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Methylation at arginine 17 of histone H3 is linked to gene activation

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The nuclear hormone receptor co-activator CARM1 has the potential to methylate histone H3 at arginine residues *in vitro*. The methyltransferase activity of CARM1 is necessary for its co-activator functions in transient transfection assays. However, the role of this methyltransferase *in vivo* is unclear, given that methylation of arginines is not easily detectable on histones. We have raised an antibody that specifically recognizes methylated arginine 17 (R17) of histone H3, the major site of methylation by CARM1. Using this antibody we show that methylated R17 exists *in vivo*. Chromatin immunoprecipitation analysis shows that R17 methylation on histone H3 is dramatically upregulated when the estrogen receptor-regulated *pS2* gene is activated. Coincident with the appearance of methylated R17, CARM1 is found associated with the histones on the *pS2* gene. Together these results demonstrate that CARM1 is recruited to an active promoter and that CARM1-mediated R17 methylation on histone H3 takes place *in vivo* during this active state.

INTRODUCTION

Covalent post-translational modifications of histone N-termini, such as acetylation, phosphorylation and methylation, play a fundamental role in chromatin structure and transcriptional regulation (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Marmorstein, 2001). Although histone methylation was first discovered more than 35 years ago (Murray, 1964), only recent studies have started to elucidate its biological significance. Recent work on methylation of lysines has shown a role for this modification in heterochromatic silencing (Rea *et al.*, 2000; Bannister *et al.*, 2001; Lachner *et al.*, 2001; Noma *et al.*, 2001) and euchromatic gene expression (Nielsen *et al.*, 2001). The function of methylation of arginine residues is much less clear. This may be partly because unlike lysine methylation, which is found to occur *in vivo* on histones H3 and H4 (Strahl *et al.*, 1999; Strahl and Allis, 2000), the occurrence of arginine methylation has been difficult to detect on mammalian histones (Gary and Clarke, 1998).

Different sets of enzymes carrying out either arginine or lysine methylation activity have been identified recently (Chen *et al.*, 1999; Rea *et al.*, 2000; Stallcup, 2001). Protein arginine (R) methyltransferases (PRMTases) share a highly conserved domain encompassing the methyltransferase activity. They have a broad spectrum of substrates, including RNA-processing proteins, RNA-transporting proteins, protein phosphatase 2A, G-proteins and histones (Aletta *et al.*, 1998). Three of the five mammalian PRMTases, PRMT1, JBP1 and CARM1 [cofactor associated arginine (R) methyltransferase 1], have been demonstrated to have histone methyltransferase activity (McBride and Silver, 2001; Stallcup, 2001). PRMT1 specifically methylates arginine 3 of histone H4 *in vivo* (Strahl *et al.*, 2001; Wang *et al.*, 2001). CARM1 was isolated through a yeast two-hybrid screen searching for proteins that interact with the p160 nuclear hormone receptor co-activator GRIP1 (Chen *et al.*, 1999). Recombinantly expressed CARM1 preferentially methylates histone H3 within a bulk histone preparation *in vitro* (Chen *et al.*, 1999). Both PRMT1 and CARM1 co-activate nuclear hormone receptor regulated gene expression in transient transfection assays, and their co-activator potential is dependent on an intact methyltransferase domain (Chen *et al.*, 1999; Koh *et al.*, 2001; Wang *et al.*, 2001). These studies suggest that methylation of arginines within histone tails may represent an activating step in mammalian gene transcription.

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A recent study revealed that PRMT1 also methylates the transcription factor STAT1 and that this methylation is required for STAT1-mediated transcriptional activation (Mowen *et al.*, 2001). This raises the question whether histones are indeed the major physiological substrate of arginine methyltransferases or whether methylation of other proteins in the transcription complexes results in a transcriptionally active promoter status, as it has already been found to be the case for acetylation (Kouzarides, 2000).

To evaluate the link between histone arginine methylation by CARM1 and active gene expression, we generated an antibody that specifically recognizes the major site of CARM1 methylation in histone H3 *in vitro.* This antibody allowed us to establish that this modification takes place *in vivo* in mammalian cells. We use chromatin immunoprecipitation analysis (ChIP) to demonstrate for the first time that an arginine methyltransferase (CARM1) is indeed recruited to an endogenous target promoter upon gene activation, a process which coincides with the appearance of arginine methylation on histone H3.

RESULTS

Arginine 17 on histone H3 is methylated *in vivo*

The nuclear hormone receptor co-activator CARM1 is able to methylate arginine residues within histone H3 *in vitro*. However, evidence that such methylation takes place *in vivo* has been difficult to obtain (Gary and Clarke, 1998; Stallcup, 2001). We therefore raised an antibody that recognizes the methylation site for CARM1 in histone H3, to establish (i) if such methylation takes place on CARM1 regulated promoters *in vivo* and (ii) whether this methylation occurs when a gene is actively transcribed.

The major site for CARM1 methylation was mapped, by radiosequencing analysis of ^{3}H-methylated recombinant H3, to arginine 17 (R17) (Figure 1A). A peptide methylated at R17 was used to immunize rabbits. Figure 1B shows that the resulting antibody (Me-R17H3) recognizes recombinant histone H3 only when it is methylated by CARM1 (compare lanes 1 and 2). Purified calf thymus histone H3 is also recognized by this antibody (lane 3) indicating that R17 is indeed methylated *in vivo*. The Me-R17H3 antibody does not recognize recombinant histone H4 methylated by PRMT1 or when H4 is purified from calf thymus (lanes 4–6), indicating that the antibody only recognizes methyl arginine in the correct amino acid context (i.e. methyl R17 of histone H3).

A recent study (Schurter *et al.*, 2001) reports that arginines in the C-terminal domain of H3 are methylated by CARM1 *in vitro*. To confirm the specificity of the Me-R17H3 antibody for R17 and the N-terminal tail of H3, we analysed tail-less histone H3 methylated *in vitro* by CARM1 and showed that this antibody does not recognize the C-terminal methylation sites of CARM1 (data not shown). The high specificity for methylated R17 on H3 was further confirmed by peptide competition with unmethylated H3, R17 methylated H3 and R3 methylated H4 peptides (Figure 1C). When total U2OS cell extract is probed with antiMe-R17H3, the only protein recognized is histone H3 (Figure 1D). Taken together these results indicate for the first time that methylation at R17 in histone H3 indeed occurs *in vivo*.

The methyltransferase CARM1 co-activates the ER-regulated pS2 promoter in a methyltransferase activity dependent manner

To establish whether methylation on R17 takes place on a promoter regulated by CARM1, we chose to study the promoter of the estrogen receptor regulated *pS2* gene. It has recently been demonstrated that p160 co-activators are recruited to the *pS2* gene promoter *in vivo* (Shang *et al.*, 2000). As shown in Figure 2A, this promoter can be stimulated by CARM1 in transient transfection assays in a manner dependent on the SRC1 co-activator to which CARM1 binds. To analyse whether the methyltransferase activity of CARM1 is necessary for this stimulation, a CARM1 mutant was generated that lacks the methyltransferase domain (CARM1 284–608). This mutant is not able to stimulate the *pS2* gene promoter (Figure 2A) even though it is still able to bind SRC1 with an affinity comparable to full-length CARM1 (Figure 2B). These data identify the *pS2* gene as a target for CARM1 co-activation.

pS2 gene activation is correlated with CARM1 recruitment and histone H3 methylation at arginine 17

The Me-R17H3 antibody and an antibody against CARM1 were used in chromatin immunoprecipitation analysis to probe the role of arginine methylation by CARM1 *in vivo* at the estrogen-dependent *pS2* gene in the human breast cancer cell line MCF-7. The *pS2* gene is a well-characterized target of the estrogen receptor in these estrogen responsive cells (Shang *et al.*, 2000). We first used our Me-R17H3 antibody to monitor changes in R17 methylation by ChIP analysis following CARM1 transfection. However, these changes were minimal, probably as a result of the low stimulatory effect of transfected CARM1 on this promoter (Figure 2A). We therefore decided to monitor the changes in chromatin methylation at the pS2 promoter after the gene is activated by the addition of two stimuli: estradiol (E2) and tetradodecanoyl phorbol acetate (TPA) (Figure 3). When each of these stimuli is added independently to MCF-7 cells, transcription of the endogenous *pS2* gene is slightly induced, as shown by northern blot analysis (Figure 3A). However, when added together, E2 and TPA have a synergistic effect on mRNA levels (Figure 3A).

When the Me-R17H3 and CARM1 antibodies are used in ChIPs, Figure 3B shows that the chromatin around the pS2 promoter has no detectable R17 methylation or CARM1 association under non-induced conditions (lane 1). In contrast, when *pS2* gene expression is stimulated by a combination of E2 plus TPA, methylation of R17 and recruitment of CARM1 are both dramatically increased (Figure 3B, lane 4). Individually, each of the stimuli have a small effect on methylation and CARM1 association, consistent with the small increase seen on mRNA levels.

In order to ensure that the enhanced histone H3 methylation associated with the stimulated pS2 promoter did not reflect a global nucleosome modification, we examined the methylation of a gene promoter that is not an estrogen receptor target, the *cdc25* gene promoter. ChIP analysis of this promoter shows a very weak R17 methylation of H3 but there is no alteration in the

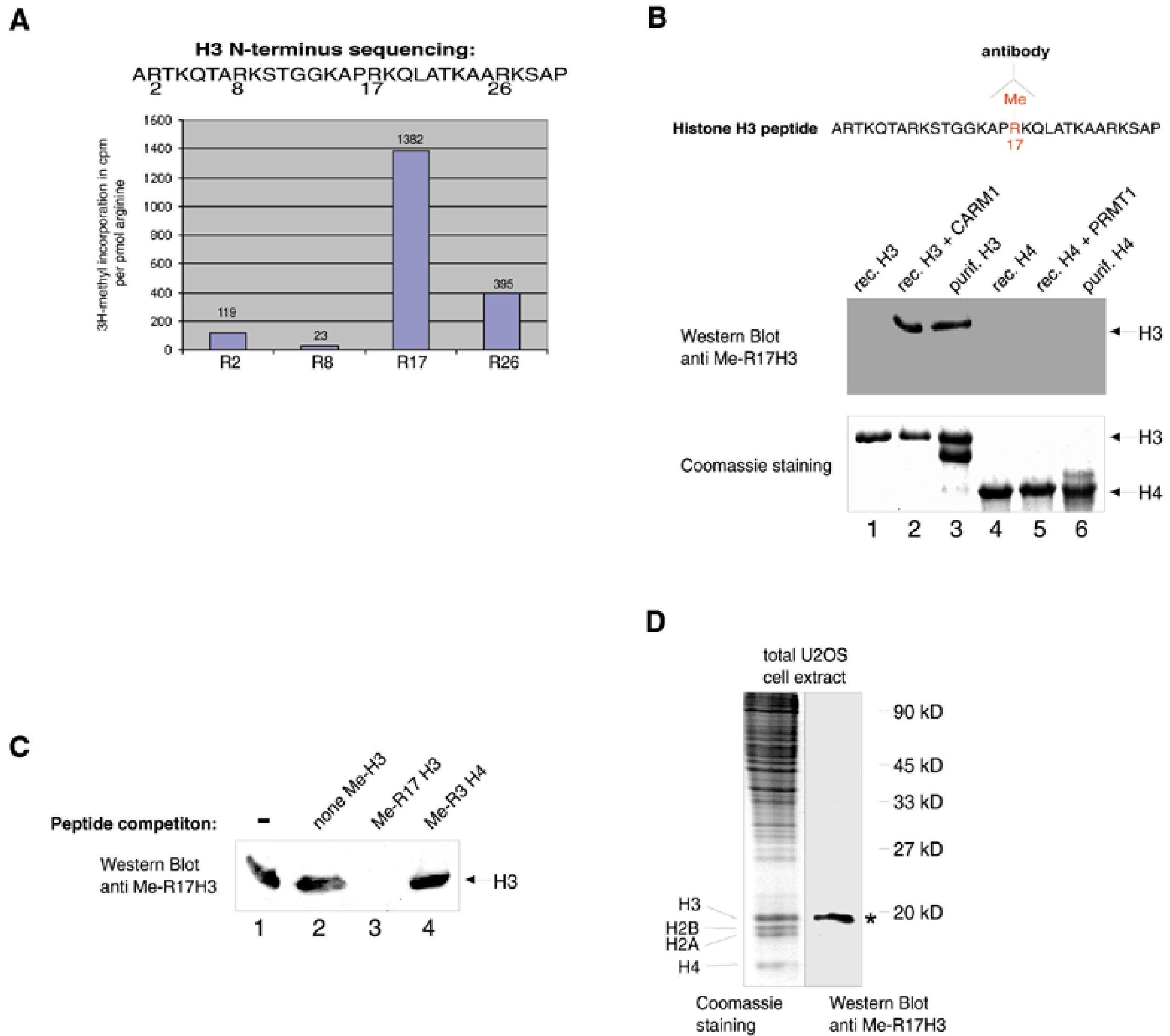

Fig. 1. Methylation of histone H3 at R17 occurs *in vivo*. (**A**) Recombinant *Drosophila* histone H3 was *in vitro* methylated by GST–CARM1 in the presence of [^{3}H]SAM and subjected to microsequencing of residues 1–30. Amino acid fractions were analysed for the presence of tritium by scintillation counting. Additionally non-radioactively methylated recombinant H3 was sequenced and the amino acid concentration in each fraction was determined by HPLC. The raw data of the radiosequencing were then corrected by calculation of the [^{3}H]methyl incorporation per pmol amino acid. The result of this refinement is shown for the four arginines (R2, R7, R17 and R26) in the H3 N-terminus which are the putative methylation sites of CARM1. The amino acid sequence of residues 1–30 of histone H3 is shown above and the positions of the four arginines are indicated. (**B**) The antiMe-R17H3 antibody (from www.abcam.com) was raised against a histone H3 peptide (aa 11–24) asymmetrically dimethylated at R17, which represents the major site of CARM1 methylation. Western blot analysis was performed using Me-R17H3 and 2 µg of unmodified recombinant *Drosophila* histone H3 (lane 1), recombinant H3 after *in vitro* methylation with GST–CARM1 protein (lane 2), purified calf thymus histone H3 (lane 3), unmodified recombinant *Drosophila* histone H4 (lane 4), recombinant H4 after *in vitro* methylation with GST–PRMT1 protein (lane 5) and purified histone H4 (lane 6). Coomassie Blue staining (lower panel) revealed the presence of approximately equal amounts of histones in each lane. The enzymatic activity of GST–CARM1 and GST–PRMT1 on histones was confirmed by *in vitro* methylation of bulk histones in the presence of [^{3}H-Me]S-adenosyl methionine, SDS–PAGE and autoradiography (data not shown). (**C**) Peptide competition experiment was performed by western blot analysis using 1 µg of purified calf thymus histone H3 and antiMe-R17H3 antibody in the presence of 1 µg/ml none methylated H3 peptide (aa 11–24, lane 2), R17 methylated H3 peptide (aa 11–24, lane 3) or R3 methylated H4 peptide (aa 1–14, lane 4). (**D**) Total U2OS cell extract was western blotted using the antiMe-R17H3 antibody. The asterisk indicates methylated histone H3. The left panel shows presence of core histones (indicated on the left) by Coomassie Blue staining. Molecular weights are indicated on the right.

methylation pattern when comparing unstimulated and stimulated MCF7 cells (Figure 3B). These data strongly argue that methylation at R17 is highly specific and takes place only on selected promoters, for example the activated *pS2* gene, and is consistent with the recruitment of the CARM1 methyltransferase to the promoter.

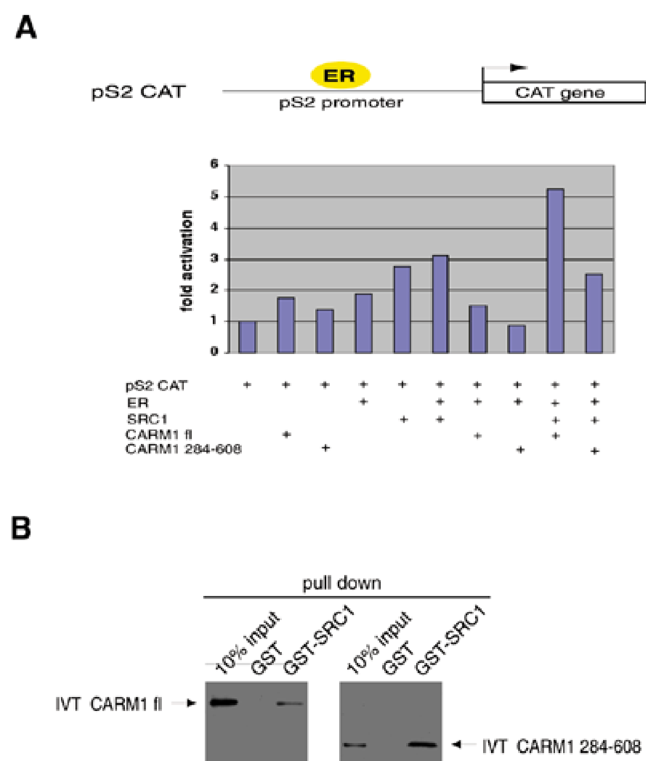

Fig. 2. CARM1 co-activates the pS2 promoter in a methylation dependent manner. (**A**) 293T cells were transiently transfected with the indicated expression plasmids. Whole-cell extracts were used in CAT assays and the results were quantified on a PhosphorImager. The basal promoter activity of the pS2 CAT reporter in the presence of empty expression vector was normalized to 1.0, and the activities of the remaining transfection reactions were expressed relative to this. The graph shows the result of one experiment which was reproduced independently several times. Equal protein expression of wild-type full-length CARM1 (CARM1 fl) or mutant CARM1 (CARM1 284–608) was confirmed by western blot analysis (data not shown). (**B**) GST alone or GST fusion protein of SRC1 (984–1441) was expressed in *E. coli*, purified and used in GST pull-down experiments with *in vitro* translated (IVT), ^{35}S-labelled CARM1 fl or mutant CARM1 284–608. Interaction between the proteins was analysed by SDS–PAGE and fluorography.

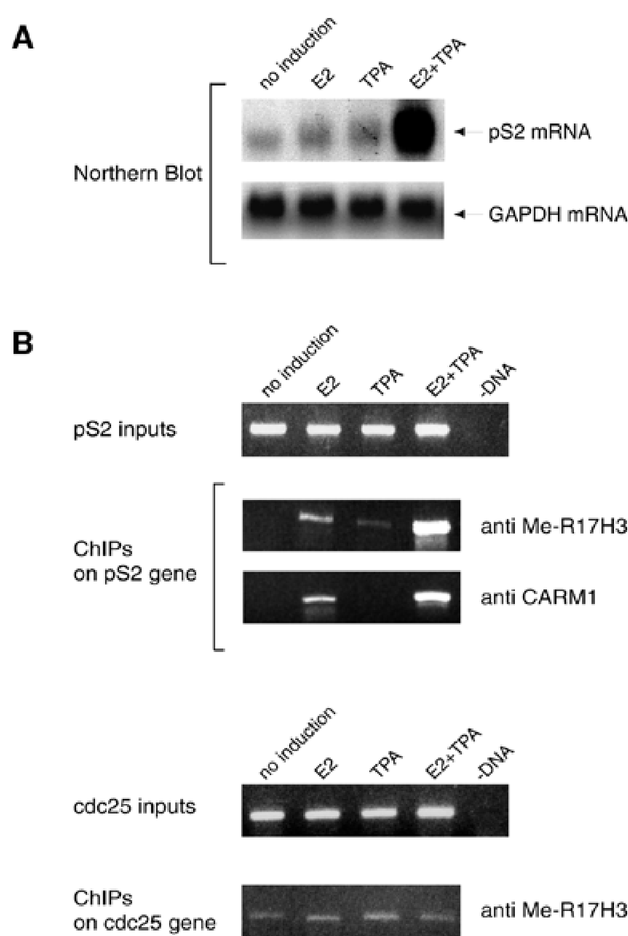

Fig. 3. Activation of the *pS2* gene promoter coincides with CARM1 recruitment and methylation of histone H3 at R17 *in vivo*. MCF-7 cells were stimulated with DMSO/ethanol (no induction control) or with 200 nM 17β-estradiol (E2), or 100 ng/ml tetradecanoyl phorbol acetate (TPA), or both (E2+TPA) for 3 h and analysed by northern blot and ChIP assay. (**A**) mRNA steady state levels of pS2 and GAPDH were determined by northern blot analysis. Total RNA was prepared from unstimulated and stimulated MCF-7 cells, and 10 µg RNA per sample separated through a 1.2% formaldehyde-agarose gel and blotted onto a nylon membrane. The pS2 and GAPDH RNA was detected by hybridization with ^{32}P-labelled pS2 or GAPDH gene probe. The GAPDH northern blot shows that equal RNA amounts were loaded. (**B**) For ChIP, genomic chromatin fragments from stimulated MCF-7 cells were immunoprecipitated either with antiMe-R17H3 or antiCARM1 (Upstate) antibodies. Immunoprecipitated chromatin was analysed by quantitative PCR for CARM1 binding and histone H3 R17 methylation in the proximal pS2 promoter region (nt –159 to –463). AntiMe-R17H3 immunoprecipitates were analysed by quantitative PCR with primers for the *cdc25* gene promoter (nt –15 to –186). PCR analysis on input chromatin confirmed that equal chromatin amounts were used for ChIPs.

DISCUSSION

The ability of CARM1 to methylate histone H3 *in vitro* and to co-activate nuclear hormone receptor regulated transcription in transient transfection (Chen *et al.*, 1999) supports the idea that arginine methylation of histones may have a stimulating effect on transcription. Our results show for the first time that an arginine methyltransferase is recruited to an endogenous promoter and that this event correlates with histone methylation and activation of transcription *in vivo*.

In contrast to a recent study, which showed that R17 and R26 are approximately equally methylated by CARM1 *in vitro* (Schurter *et al.*, 2001), our sequencing reveals R17 of histone H3 as the major methylation site for CARM1. The usage of different experimental conditions and enzyme preparations might account for the variations in the sequencing results.

To probe the role of arginine methylation by CARM1 *in vivo*, we raised an antibody that specifically recognizes the major CARM1 methylation site in histone H3, R17, based on our sequencing results. This antibody detects arginine methylation on histone H3 *in vivo*, even though methylation has previously been difficult to detect by the sequencing of bulk purified histones.

Only recently Schurter *et al.* (2001) reported the *in vivo* occurrence of arginine methylation of H3 by amino acid sequencing. However, our results go beyond the previous data because we

show site specificity of an arginine methyltransferase on histones *in vivo* using antibodies.

This raises a cautionary note for the assumption that histone sequencing recognizes all the modifications that take place *in vivo*. It may well be that many modifications are present on a small subset of promoter-bound histones, or that the modifications will only occur on the induced conditions. In either case the amounts of the modifications present on purified histones may be too small to be detected by sequencing. This then raises the possibility that many more modifications exist on histones than are currently suspected.

It has been shown recently that another arginine methyltransferase, PRMT1, methylates R3 in histone H4 and that this modification can be detected *in vivo* (Strahl *et al*., 2001; Wang *et al*., 2001). However, the role of this modification in transcriptional regulation has not been clarified so far. With the aid of ChIP analysis we have demonstrated for the first time that recruitment of the CARM1 methyltransferase onto chromatin, and concomitant R17 methylation on histone H3 takes place when an endogenous, hormonal responsive gene is activated.

Analysis of the *cdc25* control gene confirms that the estrogen induced increase in R17 methylation by CARM1 is gene specific. The fact that a cell cycle regulated gene shows a low level and constant amount of methylated R17 in its promoter opens the possibility of a broader role for arginine methylation in transcription regulation.

These data are consistent with the notion that CARM1 methyltransferase activity is essential for co-activated transcription, as predicted by transfection experiments using CARM1-methyltransferase deficient mutants (Figure 2A; Chen *et al*., 1999). Our results clearly establish that CARM1 is indeed an arginine methyltransferase whose activity is directed towards histones. The possibility also exists that CARM1 may methylate non-histone substrates such as transcription factors. Indeed, recent data show that PRMT1 is able to methylate the transcription factor STAT1 (Mowen *et al*., 2001), establishing that arginine methylation of non-histone proteins also has an important role in regulation of gene expression.

The combination of two inducers, E2 and TPA, is necessary for both maximal R17 methylation and maximal levels of mRNA expression from the *pS2* gene. The mechanism of this cooperation is unclear. The effect of E2 stimulation is likely to be the recruitment of the p160 co-activator (Shang *et al*., 2000). The TPA stimulus may affect a distinct signalling pathway which is necessary for maximal activity. It has been postulated that pre-existing modifications on histones may influence further modifications (Strahl and Allis, 2000), e.g. phosphorylation of S10 in histone H3 promotes acetylation of the neighbouring K14 by GCN5 (Lo *et al*., 2000; Berger, 2001). Recent studies, which show cooperativity between CBP/p300 and CARM1 in nuclear hormone receptor signalling, suggest that there might be a similar cross-talk between arginine methylation and lysine acetylation (Chen *et al*., 1999). Interestingly, a similar cooperativity between E2 and TPA observed here for arginine methylation has been observed for acetylation of histone H3 at the pS2 promoter (Sewack *et al*., 2001). This opens up the possibility that the cooperativity may involve a cross-talk between acetylation and arginine methylation.

It has recently become clear that the covalent modifications of histones is of central importance in the transcription regulation field. Our understanding of how these modifications are regulated is crucial towards our understanding of differential gene expression involved in many cellular processes. Our data give new insights into how arginine methylation of histones is involved in gene activity. They provide direct evidence that CARM1-mediated methylation at R17 of histone H3 takes place during transcriptional activation *in vivo*.

METHODS

Plasmids and recombinant proteins. Full-length CARM1 cDNA and the mutant CARM1 cDNA fragment (284–608) deleted of its 283 first amino acids containing the methyltransferase domain, were generated by PCR and cloned into pcDNA3-Gal4DBD. The ERα-expression plasmid (pMT2-MOR) and the SRC1e expression plasmid (pSG5-SRC1e) were gifts from David Heery and have been described previously (Kalkhoven *et al*., 1998). The *pS2* gene promoter CAT reporter (pS2 CAT) and pGEX2TK-SRC1a (984–1441) constructs were gifts from Malcolm Parker. GST and GST–SRC1 fusion proteins were expressed in and purified from *Escherichia coli* XA90 according to standard procedures.

Cell culture, transfections and reporter gene assay. MCF-7, HEK-293 and U2OS cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C and 5% CO_2. HEK-293 cells were transfected using the calcium phosphate co-precipitation method with 2 µg of pS2 CAT reporter plasmid, 1 ng pMT2-MOR, 1 µg pSG5-SRC1e and 0.5 µg of either wild-type full-length CARM1 (CARM1 fl) or mutant CARM1 (CARM1 284–608) Gal4DBD-expression plasmids. Approximately 36 h post-transfection, the cells were washed in PBS and harvested. CAT assays were performed as described previously (Hagemeier *et al*., 1993).

Histone methyltransferase assays and protein sequencing. Two micrograms of either recombinant histone H3 or recombinant H4 were incubated with either purified GST–CARM1 or GST–PRMT1 and [^{3}H-Me]S-adenosyl methionine (NEN, 80 Ci $mmol^{-1}$) in PBS at 30°C for 1 h. The reaction products were then resolved by SDS–PAGE and blotted onto nitrocellulose. For N-terminal sequencing, radiolabelled recombinant histone H3 was blotted to PVDF membrane and sequenced by Edman degradation (Protein Sequencing Facility, University of Cambridge, UK). Amino acid fractions were analysed for the presence of tritium by scintillation counting. In addition, non-radioactively methylated recombinant H3 was sequenced by Edman degradation and amino acid content in each fraction determined by HPLC.

Antibody generation. Rabbits were immunized with a histone H3 N-terminal peptide corresponding to amino acids 11–24, in which arginine 17 was asymmetrically dimethylated. Immunoreactive serum was applied sequentially to a H3 R17-unmethylated peptide and a H3 R17-methylated peptide column to affinity purify specific antibodies, since the antiserum crossreacted with unmethylated recombinant histone H3. The antibody is available from Abcam.com.

***In vitro* translations and GST pull-down assays.** *In vitro*-translated ^{35}S-labelled wild-type full-length (CARM1 fl) or mutant CARM1 (CARM1 284–608) were incubated with equal amounts of GST fusion proteins essentially as described previously (Hagemeier *et al*., 1993). Bound proteins were resolved by SDS–PAGE and viewed by fluorography.

Chromatin immunoprecipitation (ChIP) and northern blot analysis. MCF-7 cells were grown to 95% confluence in Phenol Red-free DMEM supplemented with 5% charcoal-dextran-stripped fetal calf serum and 1 nM 4-hydroxy-tamoxifen for at least 3 days. Following the addition of DMSO/ethanol as control (no induction), 200 nM 17β-estradiol (E2), or 100 ng/ml tetradecanoyl phorbol acetate (TPA), or both (E2+TPA) for 3 h, cells were either harvested for northern blot analysis, or cross-linked with 1% formaldehyde (Sigma) at room temperature for 15 min. Cells were rinsed twice with ice-cold PBS (pH 7.4) and collected in PBS and centrifuged for 5 min at 2000 *g*. ChIPs were then performed as described (Dedon *et al.*, 1991; Orlando *et al.*, 1997) with 20 µl of antiMe-R17H3 and 2.5 µl of antiCARM1 (Upstate) antibody. Immunoprecipitates were analysed for the presence of pS2 promoter fragment by PCR using previously described primers (Mazumdar *et al.*, 2001) that amplify the promoter region –463 to –159, which includes the ER-responsive element. PCR products were resolved in a 2% agarose gel and visualized with ethidium bromide.

For northern blot analysis 20 µg of total MCF-7 RNA per sample were electrophoresed in a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane (Amersham Pharmacia). The pS2 and GAPDH RNA were detected using a ^{32}P-labelled DNA probe from the corresponding gene sequence and subsequent autoradiography.

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