

Coordinated Changes in DNA Methylation and Histone Modifications Regulate Silencing/Derepression of Luteinizing Hormone Receptor Gene Transcription

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We have previously demonstrated that transcription of the luteinizing hormone receptor (LHR) gene is subject to repression by histone deacetylation at its promoter region, where a histone deacetylase (HDAC)/mSin3A complex is anchored at a proximal Sp1 site. The present studies have shown that epigenetic silencing and activation of the LHR gene is achieved through coordinated regulation at both the histone and DNA levels. The HDAC inhibitor trichostatin A (TSA) evoked robust but significantly lower activation of the LHR gene in JAR than in MCF-7 cells. This effect was localized to the 176-bp promoter region, which is highly methylated in JAR and lightly methylated in MCF-7 cells. Consequently, TSA and the DNA demethylating reagent 5-azacytidine (5-AzaC) caused marked synergistic activation of the LHR gene in JAR but not in MCF-7 cells. Multiple site-specific lysine acetylation of H3/H4 is associated with such LHR gene activation. Methylation or acetylation of H3 at K9 is present at the silenced and derepressed LHR promoter, respectively. While DNA methylation levels did not affect the histone code of the LHR gene promoter, demethylation of the promoter CpG sites was necessary for maximal stimulation of this gene. Mechanistically, the combined actions of TSA and 5-AzaC, but not either 5-AzaC or TSA alone, resulted in complete demethylation of the LHR gene promoter in JAR cells. Release of the repressive HDAC/mSin3A complex from the LHR gene promoter in both cell types required both TSA-induced changes of histone modifications and, concurrently, a demethylated promoter. Also, Dnmt1 was largely dissociated from the LHR gene promoter in the presence of TSA or TSA plus 5-AzaC, and binding of MBD2 in JAR cells was diminished upon conversion of the promoter to a demethylated state. Such changes induced a more permissive chromatin where recruitment of polymerase II and TFIIB to the promoter was significantly increased. The activated state of the LHR gene induced by TSA and 5-AzaC in JAR and MCF-7 cells was observed basally in LHR-expressing PLC cells, in which the promoter is unmethylated and associated with hyperacetylated histones. Consequently, PLC cells are unresponsive to drug treatment. These findings have elucidated a regulatory mechanism whereby concurrent dissociation of repressors and association of activators and basal transcriptional components, resulting from coordinated histone hyperacetylation and DNA demethylation, lead to derepression of the LHR gene expression.

The luteinizing hormone receptor (LHR) is a G protein-coupled receptor that has an essential role in gonadal maturation and function (8, 14). The LHR gene is expressed primarily in gonads, where its expression is tightly controlled to mediate LH signals regulating ovarian cyclic changes and testicular function. In addition, LHR gene expression has been observed in several nongonadal tissues including placenta, uterus, normal mammary glands, and breast tumor and placenta cell lines (14).

Characterization of the regulatory mechanisms for LHR gene transcription has been advanced by identification of the LHR gene promoter region in different species and the various transcription factors that are involved in its basal transcription (14, 56). LHR gene promoter activity is controlled by two activating Sp1/Sp3-binding domains and an inhibitory direct-repeat motif recognized by nuclear orphan receptors EAR2 and EAR3/COUP-TFI (19, 50–52, 57, 58). The orphan receptor-mediated repression results from a direct interaction be-

tween EAR3/COUP-TFI and Sp1 bound to the proximal Sp1 site, which perturbs the interaction of Sp1 and TFIIB and the recruitment of RNA polymerase II (Pol II) (59). Moreover, we have demonstrated that the LHR gene is subject to epigenetic regulation whereby local chromatin changes at the LHR gene promoter are critical for gene transcription (60). The proximal Sp1 site was identified as a docking site to recruit the histone deacetylase (HDAC)/mSin3A complex, with histone deacetylation causing marked silencing of gene promoter activity. It is widely accepted that different modifications of histone proteins by acetylation, methylation, or phosphorylation affect the access of regulatory factors and complexes to chromatin and influence gene expression (10, 20, 24, 28). Acetylation and deacetylation of histones H3 and H4 have been shown to induce a relaxed and competent or a condensed and inactive chromatin, respectively (20, 46). Methylation of histones at key lysine residues was found to act cooperatively with histone acetylation to encode a specific histone code to determine a heritable transcriptional state (1, 27, 40, 45). These findings include the demonstration of methylated H3 at lysine 4 (K4) enriched at active hyperacetylated euchromatin domains and methylated H3 at K9 found associated with silent hypoacetylated heterochromatin regions.

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DNA methylation by the addition of a methyl group to a cytosine residue at CpG dinucleotides is well recognized to correlate with gene silencing (5). It has been noted that DNA methylation is involved in allele-specific imprinting, X-chromosome inactivation, aberrant repression of tumor suppressor genes in cancers, and silencing of exogenous integrated genes (25, 31, 37). DNA methylation is thought to negatively regulate target gene expression by interfering with transcription factor binding or facilitating formation of an unfavorable chromatin structure (16, 26). The evidence that several methyl-CpG binding proteins (e.g., MeCP2 and MBD2) interacted with HDAC1 and HDAC2 and recruited the Sin3 corepressor protein supports a mechanism linking DNA methylation and histone modifications (6, 15, 17). However, the proposed cross talk between these mechanisms has not been fully understood, and it is not yet clear how it is triggered and precisely regulated in response to diverse physiological settings. Also, compared to substantial studies in this area for tumor suppressor genes, little is known about the role of epigenetic modulation of target genes in other categories.

The current studies have investigated the functional relevance of histone modifications and DNA methylation in the regulation of the LHR gene expression. These were initiated by characterization of the LHR gene promoter methylation status in JAR and MCF-7 cells, where the transcription of this gene is markedly silenced, and in simian virus 40-transformed normal placenta PLC cells, where LHR expression is in an active state. Utilizing the DNA demethylating reagent 5-azacytidine (5-AzaC) and the HDAC inhibitor trichostatin A (TSA), we have explored potential combinatorial requirements of histone modification(s) and DNA methylation/demethylation affecting the repression/derepression modalities of LHR gene transcription. Our results have indicated the existence of a novel gene silencing/derepression mechanism emerging from coordinated changes in histone modifications and DNA methylation/demethylation, which pertains to a different class than that derived from epigenetic studies on tumor suppressor genes.

MATERIALS AND METHODS

Cell culture and transfection. JAR cells (human choriocarcinoma cells) and MCF-7 cells (human mammary gland carcinoma cells) from the American Type Culture Collection (Manassas, VA) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Simian virus 40-transformed human placental cells (PLC) supplied by the American Type Culture Collection were cultured at 33°C in Alpha minimum essential medium (Invitrogen) with 10% fetal bovine serum.

TSA and 5-AzaC were obtained from CalBiochem (San Diego, CA) and Sigma (St. Louis, MO), respectively. Cells were treated with 5-AzaC (1 μ M), TSA, (0 to 500 ng/ml) or both or with vehicle alone (0.005% acetic acid). Cells were treated with 5-AzaC for a total of 48 h, and at every 12-h interval, the medium was replaced with freshly added 5-AzaC. TSA at indicated doses was added to the cells 12 h prior to termination of the experiments.

Transfections were carried out using Lipofectamine Plus reagents (Invitrogen), according to the procedures recommended by the manufacturer. The luciferase activities were normalized to light units/ μ g of protein and expressed as mean \pm standard error. All experiments were performed at least three times in triplicate wells.

In vitro methylation of reporter gene plasmid. CpG methylase SssI was used to methylate the CpG dinucleotides of the LHR gene promoter/luciferase reporter construct or pGL2 promoterless basic vector, based on the protocol of the manufacturer (New England Biolabs, Beverly, MA). Mock reactions were carried out in parallel without adding the methylase. The samples were then incubated with methylation-sensitive restriction enzyme BstUI or HapII, followed by

agarose gel electrophoresis. Complete methylation of the CpG sites of the constructs was verified by protection of the methylated DNA from digestion by these two enzymes. Methylated and mock-treated plasmids were purified with a QIAquick gel extraction kit (QIAGEN, Valencia, CA) and used for reporter gene analyses.

Genomic DNA isolation and bisulfite sequencing analyses. Genomic DNA from JAR or MCF-7 cells treated with or without 5-AzaC, TSA, or both was isolated by incubation of the cells with lysis buffer (200 mM NaCl, 20 mM EDTA, 40 mM Tris-HCl [pH 8.0], 0.5% sodium dodecyl sulfate, 0.5% β -mercaptoethanol) at 37°C for 3 min. This was followed by proteinase K (100 μ g/ml) digestion at 50°C for 5 h. The DNA was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and was collected by ethanol precipitation.

One microgram of genomic DNA sheared by BamHI digestion was subjected to bisulfite treatment. Briefly, DNA was denatured in 0.3 M NaOH at 37°C for 30 min and then precipitated. The denatured DNA was incubated with 3.1 M sodium bisulfite and 0.5 mM hydroquinone (pH 5.0) at 55°C for 4 h. The reaction was terminated by further incubation of the sample with 0.3 M NaOH at room temperature for 20 min, and DNA was purified using a QIAquick gel extraction kit (QIAGEN). The bisulfite-modified DNA was then used as a template in PCRs to amplify the LHR gene promoter region. The bisulfite sequencing-specific primers used were 5'GAGGTTATTGGGTTATTGGGTATT 3' (forward) and 5' CAACAACAACCTCAACAACCTACAAC 3' (reverse). The PCR products were cloned into pCR2.1-TOPO vector (Invitrogen) and analyzed by sequencing.

RNA isolation, RT-PCR, and real-time PCR. Total RNA from JAR, MCF-7, or PLC cells treated with or without 5-AzaC, TSA, or both was extracted using an RNeasy kit (QIAGEN). Prior to the reverse-transcription (RT) reaction, the total RNA was treated with DNase I to remove any possible copurified DNA. RT-PCR was carried out using a QIAGEN One-Step RT-PCR kit in which 2 μ g of total RNA of each sample was applied as template. The primers for amplification of a 475-bp fragment, which encodes the C-terminal coding region of the human LHR gene (nucleotides 1557 to 2031), were 5' GGAAACCCTCTCT CACAAGT 3' (forward) and 5'GGTGGATTGAGAAGGCTTATTG 3' (reverse). Amplification of a 620-bp fragment of the human β -actin gene was performed as an internal control. The specific primers for the β -actin gene were 5'CCTCGCCTTTGCCGATCC 3' (forward) and 5' GGATCTTCATGAGGTA GTCAGTC 3' (reverse).

For real-time quantification of LHR mRNA, 2 μ g of total RNA was reverse transcribed using a SuperScript III kit (Invitrogen) containing a mixture of oligo(dT)₂₀ and random hexamer primers. The first-strand cDNA from 100 ng of RNA was used as a template in real-time PCR with SYBER-Green Master Mix and an ABI 7500 sequence detection system (Applied Biosynthesis, Foster City, CA). The cycling program was set as follows: denature at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primers for the human LHR gene were 5' ATGGCAATCCTCATCTTCACCG3' (forward) and 5' GTG AATATTGCATACAGAAATG3' (reverse), and the specificity of the PCR products was verified by melting curve analyses at the end of the PCR. The amplified PCR product was cloned into pCR2.1-TOPO vector, and the standard curves were created by a 10-fold serial dilution (copy number of 10⁶ to 10¹) of the cloned plasmid DNA. Each sample was assayed in triplicate, and the results were normalized to the level of β -actin mRNA.

ChIP. Chromatin immunoprecipitation (ChIP) experiments were performed using a chromatin immunoprecipitation assay kit from Upstate Biotechnology (Lake Placid, NY). Briefly, 2 \times 10⁷ cells treated with or without 5-AzaC, TSA, or both were fixed by 1% formaldehyde at 37°C for 10 min and then were lysed and sheared by sonication. The soluble chromatin fraction was subjected to immunoprecipitation by antibodies against different transcription factors and modified histones as indicated. The precipitated complexes were washed sequentially with low salt, high salt, LiCl, and Tris-EDTA buffer and extracted twice with freshly prepared 1% sodium dodecyl sulfate–0.1 M NaHCO₃. The cross-linking between DNA and proteins was reversed by heating the samples at 65°C for 6 h, followed by proteinase K digestion at 50°C for 2 h. DNA was then purified by ethanol precipitation, and 2 μ l of the 50- μ l sample was analyzed by PCR with the cycle number ranging from 24 to 33, based on each individual transcription factor assayed. The primers for the LHR gene promoter region were 5'ACTGGGCACTGTGCGAGGTC3' (forward) and 5'CATGGCCGGG GAACGGGCT3' (reverse) and for the β -actin promoter were 5'TCGGATAT CCACGTGACATCCA3' (forward) and 5'GCAGCATTTTTTACCCTC3' (reverse). The ChIP-precipitated DNA and input DNA were also subjected to real-time PCR analyses using SYBR-Green Master Mix in an ABI 7500 sequence detection system, and samples from two individual ChIP assays were analyzed in triplicate.

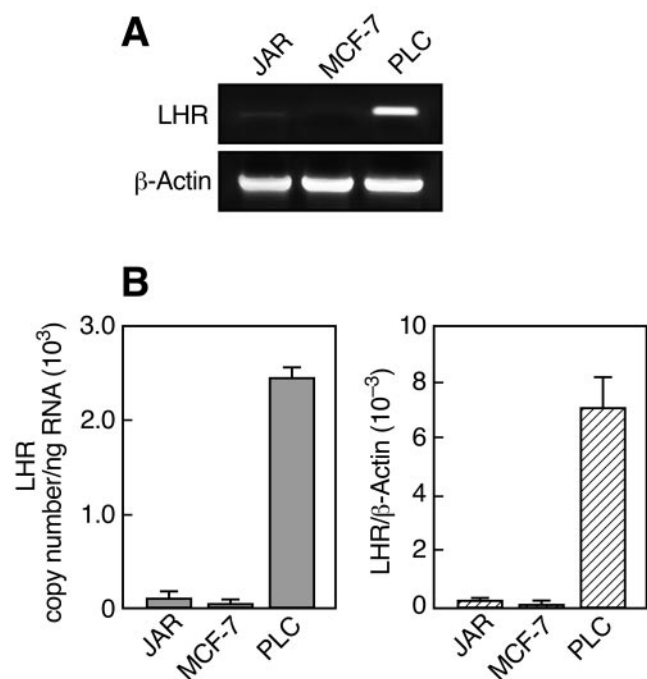


FIG. 1. Expression of LHR gene in JAR, MCF-7, and PLC cells. The LHR mRNA levels in JAR, MCF-7, and PLC cells were determined by conventional RT-PCR (A) and real-time PCR analyses (B). For quantitative measurement, the results are expressed as copy number of LHR mRNA per ng of total RNA or as the ratio to the copy number of β -actin mRNA.

Nuclear extracts isolation and Western blotting. Nuclear extracts from treated or untreated JAR or MCF-7 cells were prepared using methods described previously (13, 22). A total of 30 μ g of nuclear extracts or 2.5 μ g of histone proteins was analyzed by Western blotting using the antibodies indicated. The antibodies against different modified forms of histones were obtained from Upstate. The antibodies for transcription factors Sp1, Sp3, HDAC1/2, mSin3A, p300, GCN5, TFIIB, and RNA Pol II and the actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody for Dnmt1 was from New England Biolab, and the antibodies for MBD2 and MeCP2 were provided by Abcom (Cambridge, MA).

RESULTS

Methylation status of the LHR gene promoter in JAR, MCF-7, and PLC cells. Our previous studies have demonstrated that changes in the histone acetylation level at the LHR gene promoter region exerted a critical impact on the gene's expression in placenta carcinoma JAR cells (60). To elucidate the epigenetic mechanisms that participate in silencing or activation of LHR gene transcription, we utilized JAR and MCF-7 cells, in which the expression of the LHR gene is silenced, and a normal placenta cell line (PLC) actively expressing the LHR gene (Fig. 1). Such distinct expression patterns of the LHR gene in these cell types provided an appropriate setting for the investigation of histone and DNA modification levels associated with the state of LHR gene expression.

Analyses of the sequences at the 5' flanking region of the LHR gene (-2.8 kb) and its first exon by a CpG island searcher program indicated that the LHR gene promoter is located within a predicated single CpG island that encom-

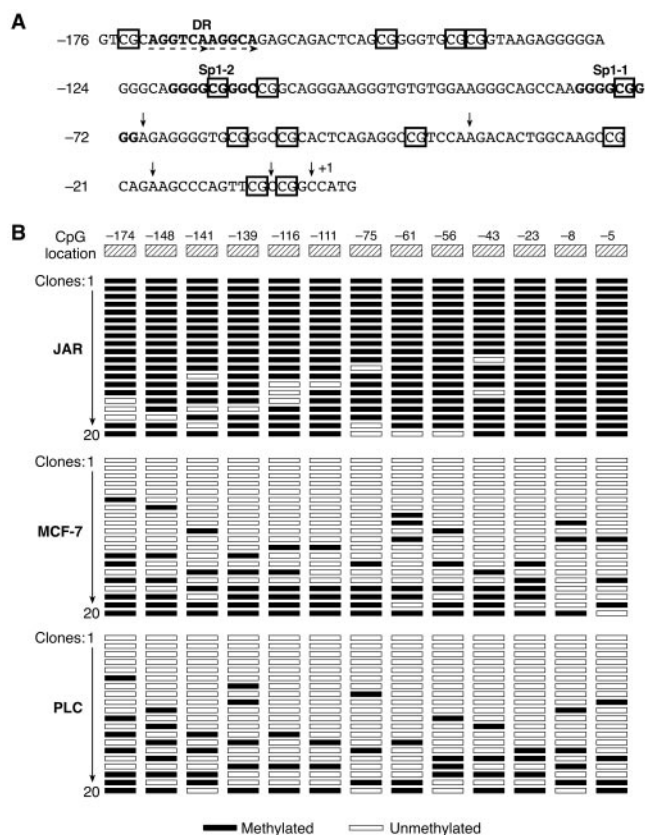


FIG. 2. DNA methylation status of the LHR gene promoter in JAR, MCF-7, and PLC cells. (A) DNA sequences of the LHR promoter with nucleotides numbered relative to ATG (+1). The transcriptional start sites are indicated by arrows. The two Sp1 domains, Sp1-1 and Sp1-2, and the orphan receptor-binding direct-repeat motif (DR) are shown in bold; the CpG dinucleotides are boxed. (B) The LHR gene promoter is highly methylated in JAR cells, whereas it is largely unmethylated in both MCF-7 and PLC cells. For each cell type, the methylation status of 20 individual clones as determined by bisulfite sequencing analyses is shown in rows 1 to 20. The filled or open blocks represent the methylated or unmethylated CpG sites, respectively.

passes nucleotides -212 to +203 relative to the ATG start codon (+1). This island was defined based on its size (all values are observed/expected; 415 bp/>200 bp), GC content (72%/>50%), and CpG dinucleotide frequency (0.66/>0.6) (49). The DNA methylation status of the LHR gene promoter in JAR, MCF-7, and PLC cells was assessed utilizing bisulfite treatment and sequencing analyses. Evaluation of 20 individual clones from each cell type revealed that 50% of the clones (10/20) in JAR cells were fully methylated at all 13 CpG sites of the promoter (100% methylation), and 45% of the clones (9/20) were methylated at 11 sites of the 13 (84.6% methylation) (Fig. 2). Even in the two clones (2/20) with the lowest methylation level, 10 CpG sites were methylated (76.9% methylation). These data demonstrated that in JAR cells the LHR gene promoter is densely methylated. In contrast, the LHR gene promoter was largely unmethylated in MCF-7 cells, with 80% of the clones (16/20) either completely unmethylated or only sparsely methylated (methylation in no more than 4 of the 13 CpG sites). Similar findings were obtained in PLC cells in

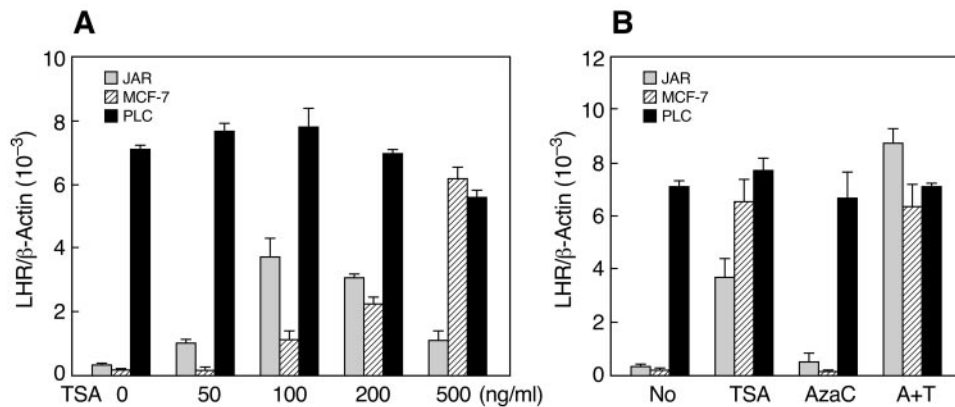


FIG. 3. TSA and 5-AzaC treatment caused differential effects on LHR gene expression in JAR, MCF-7, and PLC cells. Shown are the results of real-time PCR analyses of the LHR mRNA levels in JAR, MCF-7, and PLC cells that were treated with different doses of TSA (A) or with TSA, 5-AzaC, and the combination (B) following the procedure described in Materials and Methods. The TSA concentration utilized in panel B was 100 ng/ml for JAR or PLC cells and 500 ng/ml for MCF-7 cells. The results are presented as the ratio of the copy number of LHR mRNA to that of β -actin.

which an unmethylated promoter region was revealed. Taken together, these results demonstrated a distinct methylation state of the LHR gene promoter in JAR versus MCF-7 and PLC cells. Although in JAR and PLC cells the promoter methylation states inversely correlated with the expression levels of the LHR gene, this was not the case in MCF-7 cells. These findings indicated that the level of DNA methylation may not be solely responsible for silencing or reactivation of LHR gene expression.

Synergistic derepression of LHR gene expression by 5-AzaC and TSA in JAR but not MCF-7 cells. The coordinated effects of histone acetylation and DNA demethylation on LHR gene expression were next investigated by real-time PCR analyses of cells treated with TSA, 5-AzaC, or both (Fig. 3). The maximal concentration of TSA utilized in these experiments was 500 ng/ml. Higher doses of TSA caused marked apoptotic effects in JAR and MCF-7 cells. TSA caused a significant dose-dependent increase in expression of the LHR gene in JAR and MCF-7 cells while only minimal changes were observed in PLC cells. Maximal induction of LHR gene expression in JAR and MCF-7 cells, however, was achieved at different doses of TSA. In JAR cells, 100 ng/ml of TSA resulted in the highest increase of the LHR mRNA level, while in MCF-7 cells 500 ng/ml of TSA induced the LHR gene expression to a level similar to that observed in PLC cells (treated and untreated). 5-AzaC alone (1 μ M for 48 h) had little influence on LHR gene expression in all cell types (Fig. 3B). Extension of the treatment of cells with 5-AzaC (1 to 5 μ M) for up to 96 h gave similar results (data not shown). However, the combined treatment of cells with 5-AzaC and TSA resulted in synergistic derepression of LHR gene expression in JAR cells in which mRNA levels were slightly higher than in PLC cells. Such a synergistic effect was not observed in MCF-7 cells in which similar LHR gene expression was observed after treatment with TSA plus 5-AzaC versus TSA alone.

The relevance of the LHR gene expression patterns to the activation of its promoter activities in these cell types upon treatment by TSA and 5-AzaC were then investigated by reporter gene analyses. To mimic the endogenous methylation

status of the LHR gene promoter, an in vitro methylated LHR promoter/reporter construct was transfected into JAR cells, and the nonmodified promoter was introduced into MCF-7 or PLC cells (Fig. 4). Consistent with the gene expression analyses, TSA triggered more robust activation of the LHR promoter activity in MCF-7 cells than in JAR cells at the highest TSA dose. Also, no effect of TSA was revealed in PLC cells, as TSA also caused the same increase in induction (onefold) for the promoterless vector construct (data not shown). Furthermore, in the absence of TSA, 5-AzaC only caused a minor increase in promoter activity in JAR cells. In contrast, preincubation of the cells with 5-AzaC followed by TSA treatment elicited marked synergistic activation of LHR promoter activity. At 100 ng/ml of TSA, TSA plus 5-AzaC resulted in 120-fold stimulation, a level that was significantly higher than that induced by TSA alone (40-fold). However, such synergism was totally absent in MCF-7 and PLC cells.

The notable differential effects of TSA on the methylated (40-fold in JAR cells) versus the nonmethylated LHR promoter (180-fold in MCF-7 cells) also raised the possibility that the differential DNA methylation levels might have an impact during TSA-mediated LHR gene activation. The activities of nonmethylated and methylated LHR promoter constructs were then compared in the absence or presence of TSA (Fig. 5). Methylation of the promoter CpG sites by SssI caused substantial reduction of the basal promoter activities in both cell types ($-$ TSA). Upon TSA treatment, the unmethylated LHR gene promoter exhibited higher relative activation than its methylated counterpart ($+$ TSA), confirming that the promoter methylation state influenced the TSA-regulated LHR gene expression. Taken together, these studies have illustrated that the silenced state of LHR gene expression in JAR and MCF-7 cells can be reversed by TSA treatment. The magnitude of the TSA responses in these cells, however, was influenced by the LHR promoter methylation status. Maximal derepression of expression of the LHR gene in JAR cells required the synergistic action of 5-AzaC and TSA, indicating that promoter methylation also has an important role during TSA-mediated activation.

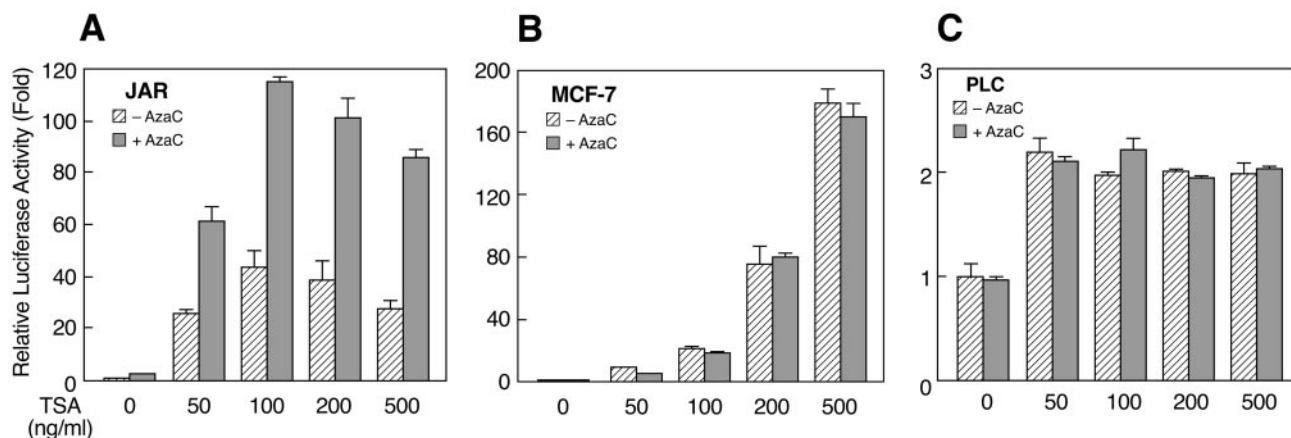


FIG. 4. Synergistic activation of the LHR gene promoter activity by TSA plus 5-AzaC in JAR but not MCF-7 cells. Results of reporter gene analyses of the LHR gene promoter activity in JAR, MCF-7, and PLC cells are shown. In vitro SssI-methylated LHR promoter construct was utilized to transfect JAR cells, and the mock-treated construct was transfected into MCF-7 or PLC cells; this step was followed by treatment with TSA and 5-AzaC. Relative promoter activities are indicated as the increase in induction (*n*-fold) over that in the absence of the treatment.

Demethylation of the LHR gene promoter in JAR cells by 5-AzaC and TSA. Based on the findings described above, it was important to determine whether the methylation state of the LHR gene promoter changed in JAR cells treated with 5-AzaC or TSA or with both reagents. Bisulfite sequencing analyses revealed that the densely methylated LHR gene promoter was only partially demethylated in the presence of 5-AzaC (Fig. 6). As expected, TSA did not alter the LHR gene promoter methylation status, while the promoter remained highly methylated after TSA treatment. However, in marked contrast, the profound methylation of the 13 CpG dinucleotides of the LHR gene promoter was largely erased upon combined treatment with 5-AzaC and TSA. The corequirement of TSA but not 5-AzaC alone for this reversal of the LHR gene promoter from the methylated to demethylated state clearly indicated the critical participation of chromatin structure during this process. Also, demethylation of the LHR gene promoter concurrently with the synergistic induction of its promoter activity by 5-AzaC and TSA illustrated that DNA methylation contributed to the LHR gene silencing in JAR cells.

Deciphering the histone code formed on the silenced or derepressed LHR gene promoter. The possibility that site-specific histone methylation works coordinately with histone acetylation during silencing or derepression of the LHR gene in the context of its promoter methylation state was next investigated by ChIP analyses with primers that encompassed the LHR gene promoter region (Fig. 7A and B). The ChIP assays were followed by quantitative analyses using real-time PCR (Fig. 8). In both JAR and MCF-7 cells, association of the LHR gene promoter with acetylated histone H3 at K9 or K14 and acetylated H4 at K12 was significantly increased in the presence of TSA or TSA plus 5-AzaC. In the PLC cells expressing LHR, the basal levels of occupancy of the LHR gene promoter by AcH3K9, AcH3K14, or AcH4K12 were similar to those found in JAR and MCF-7 cells following LHR gene activation. In contrast, methylated H3 at K9 was present prominently at the silenced LHR gene promoter in JAR and MCF-7 cells either nontreated or treated with only 5-AzaC, but it was largely absent from the activated LHR gene promoter in PLC

cells. The precise correlation of H3K9 methylation or acetylation to the LHR gene repressive or derepressive state has thus indicated that the modification status of H3K9 at the LHR gene promoter region served as a histone code for LHR gene expression. Moreover, the finding that the combined treatment of TSA and 5-AzaC did not affect the TSA-elicited H3K9 modification suggested that this epigenetic mark was independent of the promoter DNA methylation status. In addition, increased binding of MeH3K4, AcH4K8, and AcH4K16 to the activated LHR gene promoter was only noted in JAR cells, whereas enriched association of AcH4K5 was only observed in MCF-7 cells after treatment with TSA or TSA plus 5-AzaC. Such differences implied that these site-specific histone modifications might be involved in the control of LHR gene expression in a cell type-specific manner. All the observed changes associated with the LHR gene promoter have been confirmed as a gene-specific effect since the occupancy of the β -actin promoter by various types of modified histone proteins remained unaffected by the drug treatment (Fig. 7C). Taken together, these results revealed that the promoter-associated H3/H4 hyperacetylation, in particular, the conversion of methylated to acetylated H3 at lysine 9, was critically involved in TSA-induced LHR gene derepression. Moreover, the observation that the DNA methylation status of the promoter did not affect the histone code formed at the LHR gene promoter indicates that DNA methylation could regulate LHR gene transcription via a mechanism different from an action at the histone modification level.

Recruitment of transcription factors to the LHR gene promoter during silencing and derepression of LHR gene expression. ChIP analyses were further pursued to elucidate how the recruitment of transcription factors to the LHR gene promoter was regulated in correlation with the LHR gene transcriptional levels. Similar binding patterns of Sp1 and Sp3 with the LHR gene promoter were observed in JAR, MCF-7, and PLC cells at basal condition (Fig. 9). The binding remained unchanged in JAR and MCF-7 cells after the drug treatment, thus ruling out the possibility that the TSA- and 5-AzaC-mediated derepression of the LHR gene was due to the increased binding activ-

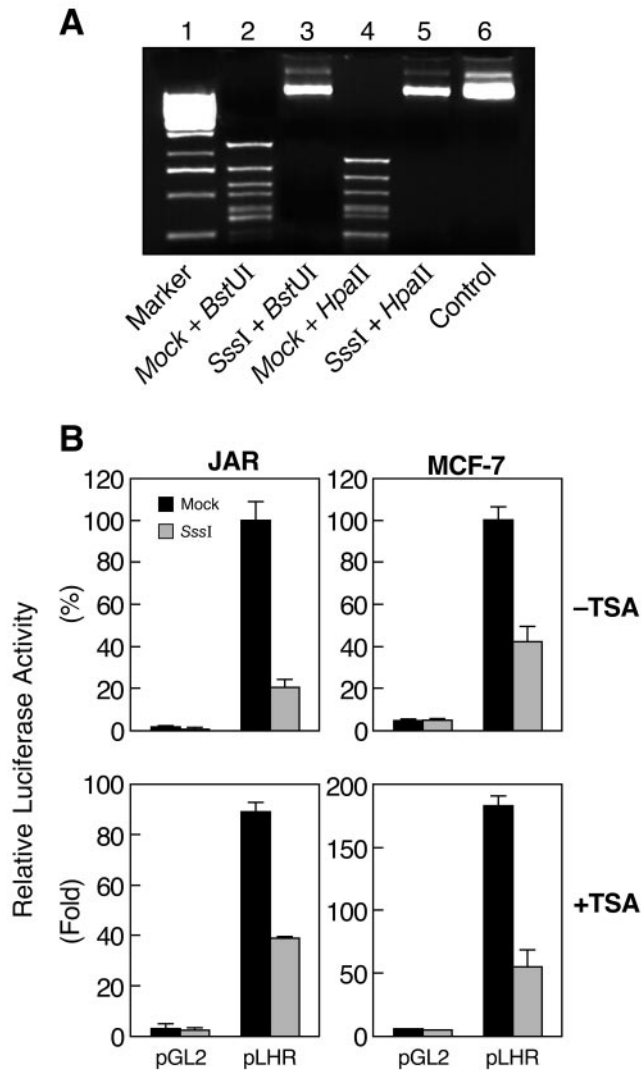


FIG. 5. DNA methylation influenced LHR gene promoter activity in the absence or presence of TSA. (A) The LHR promoter/reporter gene construct was *in vitro* methylated by SssI or mock treated, followed by digestion analyses with BstUI or HpaII. The plasmid DNA without treatment served as a control. (B) The SssI or mock-treated LHR promoter construct (pLHR) or pGL2 vector (pGL2) was transfected into JAR or MCF-7 cells, followed by treatment with (+) or without (-) TSA. The relative luciferase activities in the group without TSA are indicated as the percentage of the activity of the unmethylated pLHR construct (100%). For the TSA-treated group, the activity of each construct is indicated as the increase in induction (*n*-fold) over its activity without treatment.

ities of Sp1 and Sp3. In contrast to the observation that the HDAC1/HDAC2/mSin3A complex is largely absent from the LHR gene promoter in PLC cells, the complex was markedly associated with the repressed LHR gene in nontreated JAR and MCF-7 cells. Furthermore, release of this complex during the activation of the LHR gene depended on changes at both the histone and DNA levels. When the LHR gene promoter remained methylated in JAR cells either nontreated or treated with 5-AzaC or TSA, similar levels of association of HDAC1 and mSin3A were observed, while the binding of HDAC2 was slightly decreased by TSA. However, the complex was released

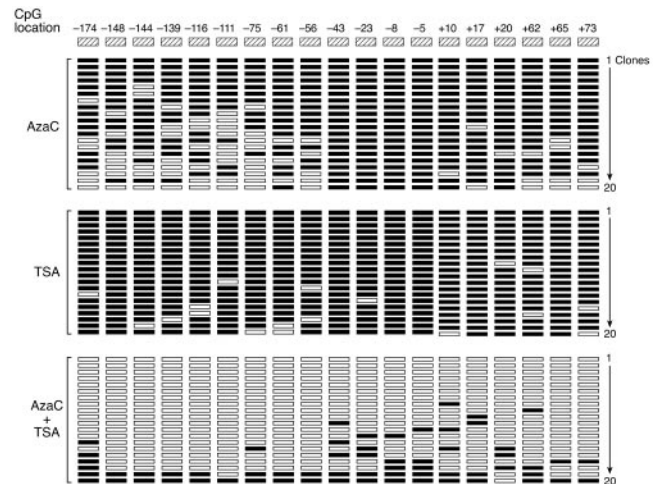


FIG. 6. Demethylation of the LHR gene promoter in JAR cells required the combined actions of TSA and 5-AzaC. The DNA methylation status of the LHR promoter in JAR cells upon treatment with 5-AzaC, TSA, or both is shown. The results of 20 clones assayed by bisulfite sequencing analyses are presented. The filled and open blocks indicate the methylated and unmethylated CpG sites, respectively.

from the demethylated promoter upon the combined action of 5-AzaC and TSA. On the other hand, TSA treatment of MCF-7 cells largely eliminated this complex from the LHR gene promoter, and no additional effect was observed in cells exposed to both 5-AzaC and TSA. Clearance of the repressive HDAC/mSin3A complex from the unmethylated but not methylated LHR gene promoter in coordination with TSA-induced changes of histones might explain the higher-magnitude activation of the LHR gene by TSA in MCF-7 cell than in JAR cells, as well as the notable synergism of TSA and 5-AzaC shown only in JAR cells.

In addition, the comparable binding of histone acetyltransferases, namely, p300 and GCNF5, to the LHR gene promoter were revealed in all three cell types treated with or without TSA and 5-AzaC. These findings indicated that dissociation of the HDAC complex rather than promotion of histone acetyltransferase recruitment was essential for disruption of the balance of histone acetylation and deacetylation within the LHR gene promoter region. Moreover, Dnmt1, the maintenance DNA methyltransferase, was found to associate with the silenced LHR gene promoter in both JAR and MCF-7 cells either nontreated or treated with 5-AzaC. However, this binding was largely reduced by TSA. TSA and 5-AzaC cotreatment caused a further decrease of this binding in JAR cells, but this appeared not to be the case in MCF-7 cells, where only residual Dnmt1 association remained after TSA treatment. In addition, among the various transcription factors analyzed in these studies, Dnmt1 was the only factor whose protein level was down-regulated by TSA or 5-AzaC and TSA treatment in JAR (Fig. 9B) and MCF-7 cells (data not shown). The potential involvement of methyl-CpG-binding proteins in this process was also investigated. MBD2 but not MeCP2 was found to associate with the highly methylated LHR gene promoter in JAR cells but was totally absent from the scarcely methylated promoter in MCF-7 and PLC cells. This association, as expected, was abolished when JAR cells were treated with TSA

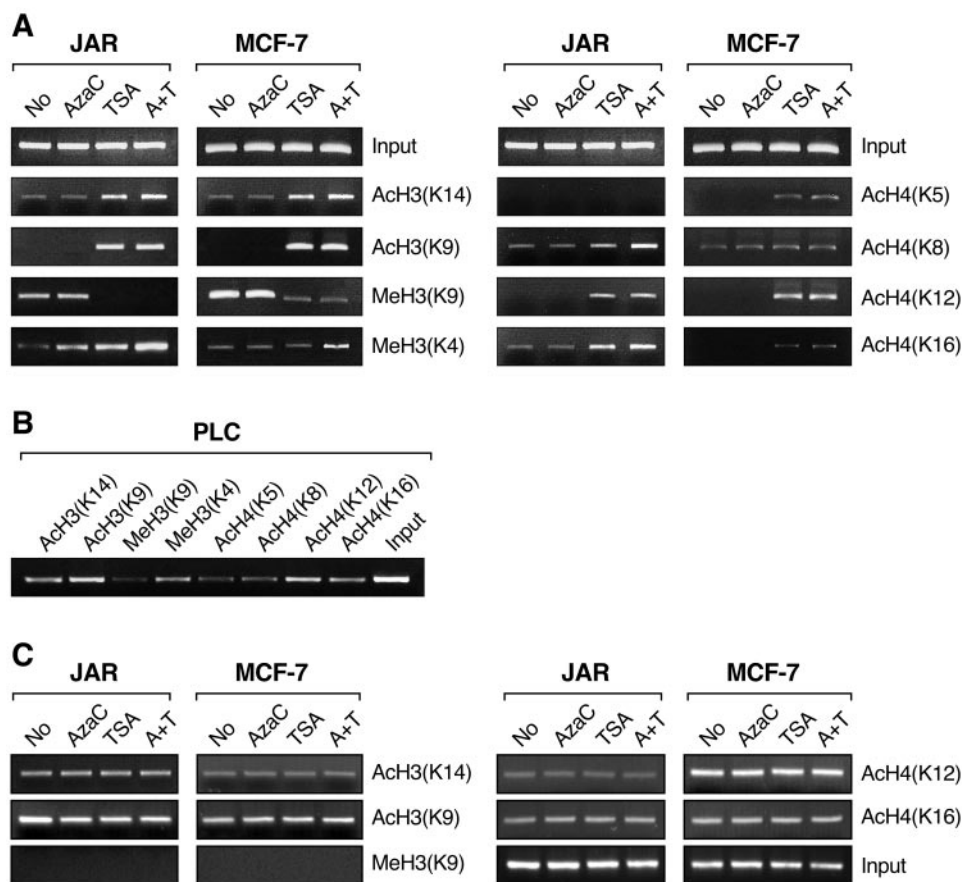


FIG. 7. ChIP analyses of the status of histone acetylation/methylation associated with the LHR gene promoter. (A) Association of the LHR gene promoter with site-specifically modified histones H3 and H4 in JAR or MCF-7 cells treated with or without TSA and 5-AzaC. (B) Recruitment of the modified H3 and H4 to the LHR gene promoter in PLC cells. (C) Association of the β -actin promoter by modified H3 and H4 in JAR and MCF-7 cells under treated or nontreated conditions. Amplification of soluble chromatin before precipitation is shown as a control (Input). No, untreated; A+T, 5-AzaC and TSA.

and 5-AzaC that converted the LHR gene promoter into a demethylated state.

Furthermore, the recruitment of Pol II and TFIIB to the LHR gene promoter was assessed in these cell types in correlation to LHR gene expression levels. In contrast to the significant occupancy of the active LHR gene promoter in PLC cells by these two general transcription factors, Pol II and TFIIB were minimally associated with the silenced state promoter in JAR and MCF-7 cells. TSA treatment caused marked enhancement of Pol II recruitment in both JAR and MCF-7 cells, and this TSA-initiated effect was further increased in JAR cells treated with TSA and 5-AzaC. Since 5-AzaC and TSA did not alter the histone code of the LHR gene over the TSA-induced changes, it is suggested that demethylated LHR promoter serves as a more favorable platform for Pol II recruitment. This became evident also for TFIIB, whose binding to the TSA-activated or 5-AzaC- and TSA-activated promoter was markedly elevated only in situations when the promoter was demethylated. Taken together, these studies have demonstrated the profound specific changes of transcription factors/complex associating with the LHR gene promoter, which resulted from the combined changes at the histone and DNA levels during silencing and derepression of the LHR gene.

DISCUSSION

The LHR is essential for reproduction in mammals. Disrupted expression and function of the LHR gene lead to compromised ovulation in females and testicular development in males (30, 39, 55). Various aspects of the LHR gene, including structure, biogenesis, expression, ligand binding/activation, and regulation of this gene, have been extensively studied (14). However, in contrast to the elucidation of the *cis* elements and *trans* factors involved in the transcriptional control of this gene, the evidence for the participation of an epigenetic mechanism was only recently revealed (56, 60).

Based on our previous report that histone hypoacetylation contributes to repression of the LHR gene, the current studies have delineated a mechanism whereby modification of histones H3/H4 assembled at the LHR promoter region and of the CpG sites of the promoter coordinately cause silencing of LHR gene expression. Derepression of this gene is achieved as a result of hyperacetylation of H3/H4 at multiple lysine residues, with a switch of methylation to acetylation of H3 at K9 and complete demethylation of its promoter region. These changes induced a chromatin environment that favored depletion of HDAC/mSin3A, Dnmt1, and MBD2 from the silenced LHR gene

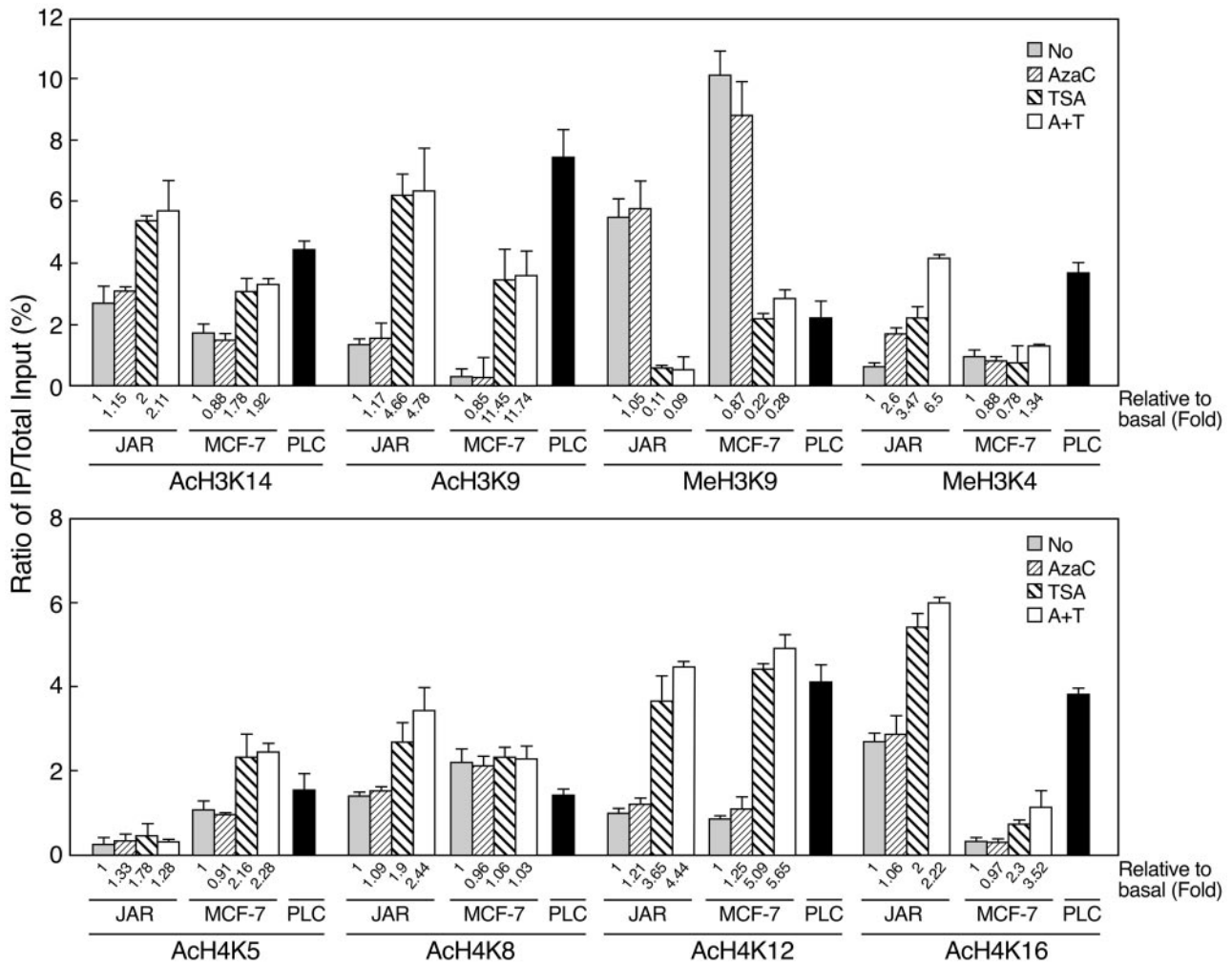


FIG. 8. Quantitative analyses of the occupancy of modified H3 and H4 to the LHR gene promoter in JAR, MCF-7, and PLC cells. The association of the LHR gene promoter with various modified forms of histones H3 and H4 in the three types of cells under treated or nontreated conditions was quantitatively determined by real-time PCR. The results are expressed as the percentage of total input DNA utilized. IP, immunoprecipitate; A+T, 5-AzaC and TSA.

promoter and promoted recruitment of Pol II and TFIIB to the activated promoter.

We have demonstrated that histone modifications have a dominant role in the control of silencing or activation of LHR gene expression, and DNA methylation and demethylation are operative under the architecture set by histone modifications. Such conclusions are derived from the following findings. (i) TSA caused marked yet differential activation of LHR gene expression in both JAR and MCF-7 cells, independent of the distinct promoter methylation status in these cells. (ii) Exposure to 5-AzaC has been known to generally lower the overall DNA methylation levels in various cell types. However, treatment with 5-AzaC alone (1 μ M for 48 h) did not elicit an effect on the LHR gene expression level in JAR cells, and extended treatment of the cells with higher doses of 5-AzaC (5 μ M for 96 h) gave similar results (data not shown). These results implied that as long as the chromatin condensation persists, the changes associated with promoter methylation are not sufficient to evoke a significant difference at the LHR gene ex-

pression level. (iii) Demethylation of the LHR promoter in JAR cells required the combined actions of TSA and 5-AzaC (but not 5-AzaC alone), supporting the concept that alteration of the chromatin structure is concurrently necessary for the DNA demethylating process in this cell type. (iv) The histone code formed at the LHR promoter is not influenced by the promoter methylation status. (v) Nevertheless, demethylation of the LHR promoter reinforced the activation of the LHR gene expression initiated by histone hyperacetylation in JAR cells, resulting in a maximal induction of the expression of this gene in the presence of TSA and 5-AzaC.

Epigenetic regulation of target gene expression was best studied for several tumor suppressor genes whose expression is often silenced in a variety of malignancies (18). These include the genes of p21^{WAF/CIP1}, p27, p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}, which encode cyclin-dependent kinase inhibitors (9, 34, 38, 62). Reactivation of the expression of the INK family members can be restored by 5-AzaC but not TSA; 5-AzaC not only demethylates these promoters but also resets the histone

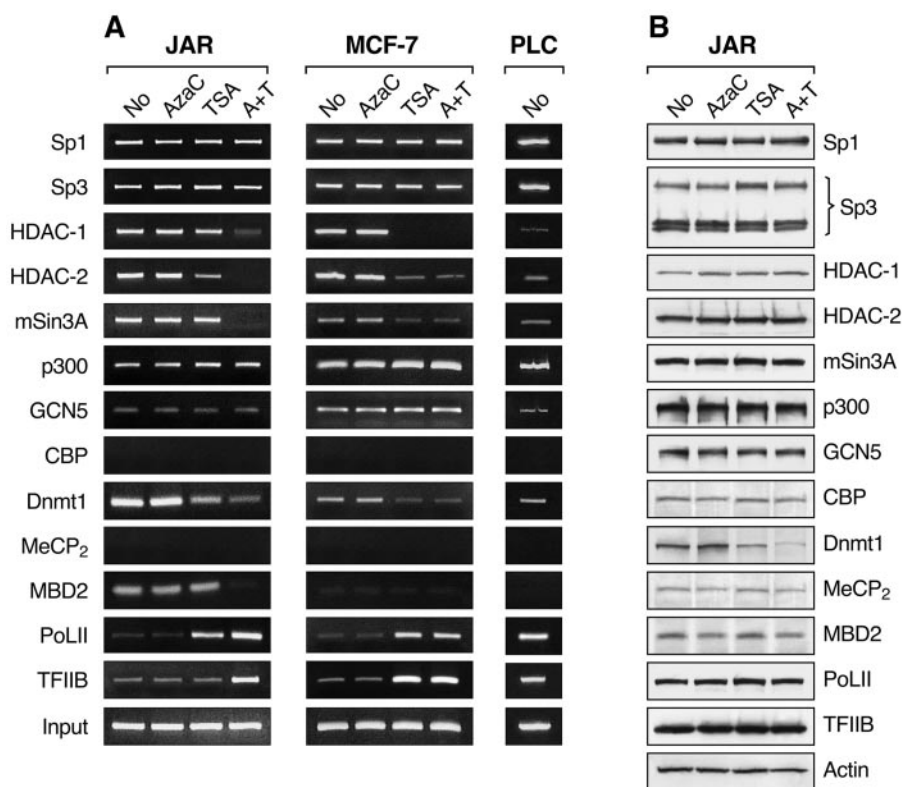


FIG. 9. Recruitment of transcription factors to the LHR gene promoter during silencing and derepression of LHR gene. (A) ChIP analyses of the association of various transcription factors with the LHR gene promoter region in JAR and MCF-7 cells treated with or without 5-AzaC, TSA, or both (A+T). Occupancy of the LHR gene promoter by these factors in PLC cells at basal condition was shown as a control (PLC). (B) Western blot analyses of treated or untreated JAR cells of the endogenous protein levels of transcription factors studied in the ChIP assays. No, untreated.

code. While TSA does not facilitate promoter demethylation, it may synergize 5-Azac-mediated gene activation, depending on the particular gene or cell type analyzed. In this case, the TSA-mediated positive regulation via histone acetylation relies on initial promoter demethylation by 5-AzaC. Such a regulatory mode also caused repression of other genes, including adenomatous polyposis coli, RASSF1 (RAS association domain family protein 1), ARH1 (Ras homologue member 1), and MDR1 (multidrug resistant gene 1) (7, 15, 36, 54). Moreover, the studies of reactivation of p21^{WAF/CIP1} gene expression by HDAC inhibitors have shown that histone acetylation-driven derepression of expression of this gene was also influenced by its promoter methylation status, such that induction was absent when the promoter was highly methylated (21, 23, 41, 44, 63). Except for the MDR1 gene, where synergistic derepression elicited by TSA and 5-AzaC was due to release of MeCP₂ and HDAC1 from its promoter (15), the mechanism engaged in silencing of the tumor suppressor genes described above has not been well understood.

Synergistic activation of the LHR gene by TSA and 5-AzaC in JAR cells depends on concerted changes of histone modifications and promoter demethylation. TSA was solely responsible for resetting the histone code during this process, since the promoter-associated histone modification patterns were unchanged by 5-AzaC and remained the same in cells treated with TSA as with TSA plus 5-AzaC. Although inhibition of HDAC activity by TSA or other inhibitors (sodium butyrate,

e.g.) was shown to cause generic acetylation of histones H3 and H4 at multiple lysine residues (21, 47), only hyperacetylated H3 at K9/K14 and H4 at K12 were present on the activated LHR gene promoter in all cells studied. Moreover, in contrast to the correlation of MeH3K9 or AcH3K9 to silenced or activated LHR gene expression, respectively, this was not the case for MeH3K4 in MCF-7 cells. These findings supported the view that this modification may not always be required for gene activation (40, 42). On the other hand, methylation of arginine residues in H3 has been linked to active transcription (27, 32). CARM1, the enzyme that methylates H3 at R17, interacts with the p160 coactivators of nuclear hormone receptor signaling pathways (33). Moreover, during estrogen-stimulated pS2 gene activation, the CARM1-induced MeH3R17 was tethered to the pS2 promoter following CBP-catalyzed acetylation of H3 at K18/23 but not K14 (11, 12). However, this appears not to be operative in the LHR gene because of the absence of MeH3R17 (data not shown) and CBP from the TSA-activated LHR gene promoter. These observations imply that modifications of histone proteins at various residues by specific modifying enzymes are likely involved in distinct regulatory modalities triggered by different stimuli (TSA versus steroid hormones).

Promoter demethylation reinforced TSA-mediated activation of the LHR gene. A less methylated LHR gene promoter, as revealed in MCF-7 cells or in an *in vitro* unmodified LHR promoter/reporter construct, responded more robustly to TSA

than its methylated counterpart. Increased binding activity of Sp1/Sp3 as a result of demethylation of its cognate binding site or adjacent elements has been indicated to augment the expression of several genes in the presence of 5-AzaC, while in some other cases 5-AzaC-mediated changes at Sp1/Sp3 protein levels contributed to 5-AzaC-upregulated gene expression (2–4, 43, 53, 63). However, these mechanisms appeared not to be the mechanism employed by the LHR gene. Unchanged Sp1/Sp3 binding to the LHR gene promoter, regardless of treatment by TSA, 5-AzaC, or both, was confirmed by ChIP analyses with assessment of the entire 176-bp LHR promoter region and by DNA affinity precipitation assays in which the bindings to the unmethylated and methylated Sp1-1 site (methylated at –75 bp CpG) or Sp1-2 site (methylated at –111 and –116 bp CpGs) were comparable (data not shown). Our studies have, however, demonstrated that upon TSA treatment, release of the HDAC/mSin3A complex and increase of TFIIB association to the LHR gene promoter occurred in a promoter demethylation-dependent manner. These findings may explain the higher magnitude of the TSA induction observed in MCF-7 cells and the synergism of TSA and 5-AzaC in JAR cells.

The further increased association of Pol II and TFIIB in JAR cells in the presence of both TSA and 5-AzaC over that of TSA alone indicates that promoter demethylation facilitates recruitment of basal transcriptional machinery components to the LHR gene. Moreover, the occupancy of Dnmt1 on the LHR gene promoter was significantly reduced in both cell types upon its activation by TSA or TSA plus 5-AzaC. This resulted from, at least in part, the decrease in the Dnmt1 protein level in cells treated by these drugs. Dnmt1 catalyzes the postreplication methylation of DNA and is responsible for maintaining the DNA methylation pattern during embryonic development and cell division (31). It has been generally accepted that Dnmt1 promotes tumorigenesis through silencing tumor suppressor genes via DNA methylation (29, 48). However, a recent study has demonstrated that knockdown of Dnmt1 activates expression of p21 and Bcl2-interacting killer genes through Sp1 sites, independent of DNA methylation and histone acetylation (35). In our studies, the similar association of this enzyme to both the unmethylated and methylated LHR gene promoter and the fact that an Sp1 site is critical for TSA-induced LHR gene expression suggest that, as for the p21 and Bcl2-interacting killer genes, Dnmt1 may participate in regulation of the LHR gene, independent of DNA methylation. Also, Dnmt1 does not appear to have a major role in the recruitment of HDAC to the LHR gene, since down-regulation of Dnmt1 binding did not coincide with dissociation of the HDAC complex during derepression of the LHR gene. Investigation of the role of Dnmt1 in the overall repression of the LHR gene would require identification of its function in this system. In addition, release of MBD2 from the demethylated LHR gene promoter in JAR cells could contribute to elimination of the HDAC complex from the promoter, due to the recognized interaction between MBD2 and HDAC (61). In MCF-7 cells, however, such facilitation does not exist since MBD2 was totally absent in the demethylated LHR gene promoter.

Taken together, these studies have revealed that the cooperative action of histone modifications and DNA methylation/

demethylation controls silencing and derepression of the LHR gene.

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