al.* (2003). Regulated recruitment of HP1 to a euchromatic gene induces mitotically heritable, epigenetic gene silencing: a mammalian cell culture model of gene variegation. *Genes Dev* **17**: 1855–1869.
31. Stewart, MD, Li, J and Wong, J (2005). Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment. *Mol Cell Biol* **25**: 2525–2538.
32. Craig, JM (2005). Heterochromatin—many flavours, common themes. *Bioessays* **27**: 17–28.
33. Kellum, R (2003). HP1 complexes and heterochromatin assembly. *Curr Top Microbiol Immunol* **274**: 53–77.
34. Schlissel, M (2004). The spreading influence of chromatin modification. *Nat Genet* **36**: 438–440.
35. Ayer, DE (1999). Histone deacetylases: transcriptional repression with SINers and NuRDs. *Trends Cell Biol* **9**: 193–198.
36. Wei, GH, Liu, DP and Liang, CC (2005). Chromatin domain boundaries: insulators and beyond. *Cell Res* **15**: 292–300.
37. Hilton, TL, Li, Y, Dunphy, EL and Wang, EH (2005). TAF1 histone acetyltransferase activity in Sp1 activation of the cyclin D1 promoter. *Mol Cell Biol* **25**: 4321–4332.
38. Workman, JL (2006). Nucleosome displacement in transcription. *Genes Dev* **20**: 2009–2017.
39. Wang, F, Koyama, N, Nishida, H, Haraguchi, T, Reith, W and Tsukamoto, T (2006). The assembly and maintenance of heterochromatin initiated by transgene repeats are independent of the RNA interference pathway in mammalian cells. *Mol Cell Biol* **26**: 4028–4040.
40. Matzke, MA and Birchler, JA (2005). RNAi-mediated pathways in the nucleus. *Nat Rev Genet* **6**: 24–35.
41. Bienvenu, T and Chelly, J (2006). Molecular genetics of Rett syndrome: when DNA methylation goes unrecognized. *Nat Rev Genet* **7**: 415–426.
42. Navarre, WW, Porwollik, S, Wang, Y, McClelland, M, Rosen, H, Libby, SJ *et al.* (2006). Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. *Science* **313**: 236–238.
43. Zhang, G, Budker, V and Wolff, JA (1999). High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther* **10**: 1735–1737.
44. Chaya, D, Hayamizu, T, Bustin, M and Zaret, KS (2001). Transcription factor FoxA (HNF3) on a nucleosome at an enhancer complex in liver chromatin. *J Biol Chem* **276**: 44385–44389.
45. Weinmann, AS and Farnham, PJ (2002). Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. *Methods* **26**: 37–47.