Hormone-dependent, CARM1-directed, arginine-specific methylation of histone H3 on a steroid-regulated promoter

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Activation of gene transcription involves chromatin remodeling by coactivator proteins that are recruited by DNA-bound transcription factors. Local modification of chromatin structure at specific gene promoters by ATP-dependent processes and by posttranslational modifications of histone N-terminal tails provides access to RNA polymerase II and its accompanying transcription initiation complex [1, 2]. While the roles of lysine acetylation, serine phosphorylation, and lysine methylation of histones in chromatin remodeling are beginning to emerge [2–5], low levels of arginine methylation of histones have only recently been documented [4, 6–9], and its physiological role is unknown. The coactivator CARM1 methylates histone H3 at Arg17 and Arg26 in vitro [7] and cooperates synergistically with p160-type coactivators (e.g., GRIP1, SRC-1, ACTR) and coactivators with histone acetyltransferase activity (e.g., p300, CBP) to enhance gene activation by steroid and nuclear hormone receptors (NR) in transient transfection assays [10, 11]. In the current study, CARM1 cooperated with GRIP1 to enhance steroid hormone-dependent activation of stably integrated mouse mammary tumor virus (MMTV) promoters, and this coactivator function required the methyltransferase activity of CARM1. Chromatin immunoprecipitation assays and immunofluorescence studies indicated that CARM1 and the CARM1-methylated form of histone H3 specifically associated with a large tandem array of MMTV promoters in a hormone-dependent manner. Thus, arginine-specific histone methylation by CARM1 is an important part of the transcriptional activation process.

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Received: **3 October 2001**
Revised: **24 October 2001**
Accepted: **26 October 2001**

Published: **11 December 2001**

Current Biology 2001, 11:1981–1985

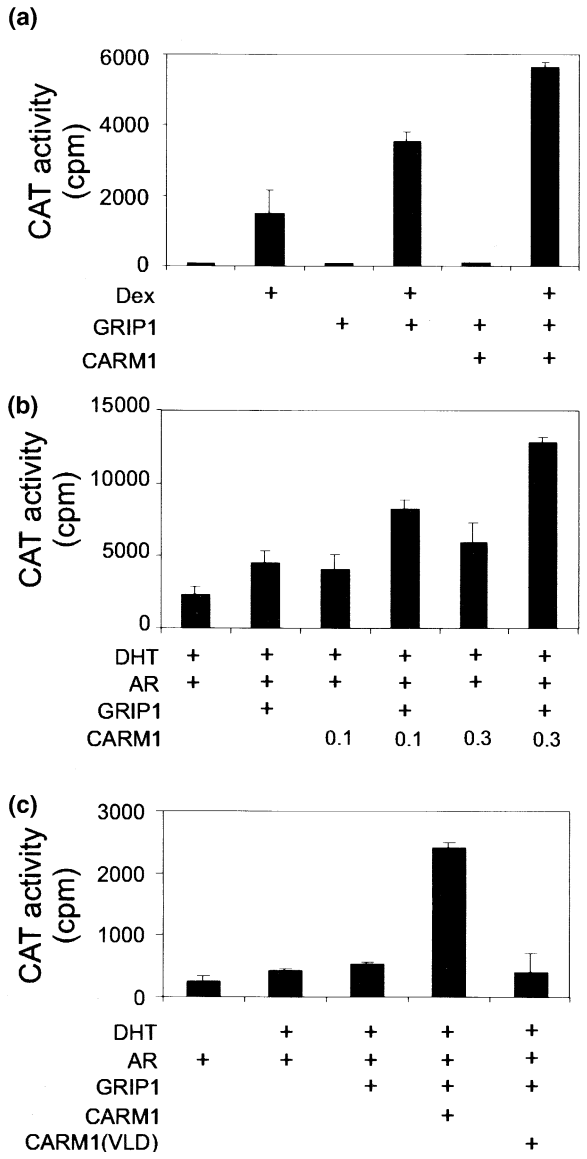
0960-9822/01/$ – see front matter

Results and discussion

CARM1 coactivator function has previously been demonstrated only with transiently transfected reporter genes [10, 11]. Therefore, we examined the ability of CARM1, in cooperation with p160 coactivator GRIP1, to enhance the ability of NRs, a family of hormone-regulated transcriptional activators [12, 13], to activate chromosomally integrated reporter genes, which have well-known differences in chromatin structure and transcriptional activation requirements compared with transiently transfected reporter genes [14]. The cell line 1471.1 contains multiple tandemly integrated copies of a chloramphenicol acetyltransferase (CAT) gene controlled by a MMTV promoter, which contains enhancer elements that bind the glucocorticoid receptor (GR) or androgen receptor (AR). CAT activity was stimulated by the glucocorticoid dexamethasone (dex), which activates the endogenous GR in these cells (Figure 1a). Coexpression of GRIP1 by transient transfection of a GRIP1 expression plasmid enhanced CAT activity 2-fold, and coexpression of GRIP1 plus CARM1 caused a 4-fold increase. All activity was hormone dependent, indicating that the activity of the coactivators was entirely dependent on hormone-activated GR. Similarly, when the cells were transfected with a vector expressing AR and treated with dihydrotestosterone (DHT) to activate AR, coexpression of CARM1 and GRIP1 together caused a synergistic 5-fold increase in CAT activity (Figure 1b,c). A CARM1 mutant (VLD) lacking methyltransferase activity failed to enhance CAT activity in cooperation with GRIP1 (Figure 1c). Thus, on a stably integrated reporter gene, the coactivator function of CARM1 required its methyltransferase activity and the coexpression of GRIP1 (Figure 1), as shown previously for transiently transfected reporter genes [10].

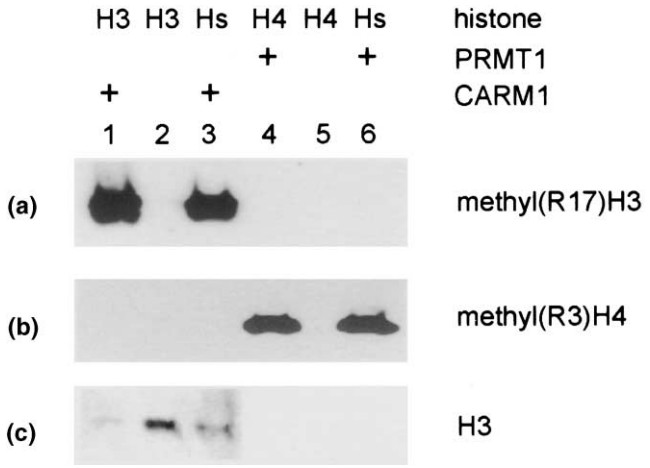
To test whether histone H3 is methylated by CARM1 in connection with promoter activation in vivo, we devel-

Figure 1



Coactivator function by CARM1 and GRIP1 with stably integrated MMTV-CAT reporter genes. Cell line 1471.1 was transfected with expression vectors for IL-2 receptor and the following proteins as indicated: GRIP1, CARM1 or CARM1 VLD-to-AAA mutant, and AR. Transfected cells were grown without hormone or with **(a)** 100 nM dex (for endogenous GR) or **(b,c)** 100 nM DHT (for transfected AR), as indicated. Cells expressing IL-2 receptor were isolated from nontransfected cells by magnetic bead sorting, and cell extracts were assayed for CAT activity.

Figure 2

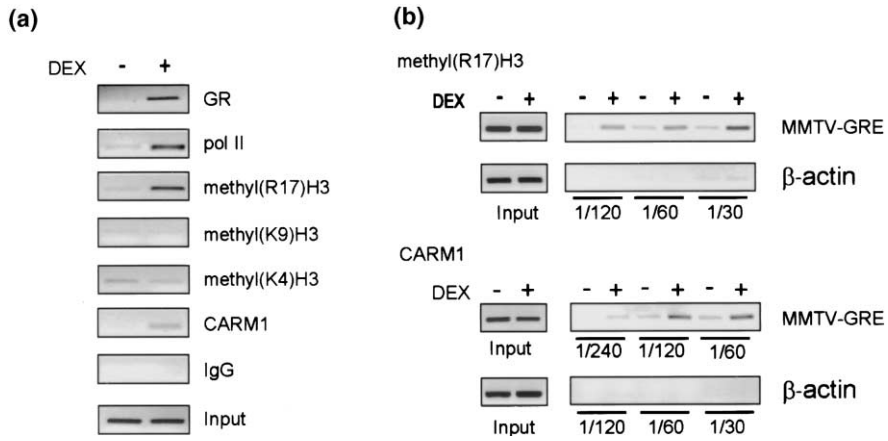


Antiserum specific for CARM1-methylated histone H3. Purified calf thymus histone H3 or H4 or unfractionated core calf thymus histones (Hs) were incubated with S-adenosylmethionine and recombinant GST-CARM1 or GST-PRMT1. Immunoblots were performed with antisera as follows: **(a)** α-methyl(R17)H3, **(b)** α-methyl(R3)H4, **(c)** against unmethylated histone H3.

oped antibodies that specifically recognize the CARM1-methylated form of histone H3, using a histone H3 peptide containing asymmetric dimethylarginine at position 17. On immunoblots, the α-methyl(R17)H3 antiserum recognized calf thymus histone H3 methylated by CARM1, but not unmethylated histones H3 and H4 or histone H4 methylated by PRMT1 (Figure 2a), another arginine-specific protein methyltransferase [4]. The α-methyl(R17)H3 antiserum also failed to recognize three different nonhistone proteins methylated in vitro by CARM1 and one nonhistone protein methylated by PRMT1 (data not shown). In contrast, antiserum α-methyl(R3)H4 [8] against PRMT1-methylated histone H4 failed to recognize the CARM1-methylated histone H3 or unmethylated histone H4, but reacted with PRMT1-methylated H4 (Figure 2b). An antibody against unmodified histone H3 was used to show that histone H3 was present in lanes 1–3 (Figure 2c). Thus, the α-methyl(R17)H3 antiserum was highly specific for CARM1-methylated histone H3.

Chromatin immunoprecipitation assays were then used to test whether CARM1 recruitment and CARM1-specific methylation of histone H3 occurred in vivo on the MMTV promoter during its activation by glucocorticoid hormone. Antibodies against various proteins were used to immunoprecipitate cross-linked, sheared chromatin fragments from dex-treated or untreated 1471.1 cells; DNA from the immunoprecipitates was then analyzed by polymerase chain reaction (PCR) using primers that bracket the glucocorticoid response elements of the MMTV promoter. As expected, GR and RNA polymerase II were associated with the MMTV promoter in a hormone-dependent manner (Figure 3a). Similarly, CARM1 antiserum and the α-methyl(R17)H3 antiserum precipitated the hormone-activated MMTV promoter more efficiently than the inactive MMTV promoter, indicating that CARM1 and CARM1-methylated histone H3 were more highly associated with the hormone-activated promoter (Figure 3a,b).

Figure 3



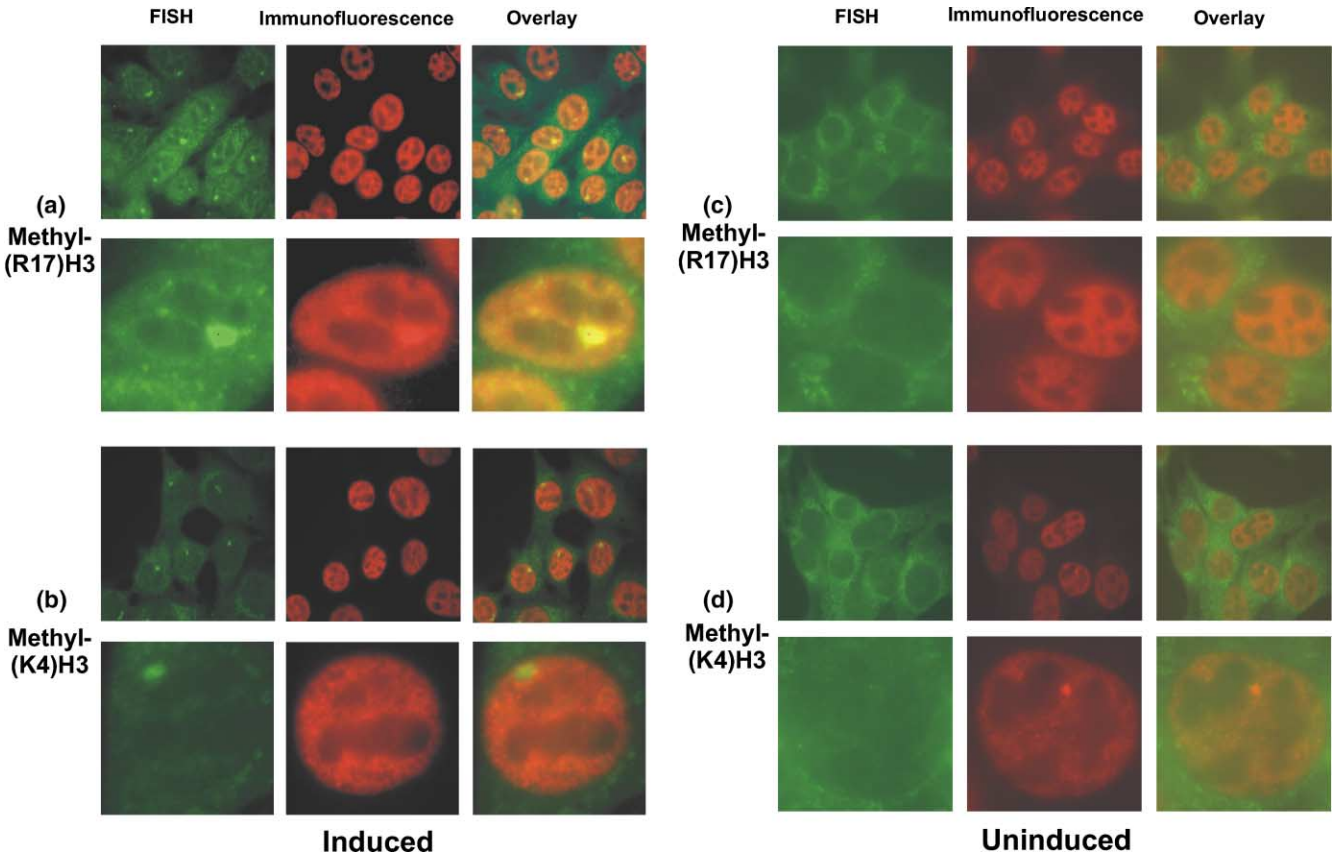
Detection of CARM1 and CARM1-specific methylation of histone H3 on the MMTV promoter by chromatin immunoprecipitation. **(a)** 1471.1 cells were incubated without or with 100 nM dex for 40 min. Sheared chromatin was prepared from cross-linked cells, and chromatin immunoprecipitation assays were conducted with antibodies against the indicated proteins, as described in the Materials and methods. DNA was isolated from the immunoprecipitates, and the presence of the nucleosome B region of the MMTV promoter (which contains the glucocorticoid response elements) was detected by PCR. Before immunoprecipitation, a fraction of the sheared chromatin was reserved, and PCR analysis of the isolated DNA was conducted (input). The negative images of ethidium bromide-stained PCR products from a single experiment are shown. The results shown are typical of three independent experiments. **(b)** The results of a second, independent chromatin immunoprecipitation experiment, using antibodies against CARM1 or α-methyl(R17)H3 antibodies against the CARM1-methylated form of histone H3, are shown. Fractions of the total immunoprecipitated DNA used for PCR are indicated below each panel. PCR was performed with primers for the MMTV promoter (MMTV-GRE) as in (a), or primers for the β-*actin* 5′-untranslated region (β-actin).

In control PCR reactions with DNA from the same immunoprecipitations with CARM1 and α-methyl(R17)H3 antisera, the 5′-untranslated region of the β-*actin* gene produced a very weak signal that was not affected by hormone treatment of the cells (Figure 3b). Only specific proteins and histone modifications were targeted to the MMTV promoter by the hormone: normal rabbit IgG failed to precipitate the MMTV promoter, and antibodies that specifically recognize histone H3 methylated at lysine 4 (α-methyl(K4)H3) or lysine 9 (α-methyl(K9)H3) produced weak signals that were not affected by dex treatment (Figure 3a). Methylation of Lys9 was recently associated with heterochromatin formation [5], while methylation of Lys4 has been suggested as a marker for transcriptionally competent chromatin [2], although its specific association with active genes has not yet been fully explored. Thus, not all methyl modifications on histones have the same functional consequences.

As a further test of hormone-dependent, CARM1-specific methylation of histone H3 in vivo, we used immunofluorescence microscopy to examine the interaction in situ of the α-methyl(R17)H3 antiserum with stably integrated MMTV promoters. In cell line 3134, approximately 200 tandemly integrated copies of an MMTV-Ras transcription unit (MMTV promoter driving a *v-Ras* gene) form a chromatin cluster or array that can be visualized in living cells by the hormone-dependent binding of GR attached to green fluorescent protein [15]. Fluorescence in situ hybridization against *v-Ras* RNA (RNA-FISH) also detected nascent transcripts on the array after dex treatment (Figure 4a,b, left panels, intense green spot in each nucleus), but not in the absence of dex (Figure 4c,d, left panels), indicating the hormone-dependent synthesis of *v-Ras* RNA at the array. The α-methyl(R17)H3 antiserum produced a general nuclear staining, but was excluded from nucleoli; in addition, it stained the MMTV-Ras arrays intensely in cells treated with dex (Figure 4a, middle panels), as indicated by the intense red spot in each nucleus at the same location as each intense green spot in the panel to the left. Overlay of the RNA-FISH and α-methyl(R17)H3 images confirmed that the region intensely stained by α-methyl(R17)H3 antiserum in each cell corresponds to the MMTV-Ras array (Figure 4a, right panels, intense yellow spot in each nucleus). In the absence of dex, the α-methyl(R17)H3 antiserum still produced the general nuclear staining (exclusive of nucleoli), but no intensely staining regions were observed (Figure 4c). Thus, the antiserum against the CARM1-methylated histone H3 recognized the MMTV-Ras array only when the array was transcriptionally active.

In control experiments, α-methyl(K4)H3 antiserum produced the same type of general nuclear staining pattern (excluding nucleoli) as the α-methyl(R17)H3 antiserum. However, in dex-treated cells, the α-methyl(K4)H3 antiserum was excluded from the active MMTV-Ras array (Figure 4b): note that there is no intense red immunofluorescence spot (middle panels) corresponding to the green RNA-FISH spot (left panels), and that, in the RNA-FISH/immunofluorescence overlay photos, the arrays are green when the α-methyl(K4)H3 antiserum was used (Figure 4b, right panels), in contrast to the yellow overlay spot observed with α-methyl(R17)H3 antiserum (Figure 4a, right panels). Thus, in dex-treated cells, the arrays contained histone H3 methylated at Arg17, but not at Lys4; this result is consistent with our chromatin immunoprecipitation data (Figure 3). Interestingly and unexpectedly,

Figure 4



Immunofluorescence analysis of CARM1-specific methylation of histone H3 in a transcriptionally active array of MMTV promoters. Cell line 3134 [16] was grown **(a,b)** with or **(c,d)** without 100 nM dex for 30 min. RNA-FISH to detect *v-Ras* RNA [15] (green, left panels) and immunofluorescence [17] (red, center panels) were performed as previously described; in the merged red and green images (Overlay, right panels), coincident staining is shown in yellow. Antisera used were (a,c) α-methyl(R17)H3 at 1:100 dilution or (b,d) α-methyl(K4)H3 at 1:100 dilution. Confocal microscopy images were obtained as previously described [17]. Images of single nuclei are extracted from the panel immediately above.

when cells were not treated with dex, the α-methyl(K4)H3 antiserum, but not the α-methyl(R17)H3 antiserum, interacted with the MMTV-Ras arrays (intense red spot in middle and right panels of Figure 4d, but not Figure 4c). This staining of the array by the α-methyl(K4)H3 antiserum disappeared after dex treatment (Figure 4b). The significance of the α-methyl(K4)H3 staining of the array in the absence (but not in the presence) of hormone is not clear but may suggest that Lys4 methylation of H3 marks chromatin that is competent for transcription but not yet fully activated.

Conclusions

Previous studies [4, 7], and the fact that α-methyl(R17)H3 antiserum fails to produce a convincingly specific signal on immunoblots with bulk mammalian histones (Figure 2 and data not shown), suggest that the overall cellular level of Arg17 methylation of histone H3 is extremely low. It therefore seems likely that CARM1-specific methylation of arginine residues in histone H3 may occur transiently in connection with transcriptional activation or on a small subset of promoters. In keeping with this idea, it has also been recently reported that the steady state levels of histone H4 methylation at Arg3 by PRMT1 in vivo are quite low [8, 9]. Nevertheless, when arrays of MMTV promoters were activated by hormone, we observed a concomitant appearance of methylated histone H3 in the array (Figures 3 and 4), indicating that arginine-specific histone methylation occurred during chromatin remodeling and transcriptional activation. The α-methyl(R17)H3 antiserum was specific for the CARM1-methylated form of histone H3 (Figure 2), and CARM1 is the only known arginine-specific histone H3 methyltransferase; this implicates CARM1 as the enzyme responsible for the observed histone methylation on the MMTV promoter. This conclusion is supported by our demonstration that CARM1 is recruited to the MMTV promoter in response to hormonal activation (Figure 3), that CARM1 (in collab-

oration with p160 coactivators like GRIP1) can serve as a transcriptional coactivator for GR on stably integrated MMTV-CAT reporter genes (Figure 1) and on transiently transfected reporter genes [10, 11], and that the methyltransferase activity of CARM1 is required for its coactivator function on both stable and transient reporter genes (Figure 1) [10]. Our findings provide the first in vivo evidence that arginine methylation of histones, catalyzed by recruited coactivators such as CARM1, occurs at target promoters as an important part of the transcriptional activation process. The parallels to the paradigm already well established for histone acetylation are obvious and suggest that histone methylation, probably on both arginine and lysine residues, is also part of the chromatin remodeling process that accompanies transcriptional activation.

Supplementary material

Supplementary material including Materials and methods with additional references is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgements

We thank Dr. Mark Danielsen (Georgetown University) for the BuGR2 antibodies against GR. This work was supported by United States Public Health Service grants DK55274 (M.R.S.), NS17269 (D.W.A.), GM53512 (C.D.A.), and GM20039 (B.D.S.) from the National Institutes of Health.

References

1. Glass CK, Rosenfeld MG: **The coregulator exchange in transcriptional functions of nuclear receptors.** *Genes Dev* 2000, **14:**121-141.
2. Rice JC, Allis CD: **Histone methylation versus histone acetylation: new insights into epigenetic regulation.** *Curr Opin Cell Biol* 2001, **13:**263-273.
3. Strahl BD, Allis CD: **The language of covalent histone modifications.** *Nature* 2000, **403:**41-45.
4. Stallcup MR: **Role of protein methylation in chromatin remodeling and transcriptional regulation.** *Oncogene* 2001, **20:**3014-3020.
5. Jenuwein T: **Re-SET-ting heterochromatin by histone methyltransferases.** *Trends Cell Biol* 2001, **11:**266-273.
6. Gary JD, Clarke S: **RNA and protein interactions modulated by protein arginine methylation.** *Prog Nucleic Acids Res Mol Biol* 1998, **61:**65-131.
7. Schurter BT, Koh SS, Chen D, Bunick GJ, Harp JM, Hanson L, *et al.*: **Methylation of histone H3 by aoactivator-associated arginine methyltransferase 1.** *Biochemistry* 2001, **40:**5747-5756.
8. Strahl BD, Briggs SD, Brame CJ, Caldwell JA, Koh SS, Ma H, *et al.*: **Methylation of histone H4 at arginine 3 occurs in vivo and is mediated by the nuclear receptor coactivator PRMT1.** *Curr Biol* 2001, **11:**996-1000.
9. Wang H, Huang Z-Q, Xia L, Feng Q, Erdjument-Bromage H, Strahl BD, *et al.*: **Methylation of histone H4 at arginine 3 facilitates transcriptional activation by nuclear hormone receptor.** *Science* 2001, **293:**853-857.
10. Chen D, Ma H, Hong H, Koh SS, Huang S-M, Schurter BT, *et al.*: **Regulation of transcription by a protein methyltransferase.** *Science* 1999, **284:**2174-2177.
11. Chen D, Huang S-M, Stallcup MR: **Synergistic, p160 coactivator-dependent enhancement of estrogen receptor function by CARM1 and p300.** *J Biol Chem* 2000, **275:**40810-40816.
12. Beato M, Herrlich P, Schütz G: **Steroid hormone receptors: many actors in search of a plot.** *Cell* 1995, **83:**851-857.
13. Mangelsdorf DJ, Evans RM: **The RXR heterodimers and orphan receptors.** *Cell* 1995, **83:**841-850.
14. Smith CL, Hager GL: **Transcriptional regulation of mammalian genes in vivo. A tale of two templates.** *J Biol Chem* 1997, **272:**27493-27496.
15. McNally JG, Muller WG, Walker D, Wolford R, Hager GL: **The glucocorticoid receptor: rapid exchange with regulatory sites in living cells.** *Science* 2000, **287:**1262-1265.
16. Kramer PR, Fragoso G, Pennie W, Htun H, Hager GL, Sinden RR: **Transcriptional state of the mouse mammary tumor virus promoter can affect topological domain size in vivo.** *J Biol Chem* 1999, **274:**28590-28597.
17. Baumann CT, Ma H, Wolford R, Reyes JC, Maruvada P, Lim C, *et al.*: **The glucocorticoid receptor interacting protein 1 (GRIP1) localizes in discrete nuclear foci that associate with ND10 bodies and are enriched in components of the 26S proteasome.** *Mol Endocrinol* 2001, **15:**485-500.