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Dual mechanisms of regulation of transcription of luteinizing hormone receptor gene by nuclear orphan receptors and histone deacetylase complexes☆

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Abstract

The luteinizing hormone receptor (LHR), a member of the G protein-coupled, seven transmembrane receptor family, is essential for normal sexual development and reproductive function. LHR are expressed primarily in the gonads, but also are found in non-gonadal and cancer tissues. LH acts through LH receptors in Leydig cells to maintain general metabolic processes and steroidogenic enzymes, and in the ovary enhances follicular development and steroidogenesis in granulosa and luteal cells. The major transcriptional start sites of the LHR gene are located within the 176 bp promoter domain. In the rat, the LHR gene is constitutively inhibited by upstream sequences (−176/−2056 bp) in several cell systems, while in the human only a minor inhibitory effect was observed in JAR and HeLa cells (>20%). The TATA-less human promoter is driven by Sp1 and Sp3 transactivators that bind to two Sp1 domains at −79 bp [Sp1(I)] and −119 bp [Sp1(II)] (from ATG) with additive effects. An imperfect estrogen receptor half-site response element direct-repeat within the LHR promoter is an inhibitory locus. Endogenous orphan receptors, EAR2 and EAR3/COUP-TFI, bind this motif and repress promoter activity by 70%. TR4 also binds this motif and stimulates promoter activity (up to 2.5-fold). This is reversed by coexpression of EAR2 or EAR3/COUP-TFI through competitive binding to this site. Comparative studies of hDR and rDR orphan receptors binding and function revealed sequence-specific requirements. The A/C mismatch between hDR and rDR is responsible for the lack of TR4 binding and function in the rat. The G 3′-adjacent to the hDR core is important for EAR2/EAR3-COUP-TFI high-affinity binding. The Sp1-1 site is critical for EAR3/COUP-TFI repression, with minor participation for EAR2, and is not involved in the TR4 effect. Interaction of EAR3/COUP-TFI with Sp1 perturbs association of TFIIB with Sp1, independently of HDACs, and caused impairment of LHR transcription. Other aspect of control is through HDAC/mSin3A mechanism. Inhibition of HDACs by TSA increases LHR promoter activity in JAR cells (40-fold), association of acetylated H3/H4 with the LHR promoter, recruitment of Pol II to the promoter, and LHR mRNA levels. A multiprotein complex is recruited to the hLHR promoter via interaction with Sp1/Sp3: HDACs dock directly to Sp1-1 bound DNA and indirectly to Sp3-1 bound DNA through RbAp48, while mSin3A interacts HDACs and potentiates HDAC1-mediated repression. Our studies have demonstrated that orphan receptor–ERE complexes, and the HDAC1–HDAC2–mSin3A complex have important roles in the regulation of LHR gene transcription by interaction with Sp1/Sp3, and by region-specific changes in histone acetylation and Pol II recruitment within the LHR promoter.
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1. Introduction

The luteinizing hormone receptor (LHR) is an essential G protein-coupled receptor for reproductive function and is predominantly located in the plasma membrane of gonadal cells. It mediates gonadotropin signaling and triggers intracellular responses that participate in gonadal maturation and function, as well as in the regulation of steroidogenesis and gametogenesis [1,2]. Luteinizing hormone through its surface receptors on the Leydig cell maintains general metabolic processes and steroidogenic enzymes to regulate the production of androgens [3]. In the ovary, LH promotes follicular development, at stages beyond early antral follicles including the formation of preovulatory follicles and corpora lutea [2,4]. LH stimulates steroidogenesis in follicular and luteal cells and also triggers ovulation. The hCG which is structurally similar to LH and binds the LH receptor with higher affinity than LH is secreted from placenta at the time

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of implantation and maintains the secretion of estrogen–progesterone by the corpus luteum of pregnancy [2,4].

Testicular LH receptors are expressed during fetal life, postnatally, at puberty and throughout adult life. In the ovary, the LH receptor is absent in fetal life and its induction is observed postnatally. Studies of the LHR null mice have indicated that sex differentiation in the rodent is not dependent on LH action, since these species at birth displayed testis and ovary, and accessory organs that were not different from the wild type [5,6]. In the absence of the LH receptor, the fetal testis can produce seemingly adequate quantities of testosterone and anti-Mullerian hormones that are essential for intrauterine masculinization. However, major changes were found after birth where growth and descent of the testis and also the growth of accessory organs was significantly inhibited. Although gametogenesis completed meiosis, it did not proceed beyond the round spermatid stage. Recent studies showed restoration of testicular function and fertility following androgen administration [7]. However, incomplete reversal was found in accessory organs, and progesterone–estrogen replacement did not restored fertility in females [7].

The LHR gene is also expressed in several non-gonadal tissues, including the prostate, uterus, placenta, fallopian tubes, uterine vessels, umbilical cord, brain and lymphocytes [8–13]. Immunocytochemistry studies have revealed the presence of LHR in the epithelial cells of the normal mammary gland, benign breast tumors, malignant breast tumors and various breast cancer cell lines (T47D, MCF7, and ZR75) [14]. Also, the LHR is expressed in human placental cell lines including a human choriocarcinoma cell line (JAR cells), and an SV40 transformed human placental cell line (PLC) [15]. The specific function of the LHR in non-gonadal tissues has not been determined. Furthermore, the pan-LHR null mice model has not provided the expected insights of LHR function in non-gonadal tissues since it cannot discriminate the intrinsic effects of the lack of LH action and those resulting by the highly reduced levels of steroids in both sexes. In addition, the highly elevated sustained levels of FSH observed in LHR null mice could provide additional yet to be identified changes. Therefore, tissue specific knockouts with preservation of normal Leydig cell, ovary function and gametogenesis could be expected to address the LHR function in non-gonadal tissues in a more definitive manner.

The LHR cDNA was cloned from pig testis, rat, mouse and human ovarian libraries [16–19], and the gene structure has been defined in the rat, human, and mouse [20–23]. The receptor is a 80–90 kDa single glycoprotein of which about 15 kDa are contributed by carbohydrate chains [2]. The LHR in the various species studied contains 11 coding exons separated by 10 introns, all of which are located in the extracellular domain [20–22]. Exons 1–10 encode the 5′-untranslated region and most of the extracellular domain, and exon 11 encodes the rest to the receptor.

The hormone binding domain has been localized to exons 1–7 within the extracellular domain of the receptor. The major transcriptional start sites of the LHR gene in rat and human genes are located within 176 bp 5′ to the ATG codon. Deletion analysis has localized the promoter to the 176 bp domain (from ATG) of the gene [24,25]. In the rat, the LHR gene is constitutively inhibited by upstream sequences (−176/−2056) in several in vitro cell lines examined [24,26]. In contrast, minor inhibitory effect was caused by the presence of upstream 5′-sequences to the human LHR promoter domain in human [25]. The LHR promoter is TATA-less and contains GC regions. Two of the several consensus Sp1 sites that are activated by Sp1/Sp3 are of central importance in the transcription of the LHR gene [24,25,27]. This activation is greatly magnified by histone acetylation within the promoter and is inhibited by HDAC complexes recruited to the promoter by Sp1/Sp3, and by orphan receptors EAR2 and EAR3/COUP TFI that bind EREhs direct repeats located 5′ to the Sp1 sites [28–30]. Transcriptional changes resulting from the participation of these regulatory modalities are caused by changes in the interaction of components of the basic transcriptional machinery with Sp1 complex and recruitment of Pol II to the promoter. We will review aspects of our recent work directed towards definition and understanding of the mechanisms involved in the regulation of the LHR gene transcription.

2. The LH receptor promoter structure: functional domains

The major transcriptional sites of the rat and human LHR gene are located within the TATA-less promoter domain residing 176 bp 5′ to the ATG codon in gonadal and non-gonadal tissues. In the rat testis and ovary, these encompass two major transcriptional start sites (TSS) at −14 or −13, and −19 bp, and a minor start site at −33 bp [24]. Five transcriptional start sites (TSS) were identified in the human ovary at −2, −6, −18, −37 and −70 bp from the translation initiation codon [15]. In the human testis and JAR cells upstream TSSs in addition of those located within the promoter domain were identified. Although putative upstream promoters could be active in these tissues these were not revealed in subsequent deletion studies where the promoter activity was shown to reside solely within the 176 bp domain 5′ from ATG in all cells examined [15,25].

In both the rat and human, the promoter activity is driven by two functional Sp1/Sp3 binding domains termed Sp1-2 and Sp1-4 in the rat, and the corresponding functional sites Sp1(I) and Sp1(II) in the human [24–27] (Figs. 1 and 2). These domains that bind Sp1/Sp3 contribute similarly to promoter activity. At difference to the Sp1 I–II in the human, the Sp1-4 domain in the rat is complex since it contains two overlapping non-identical Sp1 sites and only the 5′-GGG GTG GGG element binds Sp1/Sp3 protein while the 3′ Sp1 like domain does not bind Sp1 but binds an unidentified protein(s) that can sustain the Sp1-4 activity when the 5′ Sp1 is mutated [26]. Upstream of the two functional Sp1

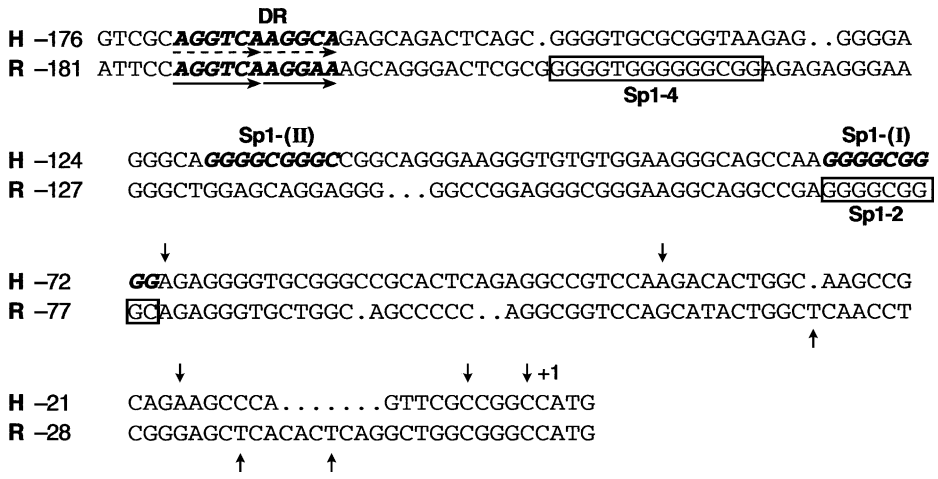

Fig. 1. Alignment of nucleotide sequences of the promoter regions of the human (H) and rat (R) LH receptor genes. DNA sequences of the human and rat LHR promoters are shown, and the nucleotides are numbered relative to the translation initiation codon (ATG, +1). The transcriptional start sites are indicated with arrows. The proximal and distal functional Sp1 sites in the human, Sp1(I) and Sp1(II) (Bold), and the corresponding sites in the rat, Sp1-2, and Sp1-4 (boxed), are indicated. The direct-repeat (DR) domains that bind nuclear orphan receptors, EAR2, EAR3 (human and rat), and TR4 (human), are underlined with arrows.

domains, an estrogen receptor (ER) direct repeat is present in both rat and human species [25,29].

The 176 bp human promoter contains two consensus Sp1 domains at −79 and −119 bp, three GC-rich AP-2 like elements (−58, −82 and −137 bp), and an ERE–DR at 171/161 with no spacing (DR0) [25,30]. Mutation of both consensus Sp1 domains reduced significantly the promoter activity by 80% in SV40 transformed human placental cell line (PLC) and 60% in JAR cells (human placenta choriocarcinoma cell line) [25]. Each domain contributed individually and in the PLC cells both accounted for most of the activity while in the JAR cells other domains appear

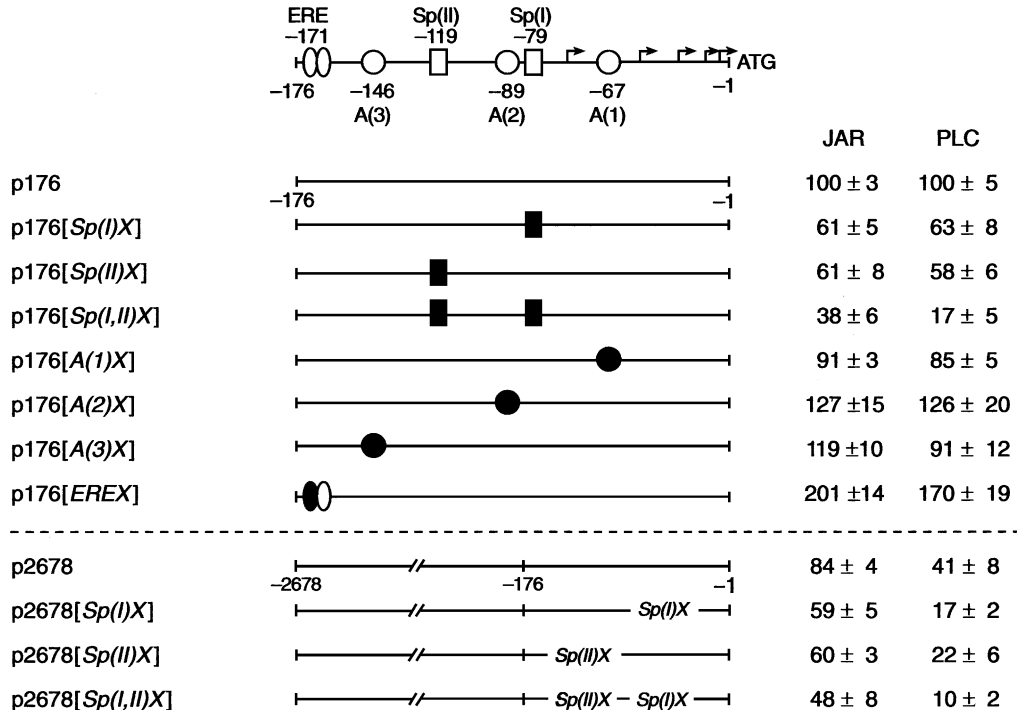

Fig. 2. Determination of functional domains of hLHR gene promoter in JAR and SV 40 transformed placental cells (PLC). (Top) Schematic diagram of the 176 bp promoter to indicate location of activating (stripped square), inhibitory (open oval), and non-functional (open circle) elements derived from mutational analyses. Arrows indicate transcriptional start sites. (Middle and bottom) Promoter activity of wild type and mutant constructs (solid square) of the −176 to −1 bp (p176) promoter domain (middle), and −2678 to −1 bp (p2678) constructs (bottom), which highlight cell specific inhibitory domain in the human. Data are expressed as the mean ± S.E. of at least three experiments performed in quadruplicate [25].

to contribute 30–40% of the basal activity. Mutations of the AP2-like elements did not significantly affect basal promoter activity. In contrast, mutation of half site of the ERE–DR resulted in a 100% increased promoter activity in both the cell types. This strongly indicated that this site was inhibitory of LHR gene transcription [25]. EMSA of the Sp1 domains revealed three retarded bands. One of them (most retarded) supershifted with the Sp1 specific antibody and the other two band complex (one closely migrating with the Sp1 band and the other faster migrating) were supershifted by Sp3 antiserum. Identical patterns of Sp1 and Sp3 associated DNA/complexes were noticed using nuclear protein extracts from JAR and PLC cells. In the most retarded bands, the Sp1 and Sp3/DNA complex likely contains the 95–115 kDa species of these proteins, and the protein present in the other band (faster) corresponds to the 80 kDa isoform of Sp3. Studies on the function of Sp1 or Sp3 protein was facilitated by their co-expression with LHR promote/luciferase reporter construct in *Drosophila Schneider* SL2 cells, that lack Sp factors [25]. Both the proteins activated LHR promoter activity. However, Sp3 was found to be less effective than Sp1 on the activation of Sp1(II) site while both activated equally the Sp1(I) site. Furthermore, co-transfection of both Sp1/Sp3 shows additive contributions. Also, EMSA studies indicated the presence of multiple ERE–DR protein binding species in PLC and JAR cells and initial studies excluded binding to monomeric orphan receptors SF1, hERR1 or dimeric estrogen receptor species, and subsequent studies demonstrated binding of dimeric orphan receptors to this site (see below and [25,29,30]).

In the rat, the LHR gene appears to be constitutively inhibited by upstream sequences (−176/−2056 bp) in several in vitro cell systems, including mouse Leydig tumor cells (mLTC1-4), CHO, Hela cells [2,24] and primary Leydig cells (Tsai-Morris, unpublished observations). In contrast, only minor inhibitory effect (15–20%) was caused by the presence of the upstream sequence 5′ to the human LHR promoter (176 bp) in both JAR and Hela cells (Fig. 1) [15,25]. However, a more prominent decrease in activity (by 60%) was observed in PLC cells. These results in the human indicate a cell type specific upstream inhibition. The differences observed between the human (minimally or less inhibition) versus the rat (with nearly complete abolition of promoter activity) may be related to the presence of cell specific regulatory proteins and/or sequence differences between the 5′-flanking of LHR gene between species.

3. Regulation of transcription of LH receptor gene by nuclear orphan receptors

3.1. Dual regulation of human LHR gene transcription by EAR2, EAR3/COUP-TFI, and TR4

Identification of an imperfect direct-repeat motif as a strong inhibitory domain for the hLHR gene transcription in normal human placenta or placenta carcinoma JAR cells indicated that putative repressor protein(s) occupied the DR element [25]. Gel mobility shift analyses of incubation of nuclear extracts from JAR cells or human gonadal tissues with a DR motif probe displayed multiple specific binding complexes, indicating the participation of more than one protein in the regulation of hLHR gene transcription through this DR element. Yeast one-hybrid screening of a human placenta library by tandem copies of human DR sequences (4X) revealed nuclear orphan receptors, EAR2, EAR3/COUP-TFI, and TR4, as specific interacting proteins [30]. These orphan receptors are closely related members of the nuclear receptor superfamily with particularly high homology noted at their DNA binding domains [31–33]. EAR2 is a subtype of EAR3/COUP-TFI, with which it shares 86% amino acid sequence homology at its DNA binding domain [34–36]. TR4 shares 70 and 50% sequence similarity with EAR3/COUP-TFI at DNA binding domain and putative ligand binding domain, respectively [37,38]. The conserved DNA binding domains confer their ability to recognize an identical or similar element that is composed of a direct-repeat of AGGTCA core sequence with variable spacing between the two-half sites ([39], review). Structurally, EAR2, EAR3/COUP-TFI, and TR4 share similarity with non-steroid hormones type II receptors (RAR, RXR, TR, VDR, PPAR) [31,40–42]. However, these are orphan receptors distinct from non-steroid hormone or steroid hormone-regulated receptors for which no ligand is known [39]. EMSA analyses carried out to determine the binding activities of these orphan receptors to the hLHR gene promoter showed that in vitro translated EAR2, EAR3/COUP-TFI and TR4 bound specifically to the hDR motif (Fig. 3A–C). This binding was abolished by the wild type (WT) unlabeled DR sequence but not by DNA competitors with mutation at either half site (*m1* and *m2*). Moreover, the finding that specific antibodies against these individual orphan receptors caused supershift of the retarded complexes further confirmed the binding specificity to the hDR domain.

Accumulated evidence support that members of the orphan receptor family play an important role in development of gonadal and non-godadal tissues [43–47]. Therefore, it is of relevance that these orphan receptors are recognized as participants in the transcriptional regulation of hLHR gene. Functional studies have demonstrated that EAR2 and EAR3/COUP-TFI caused up to 70% inhibition of LHR gene promoter activity in a dose-dependent manner (Fig. 3E). Consistent with the binding specificity of these two orphan receptors, their function (repression) was dependent on the presence of an intact DR element. Mutation at either half site to disrupt binding of EAR2 or EAR3/COUP-TFI abolished the inhibition. These results confirmed that EAR2 and EAR3/COUP-TFI bind to a direct-repeat element as dimer [30,48,49]. EAR2 and, particularly, EAR3/COUP-TFI were generally noted as repressor proteins regulating an array of different target genes ([49,50], reviews). Several mechanisms have been proposed for their inhibitory action

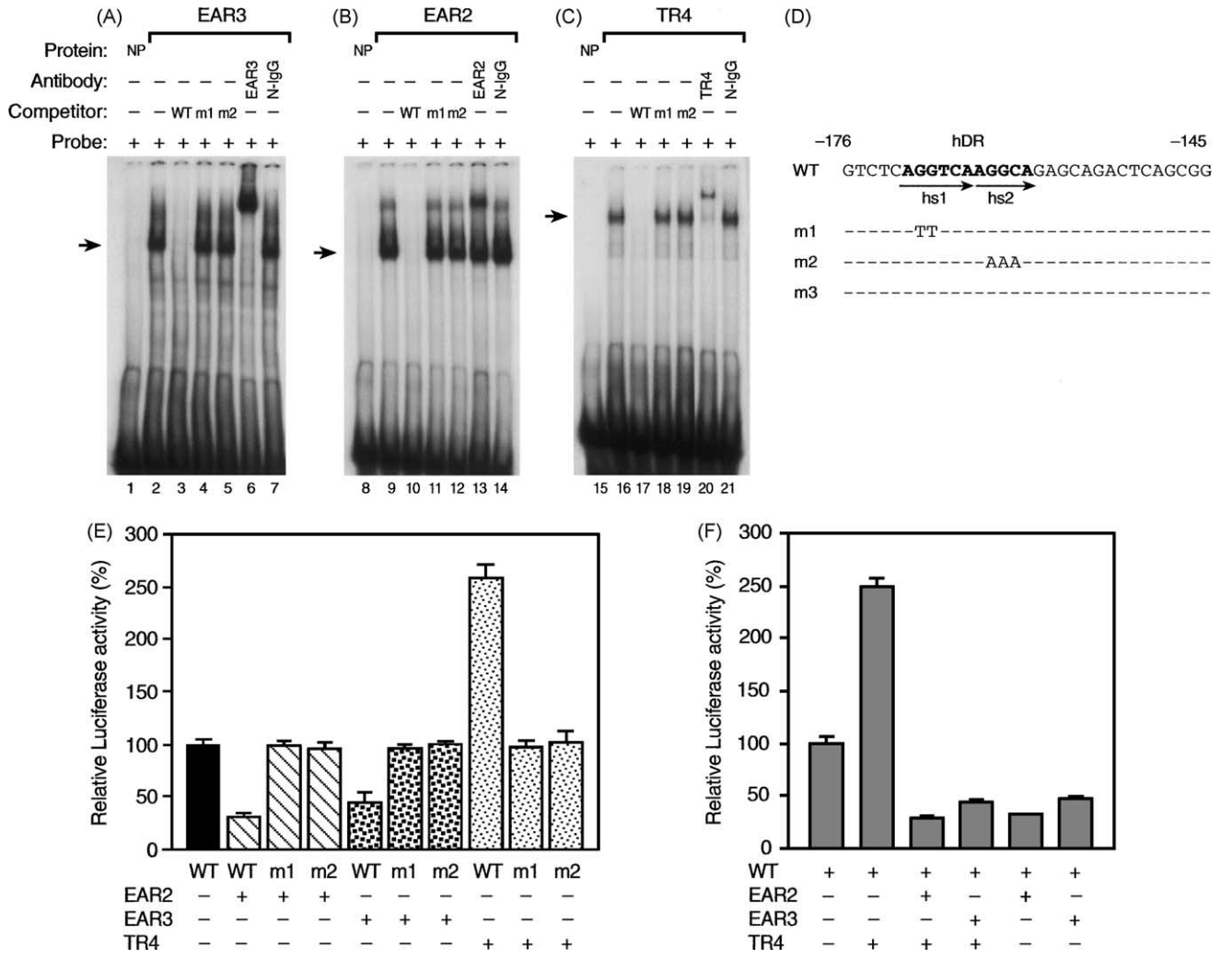

Fig. 3. Functional regulation of hLHR gene transcription by nuclear orphan receptors through binding to the hDR motif. (A, B and C) EMSA analyses of binding of in vitro translated EAR3 (A), EAR2 (B), and TR4 (C) to the hDR domain. Bindings of unprogrammed reticulocyte lysate (NP) or the nuclear orphan receptors were performed in absence (lanes 1, 2, 8, 9, 15, 16) or presence of unlabeled 100-fold excess wild-type (lanes 3, 10, 17) or mutated oligomers (lanes 4, 5, 11, 12, 18, 19), or in the presence of normal rabbit IgG (N-IgG, lanes 7, 14, 21), or antibodies of EAR3 (lane 6), EAR2 (lane 13) and TR4 (lane 20). (D) DNA sequences used in EMSAs as probe or as unlabeled competitors corresponding to wild type (WT) or mutant DR domain (*m1* and *m2*). (E) Cotransfection studies of EAR2, EAR3, or TR4 with wild type (WT) or DR domain-mutant (*m1* and *m2*) hLHR promoter constructs in CV-1 cells. (F) The wild type hLHR promoter was cotransfected into CV-1 cells with TR4 in the absence or presence of coexpression of EAR2 or EAR3. Relative promoter activities in (E) and (F) are indicated as percentage of luciferase activity (100%) from the wild type promoter in the absence of nuclear orphan receptors. Results were normalized with β-galactosidase activity and expressed as the mean ± S.E. of three independent experiments in triplicate wells [30].

including active inhibition of basal or activated transcription, quenching a transactivator-regulated transcription, and transrepression [51–55]. The repression of hLHR gene transcription appears to result from the active silencing function of EAR2 and EAR3/COUP-TFI rather than competing with, quenching, or titrating out a hormonal partner [56,57].

Our more recent results have revealed a novel mechanism for EAR2- and EAR3-COUP-TFI-mediated silencing of LHR gene transcription [58]. The proximal Sp1 site of LHR gene promoter in both human and rat species was identified to be critical for the repression. Mutation of this site significantly released the inhibition by EAR2 and abolished inhibition by EAR3/COUP-TFI. Moreover, Sp1 and Sp3 were shown to be both required for the silencing effect as no repression was observed in absence of Sp1 or Sp3. Protein–protein interaction was observed between the Sp1/Sp3 bound to the Sp1(I) site and the DR-bound EAR3, and provides a molecular basis for the observed functional connection. Evidence for a repression mechanism resulting from a negative impact on the basal transcription machinery were derived from the DNA affinity precipitation assays (DAPA), which demonstrated that the basic transcription factor TFIIB was able to interact with EAR3/COUP-TFI and Sp1/Sp3 besides its binding to the TATA-less promoter core region. Furthermore, EAR3/COUP-TFI induced a dose-dependent decrease of association of TFIIB to the

Sp1/Sp3–Sp1 site complex. Taken together, these findings demonstrate a selective Sp1-site dependent repression of transcription of a target gene by nuclear orphan receptors, particularly by EAR3/COUP-TFI. This occurs through perturbation between the communication of the activation mediated by Sp1/Sp3 and the basal transcription machinery via TFIIB.

In contrast to the action of EAR2 and EAR3/COUP-TFI, TR4 was identified to function as an activator for the hLHR promoter activity at the DR motif (Fig. 3E and F). Recognition of the same response element by three nuclear orphan receptors with apparent opposite functions implies that the net outcome of regulation of hLHR gene transcription is determined by availability or relative binding activities of these orphan receptors present within a specific cell type at a given physiological stage. Cotransfection studies demonstrated that the TR4 induced up-regulation of hLHR gene promoter activity was reversed by EAR2 or EAR3/COUP-TFI coexpression, indicating that these orphan receptors competitively occupy the DR domain of hLHR promoter (Fig. 3F). Consistent with the inhibitory role that hDR domain played in JAR cells, the hLHR gene promoter was found to bind endogenous EAR2 and EAR3/COUP-TFI in these cells. Moreover, binding of the hDR by EAR2, EAR3/COUP-TFI, and TR4 were readily observed in human gonadal tissues, indicating the existence of opposing physiological regulation of hLHR gene transcription by these orphan receptors.

3.2. Repression of rat LH receptor gene by EAR2 and EAR3/COUP-TFI

Sequence comparison of LHR gene promoter in rat with its human counterpart reveals that rLHR gene promoter harbors a conserved imperfect DR motif (rDR) as a putative binding site for the nuclear orphan receptors EAR2, EAR3/COUP-TFI, and TR4. However, differences were noted between the DR domains in the two species, specifically a single nucleotide mismatch (A/C) was observed at the second-half site, and low similarity was shared at 5′- or 3′-flanking sequences to the DR core region. Studies of functional regulation of the nuclear orphan receptors on the rat LHR gene transcription have provided insights on the modulation of LHR gene transcription by these orphan receptors in these species. Studies in the rat were carried out utilizing primary cultures of rat ovarian granulosa cells. This alternative system provided a setting where LHR gene expression is known to be tightly and dynamically controlled [59,60], and is of physiological relevance to study the regulation of the LHR gene transcription/expression during ovarian cell development and differentiation.

EMSA binding analyses identified that the rDR domain is a specific binding site for EAR2 and EAR3/COUP-TFI but not for TR4. As in the human, EAR2 and EAR3/COUP-TFI's binding in the rat was dependent on the two-half sites of the rDR element. However, no binding of TR4 for the rDR was detected [29]. Determination of EAR2 and EAR3/COUP-TFI's binding parameters for the rat and human DRs by Scatchard analyses showed that the two orphan receptors displayed significantly lower binding affinities for the rDR than for the hDR, while no differences in binding capacities were observed (Fig. 4). These findings thus indicate that the three nuclear orphan receptors possess differential binding characteristics in their recognition of rat and human LHR gene promoters.

Recent studies have shown partial restoration of TR4 binding for a probe derived from the rDR domain containing an A/C switch mutation, while the binding of EAR2 or EAR3/COUP-TFI was not affected by this change. These results indicate that the A/C single nucleotide difference critically impaired the binding of TR4 but had no influence on the binding of EAR2 or EAR3/COUP-TFI. DNA binding inhibition analyses further illustrated that component(s) of adjacent sequences to the DR domain play an important role in the binding activities of EAR2 or EAR3/COUP-TFI. The significant decrease on binding affinities of EAR2 or

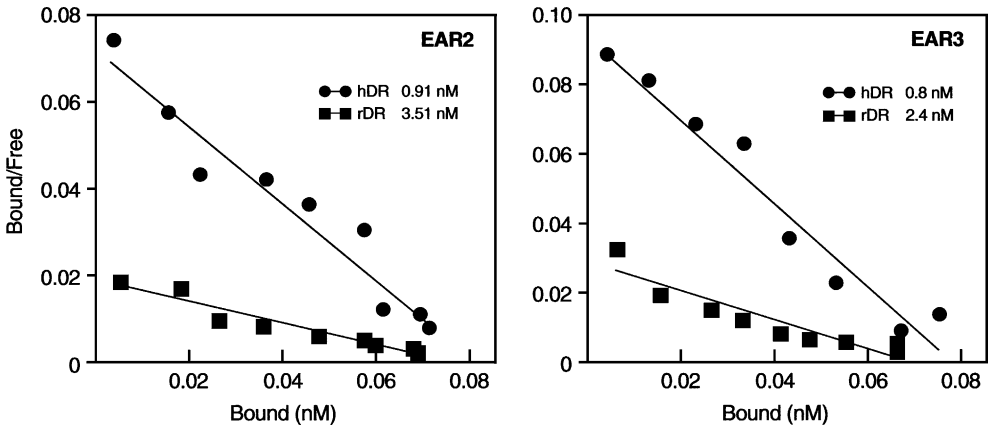

Fig. 4. Determination of binding parameters of EAR2 and EAR3 to the human and the rat DR domains by Scatchard analysis. Binding of a constant amount of in vitro translated EAR2 (left) or EAR3 (right) to various doses of ^{32}P labeled hDR (●) or rDR (■) probe were resolved in EMSAs. The binding parameters (dissociation constant, K_d (nM)) were obtained by Scatchard analyses [29].

EAR3/COUP-TFI for the rDR domain was caused by absence of a guanine residue (G) at the flanking sequence 3′ to the rDR core motif. Replacement of the rDR 3′-flanking region with the hDR 3′-region, or insertion of a G to the rDR 3′-sequence restored the binding of EAR2 or EAR3/COUP-TFI to levels comparable to their binding activities for the hDR domain (Fig. 5). It is generally accepted that the binding of a nuclear receptor to a particular target site is primarily determined by the hormone response element core sequence, which is composed of hexameric base pairs in single or repeated configuration. However, several lines of evidence indicated that nucleotides adjacent to the core sequence also contribute significantly to the binding activity [61–63]. Moreover, it was shown that specific DNA–protein interactions often resulted in conformational change in protein(s), DNA, or both [64,65]. The lack of the 3′-G next to the rDR core may induce a conformational change(s) on the EAR2 and EAR3/COUP-TFI that cause a less tight binding of the receptors for the rat LHR gene.

Cotransfection studies have demonstrated that EAR2 and EAR3/COUP-TFI exerted strong inhibition of the rat LHR gene transcription, although the repression is less profound than for the hLHR gene due to the reduced the binding affinity. It was also not unexpected that TR4 did not affect the rLHR gene promoter activity. Inhibition of rLHR gene expression by EAR2 and EAR3/COUP-TFI was further

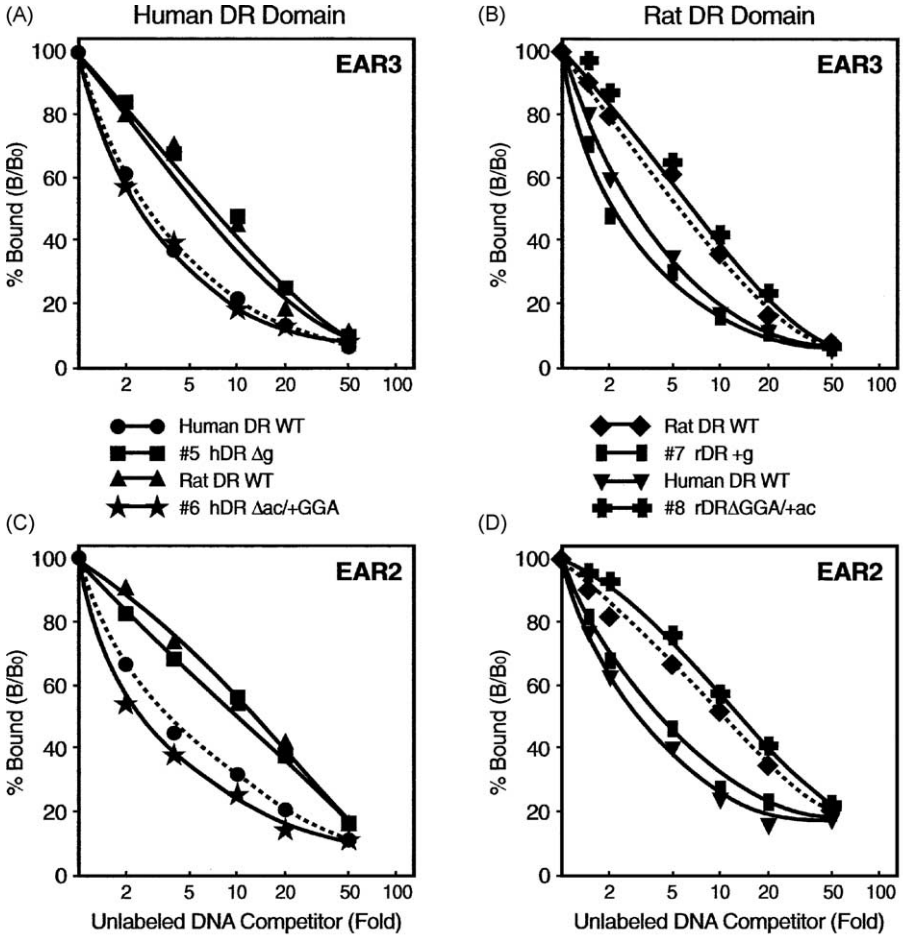

Fig. 5. Identification of a guanine residue (G) absent at 3′ rat DR domain attributable to differential binding activities of EAR2 and EAR3. (Top) Binding curves of in vitro translated EAR3 (A and B) or EAR2 (C and D) for the human (A and C) and rat (B and D) DR domains. The amount of unlabeled DNA competitors used is indicated as a fold-ratio of unlabeled/labeled, and the specific binding is expressed as relative percentage of the binding in the absence of unlabeled DNA. Displacements curves were derived from the EMSAs using labeled wild type rDR or hDR probe, and wild type rDR, hDR, or hybrid sequences #5–8 as unlabeled competitor DNAs. (Bottom) DNA sequences for the wild type rat and human DR domains containing the core motifs (underlined) and adjacent flanking regions. The highlighted box area shows the sequences shared by the two DR domains at their 3′-regions. The guanine residue (g) 3′ next to the hDR core but absent in the rDR is indicated with a (●) symbol. Deletion (#5) or insertion (#7) of a G in corresponding sequences is indicated with "Δg" or "+g". The substitution of *GGA* with *ac* is indicated as "ΔGGA/+ac" (#8), while replacement of *ac* by *GGA* is stated as "Δac/+GGA"(#6) [29].

observed in rat granulosa cells, in which the rDR motif occupied by endogenous EAR2 and EAR3/COUP-TFI functioned as a potent inhibitory domain. Gonadotropin plays an obligatory functional role in ovarian follicle maturation and function and significantly activates LHR gene expression in granulosa cells [2,4]. Therefore, the subsequent studies addressed the potential role of gonadotropin (e.g. hCG) in EAR2- and EAR3/COUP-TFI-mediated repression of the rLHR gene transcription in these cells. The rLHR gene promoter activity from either wild type or rDR motif mutated construct was significantly enhanced in granulosa cells treated with hCG for 24 h (Fig. 6A). This is consistent with the notion that both human and rat LHR gene transcription are activated by hCG/cAMP treatment (our unpublished results and [66]). Moreover, it is noted that the mutant promoter still showed a minor increase in activity over the wild type after hCG treatment (+12%) ($P < 0.05$) but this was much less marked than in parallel cultures in the absence of hormone (+84%). In granulosa cells cultured for 48 h without hCG, a 60% increase in promoter activity was observed due to the mutated DR element. However, negative regulation of the rLHR gene through the rDR domain was not present in cells treated with hCG for 48 h. These findings indicate that hCG treatment releases the DR domain-mediated inhibition of rLHR gene transcription. Furthermore, the expression of EAR2- and EAR3/COUP-TFI was significantly down-regulated by hCG in these cells (by 45% for EAR2, and by 40% for EAR3/COUP-TFI at 48 h treatment, respectively) (Fig. 6B). Therefore, these results demonstrated that the hCG-induced decrease at EAR2 and EAR3/COUP-TF protein expression level correlated with derepression of rLHR gene transcription in the presence of hormone. Taken together, these findings indicate that down-regulation of EAR2/EAR3 expression by gonadotropin with consequent derepression of rLHR gene transcription contributes to the elevated LHR expression for progression of granulosa cell

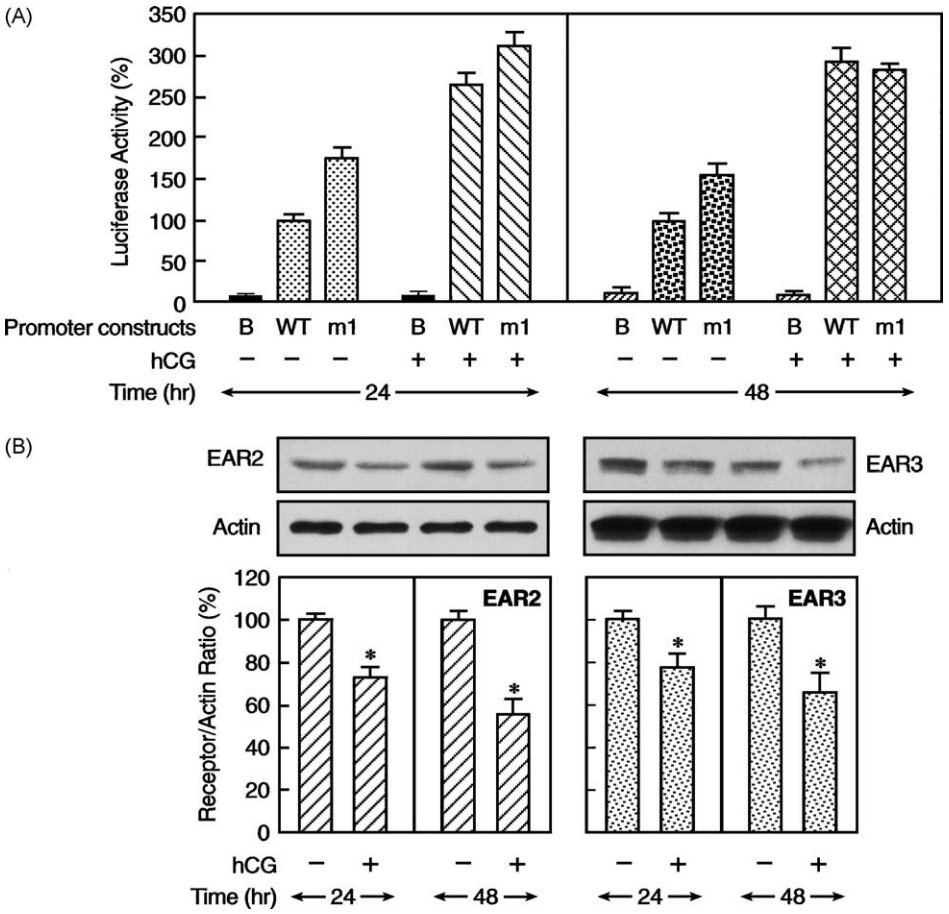

Fig. 6. Effect of gronadotropin (hCG) on the rLHR gene promoter activity and the expression of EAR2 and EAR3 in granulosa cells (A) basic (B), wild type (WT), or hs1 mutant (*m1*) rLHR promoter constructs were transfected into granulosa cells followed by treatment with or without hCG (0.5 μg/ml) for 24 h (left) or 48 h (right). Within each group, the relative promoter activities are indicated as the percentage of luciferase activity of the wild-type promoter at −hCG condition. The results were normalized by β-galactosidase activity and expressed as the mean ± S.E. of three experiments in triplicate. (B (top and middle)) Western blot analyses of endogenous EAR2 (left) and EAR3 (right) proteins in granulosa cells treated with or without hCG (0.5 μg/ml) for 24 or 48 h. Actin signals are also shown. (Bottom) EAR2 and EAR3 signals normalized by actin signals at each time point. The relative intensities of the signals are indicated as percentage of the receptor/actin ratio at −hCG condition. Data were expressed as the mean ± S.E. of three experiments. ($^{*}P < 0.01$) [29].

maturation. Such reduction of expression of these orphan receptors presumably reduces their influence in Sp1/Sp3—TFIIB communication (see pp. 11–12) and, therefore, increases transcription/expression of the LH receptor at critical stages of the cycle.

4. Silencing of transcription of human LH receptor gene by a histone deacetylase–corepressor complex

Modifications of histone proteins, particularly through reversible histone acetylation and deacetylation, have been recognized as an important mechanism in regulation of a target gene expression [67,68]. Disruption of the equilibrium maintained by the activities of histone acetyltransferases (HATs) and deacetylases (HDACs) is thought to elicit changes at local chromatin structures as well as at levels of protein–protein interactions, therefore, inducing an accessible or unfavorable chromatin status to accelerate or repress gene transcription [68,69].

Studies aimed to determine whether LHR gene transcription is subject to modulation by histone acetylation–deacetylation revealed that when the HDAC activity was blocked by treatment of JAR cells with the HDAC inhibitors, trichostatin A (TSA) or sodium butyrate (NaB), the hLHR gene promoter activity was markedly activated (Fig. 7A) [28]. Up to 40-fold induction was noted in the presence of TSA, indicating that inhibition of HDAC activities suppressed potent constitutive repression of hLHR gene transcription. Dose-response and temporal studies of the TSA effect have shown that the maximal induction of hLHR promoter activity was observed at 100 ng/ml (330 nM) TSA, where significant activation was detected at 6 h post-treatment and reached its peak at 24 h. Furthermore, TSA caused potent increase of endogenous LHR gene expression, which is under control of its natural promoter (Fig. 7B). This robust activation/derepression of endogenous LHR gene expression, in contrast to the unchanged level of the human β-actin gene mRNA, indicated that the TSA-mediated modulation is a gene-selective effect. Several lines of evidence showed that only a small fraction of cellular genes (2–5%) was up or down regulated by TSA treatment [70]. Therefore, the notable magnitude of activation of hLHR gene expression by TSA suggests that hLHR gene may be uniquely sensitive to the degree of histone acetylation in chromatin.

Disruption of the balance between HAT and HDAC activities by TSA caused time-dependent accumulation of acetylated histone proteins (H3 and H4) in the TSA-treated cells, when analyzed by Western blots [28]. Strong immuno-signals of acetylated H3 and H4 were clearly detected at 24 h of treatment. To elucidate how these acetylated histones assemble with DNA, chromatin immunoprecipitation assays were employed to address recruitment of acetylated H3 and H4 to the hLHR gene promoter region (Fig. 8C, region 1), and to the three flanking regions 5′ to the promoter (Fig. 8C, regions 2–4). Markedly enhanced association of acetylated H3 and H4 for the region 1 but not for the other regions was observed, indicating that TSA triggered recruitment of acetylated histones, specifically to the hLHR promoter region. This promoter-restricted localization of histone acetylation indicated that the local chromatin environment at the hLHR gene promoter region could be changed by TSA to facilitate gene transcription (Fig. 8A). In this regard, profound increase of recruitment of RNA Pol II to the hLHR gene promoter was observed in the presence of TSA, in agreement with the notion that chromatin decondensation positively modulates gene expression [68,69].

In an effort to elucidate the mechanism that mediate assembly of non-sequence specific binding histone proteins

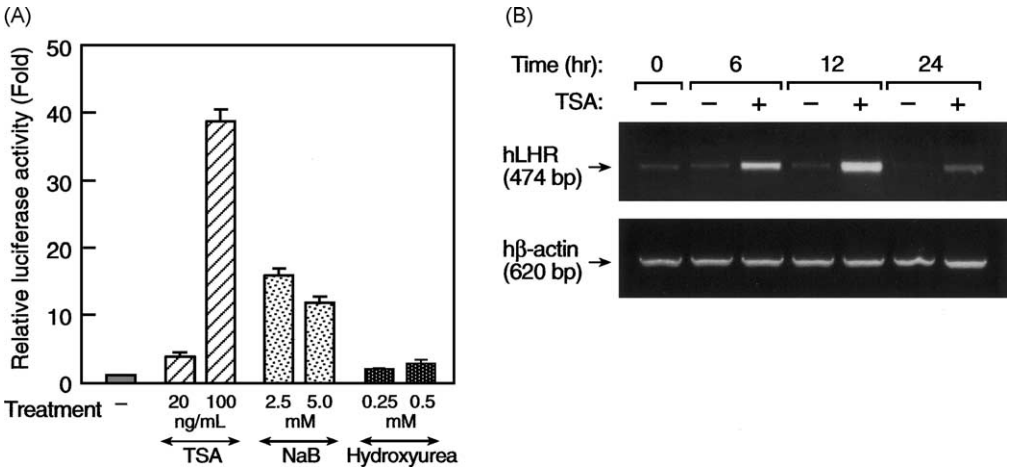

Fig. 7. Effect of histone deacetylase inhibitors on transcription of human LH receptor gene. (A) The wild type hLHR promoter was transfected into JAR cells. At 24 h of post-transfection, the cells were treated with TSA, NaB, or Hydroxyurea at the indicated doses for 24 h. The results were normalized to light units per μg protein and expressed as the mean ± S.E. of four experiments in triplicate wells. (B) RT-PCR analyses of total RNA isolated from the JAR cells treated with or without 100 ng/ml of TSA for 0, 6, 12, or 24 h. The primers were designed to amplify a 474 bp DNA fragment of the C-terminal coding region of the hLHR gene (nt 1557–2031). Amplification of a 620 bp cDNA fragment of hβ-actin gene is shown as control [28].

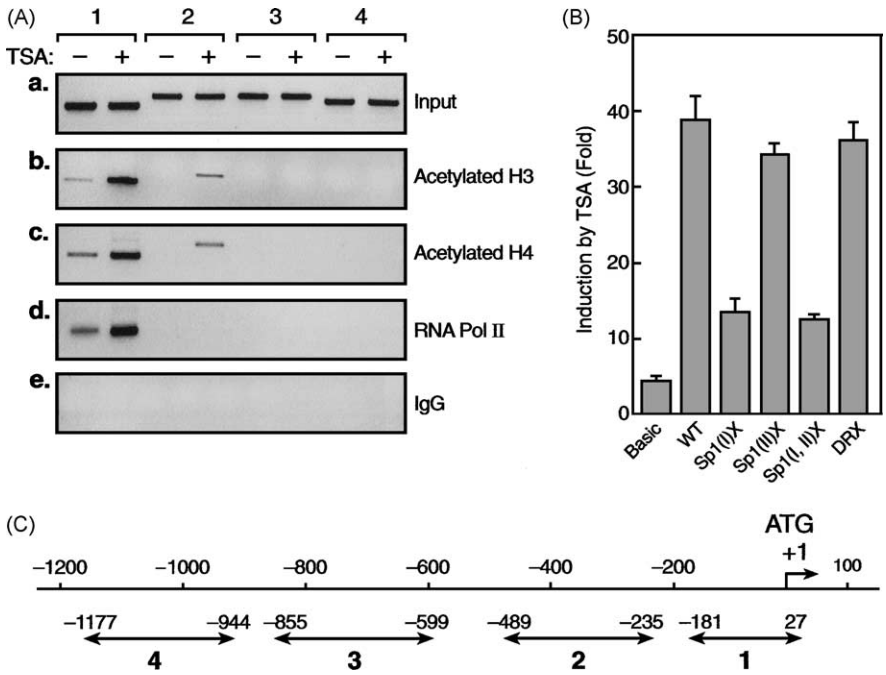

Fig. 8. Determination of recruitment of acetylated H3, H4, and RNA Pol II to the hLHR gene promoter, and identification of domain(s) critical for the TSA effect. (A) Chromatin immunoprecipitation was performed with chromatin prepared from JAR cells treated with or without TSA (100 ng/ml, 24 h) using antibodies against acetylated H3, H4, or RNA Pol II, or normal rabbit IgG (a–e). Numbers 1–4 refer to regions analyzed in PCR following the immunoprecipitation (see (C)). Results of amplification of soluble chromatin before precipitation are shown as control (Input). (B) The promoter-less (basic), wild type (WT), Sp1 site mutant constructs (*Sp1(I)X*, *Sp1(II)X*, *Sp1(I,II)X*), or the DR domain mutant construct (DRX) were transfected into JAR cells, which were treated with or without TSA (100 ng/ml) for 24 h. Relative luciferase activities are represented as fold-induction of the activity in the presence of TSA over that in the absence of TSA for the constructs indicated. (C) Schematic representation of the hLHR gene promoter (from −176 to +1) and its 5′-flanking regions. Inverted arrows show the regions (1–4) analyzed by the PCR using specific pairs of primers, which are indicated with numbers relative to the translation initiation codon (*ATG*, +1) [28].

specifically to the hLHR gene promoter locus, the Sp1(I) site was identified to be critical for the TSA effect (Fig. 8B). Mutation of this site largely abolished the TSA-mediated activation of the hLHR gene promoter activity, whereas mutation at other domains including DR motif did not affect the action of TSA. The lack of participation of the DR-bound nuclear orphan receptors in this TSA process indicates that the mechanism of silencing hLHR gene expression through alteration of HDAC activities is independent of the pathway involving unliganded hormone receptors–orphan receptors, corepressors NcoR/SMRT, and HDACs complexes [71,72]. Moreover, the Sp1 site-specific effect was shown not to be due to a change at Sp1/Sp3 binding activities after TSA treatment, since similar binding patterns were observed in the absence or presence of TSA. These findings thus reveal a novel function for the Sp1 domain besides its essential role in maintaining the basal promoter activity. Recognized primarily as a constitutive regulatory element in modulation of many target gene expression, Sp1 sites have been found to be involved in tissue-specific gene expression and in control of transcription in response to a number of different stimuli [73,74].

Furthermore, the Sp1(I) site-dependent derepression of hLHR gene transcription upon suppression of HDAC activities suggested that HDAC had a direct role in the regulation of hLHR gene with the Sp1(I) site as a putative docking locus. A multiple-protein complex was identified in the DAPA assays to associate specifically with Sp1(I) site (Fig. 9A). The complex, besides Sp1/Sp3, is composed of two histone decetylases, HDAC1 and HDAC2, a putative corepressor protein, mSin3A, and a known HDAC associating protein, RbAp48. The undetected NcoR signal within the complex in contrast to its expression readily observed in the Western blot analyses (WB) further confirmed that the orphan receptors/NcoR/HDAC pathway was not involved in the TSA-regulated hLHR gene expression. The order by which these proteins associated with each other within the complex determined by coimmunoprecipitation analyses demonstrated that direct interactions between Sp1 and both HDAC proteins and RbAp48 (Fig. 9B). Sp3 did not interact directly with either HDAC1 or HDAC2, but a weak interaction between Sp3 and RbAp48 was detected. On the other hand, although mSin3A did not associate directly with either Sp1 or Sp3, its interaction with HDAC1 or HDAC2 was clearly noted (Fig. 9C). The interaction of RbAp48 with the two HDACs confirmed this protein as a HDAC associating partner. These findings thus indicate that Sp1 and Sp3 play differential roles in recruitment of HDAC1–HDAC2–mSin3A complex to the hLHR gene promoter. We have employed a model to represent the assembly of the histone deacetylase

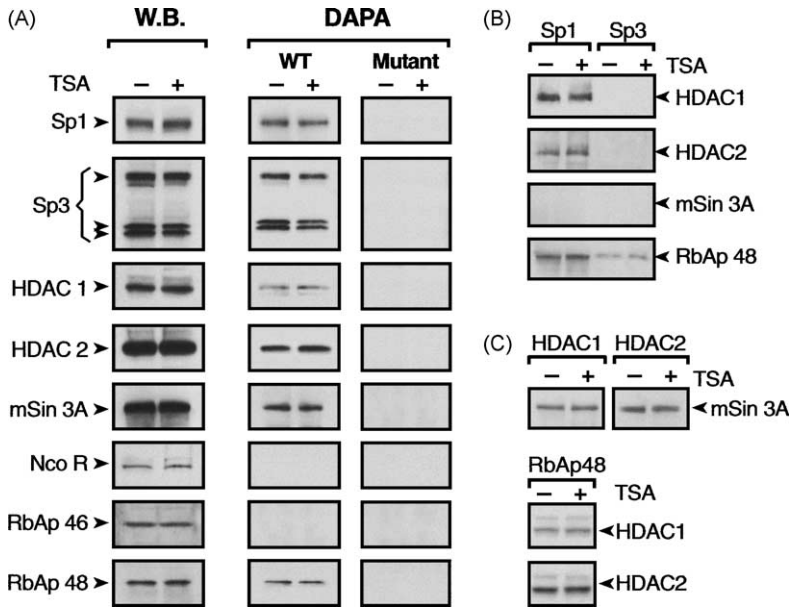

Fig. 9. Identification of recruitment of a HDAC/mSin3A complex to the Sp1(I) site, directly by Sp3 and indirectly by Sp3. (A (left)) Western blot analysis (WB) of expression of putative transcription factors in JAR cells treated with or without 100 ng/ml TSA for 24 h. Arrowheads indicate immunosignals of the transcription factors analyzed. (Right) The same cell extracts were subject to DAPA analyses using 5′-biotin-labeled wild type (WT) Sp1(I) probe or a mutant probe devoid of Sp1/Sp3 binding (mutant). The avidin precipitated protein complexes were analyzed in Western blot using antibodies against Sp1, Sp3, HDAC1, HDAC2, mSin3A, NcoR, RbAp46, or RbaAp48. (B) Coimmuno-precipitation (Co-IP) was carried using Sp1 or Sp3 antibody followed by Western blot for detection of HDAC1, or HDAC2, or mSin3A, or RbAp48, of which the signals were indicated by arrowheads. (C) Co-IPs were performed with HDAC1 or HDAC2 antibody followed by immunodection of mSin3A; or Co-IP was carried out with RbAp48 antibody for detection of HDAC1 or HDAC2 [28].

complex to the hLHR gene promoter: direct recruitment by Sp1, and indirect recruitment via Sp3 through a tethering effect of RbAp 48 (Fig. 10).

Functional analyses proved that HDAC1–HDAC2 were repressors for the hLHR gene transcription. Overexpression of HDAC1 or HDAC2 counteracted TSA-mediated dose-dependent activation of hLHR gene promoter activity [28]. Moreover, mSin3A was identified as corepressor for HDAC1 since coexpression of mSin3A with HDAC1 potentiated HDAC1-repressed hLHR gene promoter activity. Taken together, these studies have identified the hLHR gene as a target for tonic repression by the HDACs–mSin3A

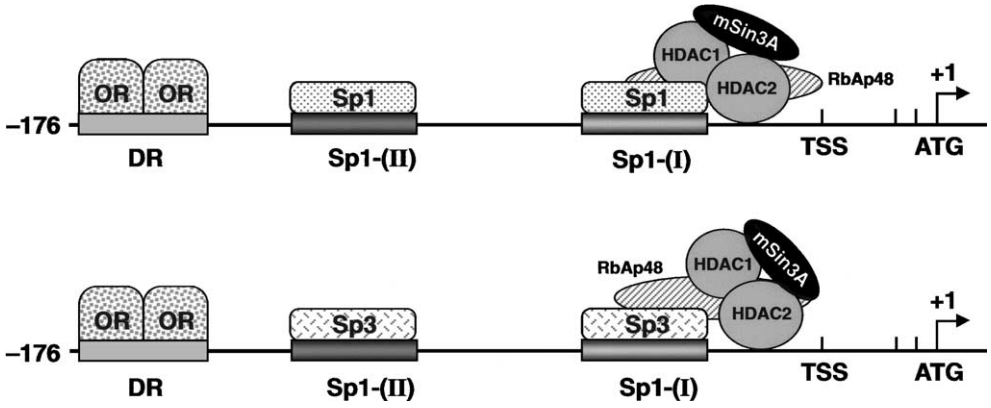

Fig. 10. The model representing the recruitment of HDACs/mSin3A corepressor complex to the hLHR gene promoter. The diagram illustrates the hLHR promoter region (from −176 to +1 at ATG), which harbors two activating Sp1/Sp3 bound Sp1(I) and Sp1(II) sites, and an inhibitory orphan receptors (OR)-bound direct-repeat (DR) motif. TSS represents the transcription start sites indicated by vertical bars. (Top) Direct recruitment of HDACs/mSin3A corepressor complex to the hLHR gene promoter by Sp1. This is achieved through direct interaction between Sp1(I) site-bound Sp1 protein and two HDAC enzymes, and through addition link via interactions of Sp1/RbAp48/HDAC1/HDAC2. The mSin3A is attached to the promoter through its interaction with HDAC1/HDAC2. (Bottom) Indirect recruitment of HDACs/mSin3A corepressor complex to the hLHR promoter by Sp3. It is mediated through tethering effect of RbAp48, which interacts with both Sp1(I)–Sp3 protein and HDAC1/HDAC2. The mSin3A is associated with the complex by its interaction with the two HDACs.

complex via region-specific changes in histone acetylation status and Pol II recruitment within the hLHR gene promoter. The regulated derepression of such control of hLHR gene, through as yet unidentified signal inputs, is of major relevance in functional control of induction and cyclic changes of LHR gene expression during differentiation, growth, and development of gonadal cells. Histone acetylation and deacetylation integrate the modulation of an array of genes that are actively involved in cell proliferation and differentiation. The recent progress in transcriptional repression of hLHR gene by HDAC-mediated regulation extends our understanding of the essential role of the LHR gene in its contribution to gonadal cell development and differentiation.

In conclusion, our studies on transcription of the luteinizing hormone receptor gene have identified two independent mechanisms that participate in silencing Sp1/Sp3 activated LHR gene promoter activity. The orphan receptors-DR complexes, and the HDAC1–HDAC2–mSin3A complex, have important roles in the regulation of LHR gene transcription by perturbance of Sp1/Sp3 activation, and by region-specific changes in histone acetylation and Pol II recruitment within the LHR gene promoter. Down-regulation of EAR2, EAR3/COUP-TFI expression by gonadotropin, with consequent derepression of LHR gene transcription, may contribute to the elevated LHR gene expression for the progression of granulosa cell maturation.

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