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Anti-steroidogenic Factor ARR19 Inhibits Testicular Steroidogenesis through the Suppression of Nur77 Transactivation*

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ARR19 (androgen receptor corepressor-19 kDa), a leucine-rich protein whose expression is down-regulated by luteinizing hormone and cAMP, is differentially expressed during the development of Leydig cells and inhibits testicular steroidogenesis by reducing the expression of steroidogenic enzymes. However, the molecular events behind the suppression of testicular steroidogenesis are unknown. In the present study, we demonstrate that ARR19 inhibits the transactivation of orphan nuclear receptor Nur77, which is one of the major transcription factors that regulate the expression of steroidogenic enzyme genes in Leydig cells. ARR19 physically interacts with Nur77 and suppresses Nur77-induced promoter activity of steroidogenic enzyme genes including StAR, P450c17, and 3β-HSD in Leydig cells. Transient transfection and chromatin immunoprecipitation assays revealed that ARR19-mediated reduced expression of steroidogenic enzyme genes was likely due to the interference of SRC-1 recruitment to Nur77 protein on the promoter of steroidogenic enzyme genes. These findings suggest that ARR19 acts as a novel coregulator of Nur77, in turn regulating Nur77-induced testicular steroidogenesis, and may play an important role in the development and function of testicular Leydig cells.

Steroidogenesis in testicular Leydig cells is primarily regulated by the pituitary gonadotropin luteinizing hormone (LH)[2] through the production of the intracellular second messenger cAMP. LH/cAMP stimulates steroidogenesis by increasing the expression of steroidogenic enzyme genes (1). Recently, it was demonstrated that LH induces Nur77 gene expression in Leydig cells (2), and Nur77 in turn regulates the expression of steroidogenic enzyme genes (3–6) such as cytochrome P450 protein 17, the steroid 21-hydroxylase, and 20-α-hydroxysteroid dehydrogenase (7, 8), suggesting an important role for Nur77 in testicular steroidogenesis.

Nur77 is a member of the Nur77 gene family, which also contains the orphan nuclear transcription factors Nurr-1 and NOR-1. These factors have similar structural features of the conserved DNA-binding domain (DBD) and ligand-binding domain (LBD) but retain a variable sequence in the N-terminal activation function (AF)-1 domain (9, 10). Nur77 family members behave as end point effectors of the protein kinase A signaling pathway acting through dimmers, and the AF-1 domain of Nur77 plays a major role in transcriptional activation, cofactor recruitment, and intra- and intermolecular interactions (11–13). Although Nur77 has been well characterized as an immediate early response gene (2, 14–17) and for its post-translational modifications (18–22), coregulators involved in Nur77 transactivation are not fully characterized. Recently, steroid receptor coactivator (SRC)-1 and silencing mediator for retinoid and thyroid hormone receptors have been shown to regulate Nur77 transactivation through direct protein-protein interactions (11, 23).

ARR19 (androgen receptor corepressor-19 kDa), also known as Cklfsf2a, is a member of the chemokine-like factor superfamily (CKLFSF), a group of novel proteins that operate as a functional bridge between chemokines and the members of the transmembrane 4 superfamily. In humans, CKLFSF2 is expressed abundantly in the testis and has two counterparts in the mouse, Cklfsf2a and Cklfsf2b. Cklfsf2a and Cklfsf2b have similar expression patterns and amino acid identities of 47.6 and 45.5%, respectively, with human CKLFSF2 (24). Mouse Cklfsf2a/ARR19 was originally cloned as a potential androgen response gene in the testis and was further characterized as a novel androgen receptor (AR) corepressor (25). The ARR19 gene encoding a hydrophobic leucine-rich 19-kDa protein is abundantly expressed in the testis and moderately in other male reproductive organs such as the prostate (24, 25).

Previously, we reported that ARR19 is differentially expressed during the development of testicular Leydig cells. It is highly expressed in the earlier stages of Leydig cell development, with progressively less expression at later stages. The expression of the ARR19 gene is regulated by LH/cAMP signaling via the GATA-1 transcription factor together with cAMP response element-binding protein-binding protein (26). When overexpressed in adult testis, ARR19 acts as an anti-steroidogenic factor by inhibiting the expression of steroidogenic enzyme genes, which results in a decrease of steroidogenesis (26). However, the mechanisms underlying the inhibition of steroidogenesis by ARR19 are unknown.

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[2] The abbreviations used are: LH, luteinizing hormone; AR, androgen receptor; CKLFSF, chemokine-like factor superfamily; SRC, steroid receptor coactivator; hCG, human chorionic gonadotrophin; RIA, radioimmunoassay; ChIP, chromatin immunoprecipitation; Ad, adenovirus; GFP, green fluorescent protein; RFP, red fluorescent protein; HA, hemagglutinin.

In the present study, we propose a molecular mechanism by which ARR19 inhibits the steroidogenesis in testicular Leydig cells. In mouse testis as well as Leydig cell lines, ARR19 inhibited the production of testosterone via down-regulating the expression of steroidogenic enzyme genes. Further studies showed that ARR19 repressed Nur77-induced promoter activity of steroidogenic enzyme genes through physical interaction with Nur77, which interfered with the binding of Nur77 to its coactivator SRC-1. These findings suggest that ARR19 acts as a corepressor of Nur77 and inhibits testicular steroidogenesis by suppressing the Nur77-induced expression of steroidogenic enzyme genes and that ARR19 may play an important role in the development and function of testicular Leydig cells.

EXPERIMENTAL PROCEDURES

Animals and Treatment—FVB mice (postnatal days 14, 21, 24, 28, 42, and 56) were purchased from Daehan Laboratories (Daejeon, Republic of Korea). The selection of mouse ages was based on previous reports of the development of adult Leydig cells (27). The animals were sacrificed by CO_2 asphyxiation, and the testes were extracted for several analyses. For Western blot analysis, 14-day-old mice were injected intraperitoneally with 10 IU of human chorionic gonadotrophin (hCG; Sigma-Aldrich) for 6 h. For Western blot analysis, radioimmunoassay, and chromatin immunoprecipitation (ChIP) assay, 42-day-old mouse testes were infected with 5×10^7 particles of recombinant adenovirus harboring green fluorescent protein (Ad-GFP) or ARR19 (Ad-ARR19) in a phosphate-buffered saline (0.01 M, pH 7.2) for defined time periods. Adenovirus was delivered to the testes under a dissecting microscope using glass micropipettes. Ethical treatment of the animals followed National Institutes of Health standards.

Plasmids—Mammalian expression vectors of ARR19, red fluorescent protein (RFP)-ARR19, Nur77, GFP-Nur77, Nur77ΔAF2, SF-1, SRC-1, and SRC-2 and reporter plasmids NurRE-luc, SF-1RE-luc, and Gal4-tk-Luc have been previously described (25, 28), as have mouse StAR(−2200/+3)-Luc, rat P450c17(−1561/+1)-Luc, and mouse 3β-HSD(−4700/+40)-Luc (29).

Cell Culture and Transient Transfection Assay—MA-10 mouse Leydig cells were maintained in RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 15% horse serum, and antibiotics. R2C rat tumor cells were maintained in F10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. All of the cells were cultured at 37 °C in an atmosphere containing 5% CO_2. For hormone treatments, MA-10 cells were plated in medium containing 10% charcoal-stripped serum and 8-bromo-cAMP (Sigma-Aldrich), or LH was added to the medium 48 h later. Transfections were conducted using Lipofectamine Plus (Invitrogen) for MA-10 and HeLa cells in accordance with the manufacturer's recommendations. The cells were plated in 24-well plates and were transfected with expression vectors, reporter gene, and the lacZ expression plasmid, pCMVβ (Clontech, Sunnyvale, CA), as a control for transfection efficiency. The total amount of DNA was kept constant by the addition of appropriate amounts of pcDNA3 empty vector. After 24 h of incubation, the cells were harvested in a lysis buffer containing 0.1% Triton X-100 and 0.2 M Tris-HCl (pH 8.0). Luciferase and β-galactosidase activities were assayed as described previously (13). The levels of luciferase activity were normalized by β-galactosidase activity.

Purification of Primary Leydig Cells—Purification of mouse Leydig cells was carried out as described previously (30) with some modifications. The animals were anesthetized prior to decapitation. Six testes/set (14, 21, 24, 28, and 56 days) were removed, and the testicular cells were dispersed by treating the decapsulated testes with collagenase (0.25 mg/ml; Sigma-Aldrich) in M199 medium (Invitrogen) at room temperature for 20 min with gentle shaking. After incubation, the dispersed tissues were diluted with M199 medium, and the solution was filtered. Interstitial cells were precipitated by centrifugation of the filtrate and washed once with M199 and twice with phosphate-buffered saline (28).

Preparation of Recombinant Adenovirus—For the ectopic expression of mouse ARR19, an adenoviral delivery system was used (31). Briefly, HA-tagged ARR19 cDNA was cloned into pAdTrack-CMV shuttle vector. Homologous recombination was performed by transforming adEasy-BJ5138-competent cells with pAdTrack-CMV-ARR19 together with adenoviral gene carrier vector. The recombinant viruses were selected, amplified in HEK-293 cells, and purified by cesium chloride density centrifugation. The number of virus particles was calculated by measuring optical density at 260 nm (A_{260}) as previously reported (26).

Western Blot Analysis—The cell lysates were prepared in radioimmune precipitation assay cell lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, pH 8.0, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 g/ml aprotinin, 0.1 g/ml leupeptin, 1 g/ml pepstanin, and 0.1 mM phenylmethylsulfonyl fluoride) and were separated via SDS-PAGE. The proteins were transferred onto nitrocellulose transfer membranes and were subsequently subjected to Western blot analysis using anti-ARR19, anti-P450c17, anti-P450scc, anti-3β HSD, anti-StAR, anti-Nur77, anti SF-1, anti-SRC-1, or anti-actin (Santa Cruz Biotechnology, Inc.) antibody. The signals were then visualized using an ECL kit (Amersham Biosciences). Actin signals were used as a loading control.

Coimmunoprecipitation—MA-10 cells were treated with 300 μM of 8-bromo-cAMP for 2 h, and nuclear extract was prepared and preincubated with anti-Nur77 or anti-ARR19 antibody for 4 h at 4 °C. Protein A/G-agarose beads were added, and the mixture was incubated for 4 h at 4 °C. Antibody complexes were pelleted and washed three times with radioimmunoprecipitation assay buffer containing protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The pellets were resuspended in loading buffer and analyzed by SDS-PAGE and subsequent immunoblotting using anti-ARR19 and anti-Nur77 antibodies.

ChIP Assay—Testes from 6-week-old mice infected with Ad-ARR19 or Ad-GFP for 0, 6, and 12 h, as well as noninfected mice were dissected out, chopped up, and cross-linked with 1% formaldehyde for 15 min at room temperature on a rotating platform. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M for 5 min at room tempera-

ture (32). R2C cells infected with Ad-ARR19 or Ad-GFP for 1 and 6 h were cross-linked with 1% formaldehyde. After incubating with TSE I buffer (100 mM Tris-HCl, pH 9.4, and 10 mM dithiothreitol) at 30 °C, the cells were washed and processed for ChIP assays as described previously (29). Anti-Nur77, anti-SRC-1, and anti-ARR19 were used for immunoprecipitation. Immunoprecipitated DNA was then subjected to PCRs using either rat P450c17 primers (sense, 5′-GATCTGAATGGCTCCTATGC-3′, and antisense, 5′-ATCCTCCCAGAGGCAAATGC-3′), which amplify a 253-bp region (−553 to −281) spanning the Nur77/SF-1-binding site of the rat P450c17 gene promoter, or mouse P450c17 primer pairs (sense, 5′-TTTCAGGGGCCAGAAGGTG-3′, and antisense, 5′-TCCTCCCAGAGGCAAATGC-3′), which amplify a 541-bp region (−837 to −296) spanning putative Nur77- and SF-1-binding sites of the mouse P450c17 gene promoter. As a negative control, PCR was done using β-actin primer pairs (sense, 5′-GAGACCTTCAACACCCCAGCC-3′, and antisense, 5′-CCGTCAGGCAGCTCATAGCTC-3′), which amplify a 362-bp region spanning exon 4 of the β-actin gene (29).

Radioimmunoassay—The testosterone concentrations were measured by RIA. The dissected testes were homogenized in phosphate-buffered saline, and steroids were extracted three times with three volumes of diethyl ether. Exponentially growing R2C cells were cultured in F10 medium supplemented with 15% charcoal-stripped fetal bovine serum. Culture medium was collected for RIA at several time points following infection with Ad-GFP or Ad-ARR19. In animal experiments, the testes were isolated from 6-week-old mice 24 h after infection of testis with Ad-GFP or Ad-ARR19. The experiment was repeated three times, and the assay procedures were followed as described previously (29).

Real Time PCR—Real time PCR was performed using a LightCycler as described previously (33).

RESULTS

ARR19 Inhibits the Expression of Steroidogenic Enzymes in R2C Leydig Cells and Mouse Testis—We have previously suggested that ARR19 functions as an anti-steroidogenic factor (26). To investigate the regulation of steroidogenic enzyme expression by ARR19 in detail, we attempted to determine the effect of adenovirus-mediated overexpression of ARR19 in R2C rat Leydig cells, which are constitutively steroidogenic. As shown in Fig. 1, the levels of steroidogenic enzymes P450c17 and P450scc began to decrease 2–4 h following infection with Ad-ARR19. Thereafter, the decrease was progressive, and enzyme activities were nearly undetectable at 24 h post-infection. In contrast, the protein level of ARR19 was increased at 2 h post-infection with Ad-ARR19 and was maximal at 4–6 h post-infection. Interestingly, StAR and 3β-HSD protein levels rapidly decreased upon expression of ARR19 protein, reaching the lowest point at ~4 h following Ad-ARR19 infection. Thereafter, their expression recovered closely to preinfection levels by 24 h. However, levels of Nur77 protein, a key regulator of the expression of steroidogenic enzymes, and its coactivator SRC-1 protein were not affected by the overexpression of ARR19. We also examined the effect of ARR19 overexpression on the synthesis of testosterone by infecting R2C Leydig cells with Ad-

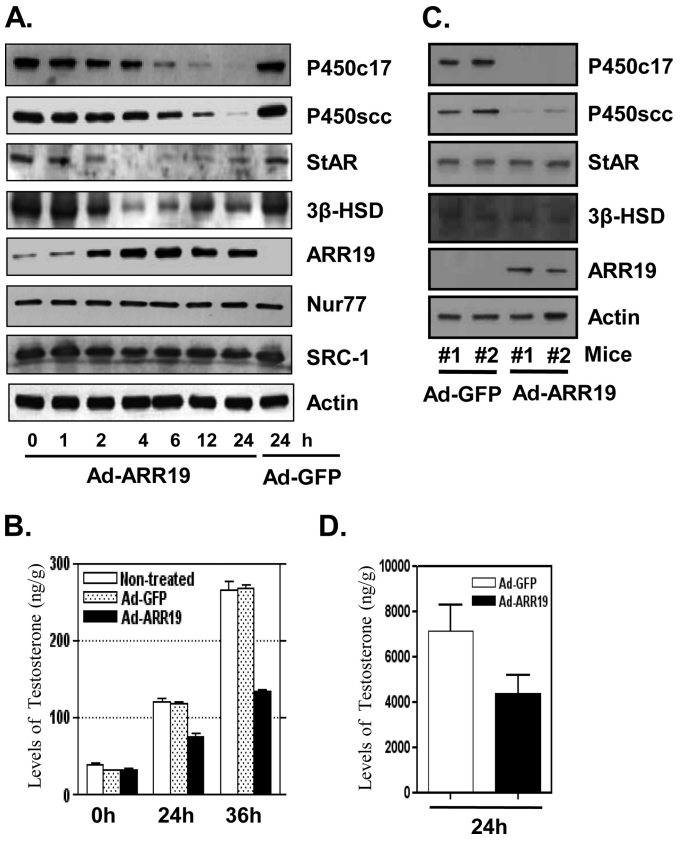

FIGURE 1. **Inhibition of the expression of steroidogenic enzymes by adenovirus-mediated overexpression of ARR19 in R2C Leydig cells and mouse testis.** *A*, the expression of steroidogenic enzymes was inhibited by ARR19 overexpression in R2C cells. R2C cells were infected with Ad-ARR19 or control Ad-GFP for the indicated time periods. Whole cell lysates were subjected to Western blot analysis with anti-P450c17, anti-P450scc, anti-StAR, anti-3β HSD, anti-ARR19, anti-Nur77, anti-SRC-1, and anti-actin antibodies. *B*, testosterone biosynthesis was inhibited by ARR19 overexpression in R2C cells. The cells were grown in medium supplemented with 15% charcoal-stripped serum that was either noninfected or infected with Ad-ARR19 or control Ad-GFP. The media were collected at different time points (0, 24, and 36 h) for RIA. *C*, the expression of steroidogenic enzymes was down-regulated by the overexpression of ARR19 in mouse testis. Testes of two 6-week-old mice (*lanes #1* and *#2*) were infected with Ad-ARR19 or control Ad-GFP at a concentration of 5×10^6 virus particles/testis and were dissected out after 24 h. Whole testis extracts were prepared and subjected to Western blot analyses with the indicated antibodies. *D*, the biosynthesis of testosterone was inhibited by ARR19 overexpression in mouse testis. Testes of 6-week-old adult mice were infected with Ad-ARR19 or control Ad-GFP for 24 h as in *C*. Testis extracts were subjected to RIA to measure testicular testosterone levels.

ARR19 or Ad-GFP as a control (Fig. 1*B*). As expected, the testosterone level in the cultured medium of Ad-ARR19-infected R2C cells was lower than that of the controls (Ad-GFP and noninfected), reaching ~50% of the control levels after 36 h of viral infection.

In an attempt to confirm the effect of ARR19 protein on the expression of steroidogenic enzyme genes and the synthesis of testosterone *in vivo*, we infected the testis of 6-week-old mice with Ad-ARR19 or the control Ad-GFP for 24 h (Fig. 1*C*). As expected, the protein levels of P450c17 and P450scc were almost completely abrogated at 24 h of Ad-ARR19 infection. In contrast, 3β-HSD and StAR protein levels were little changed at 24 h, probably because of regaining the protein expression of 3β-HSD and StAR at later time points after Ad-ARR19 infection, as seen in Ad-ARR19-infected R2C cells (Fig. 1*A*). There

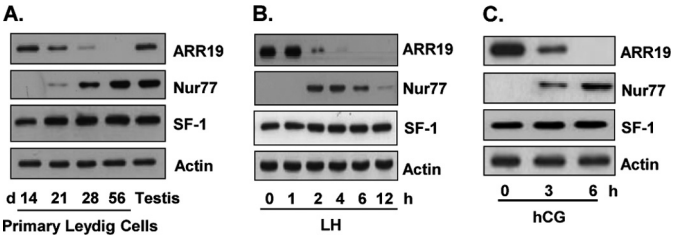

FIGURE 2. **Inverse relationship between ARR19 and Nur77 expression in testicular Leydig cells.** *A*, ARR19 expression was down-regulated during the development of Leydig cells, whereas the expression of Nur77 was up-regulated. Primary Leydig cells were isolated from 14-, 21-, 28-, and 56-day-old mouse testes, and Western blot analyses were performed using anti-ARR19, anti-Nur77, and anti-SF-1 antibodies, together with anti-actin antibody as a control. *B*, inverse regulation of ARR19 and Nur77 expression by LH in MA-10 Leydig cells. MA-10 cells were treated with 50 ng/ml of LH and harvested at different times for Western blot analyses with the indicated antibodies. *C*, inverse regulation of ARR19 and Nur77 expression by hCG/LH in the testis. Fourteen-day-old mice were injected with hCG for 0–6 h, and whole testis extracts were prepared for Western blot analyses.

were marginal changes in the levels of endogenous ARR19 protein, which is abundantly expressed in the haploid germ cells of adult testis (25) and 2–3 kDa smaller than the HA-tagged exogenous form expressed from Ad-ARR19 (data not shown). In Ad-ARR19-infected testes, the level of testosterone was significantly reduced to ~60% that of the control Ad-GFP-infected testes by 24 h of viral infection (Fig. 1*D*). Collectively, these results support the suggestion that ARR19 inhibits the production of testosterone in Leydig cells through the suppression of steroidogenic enzyme gene expressions.

ARR19 and Nur77 Expression Is Inversely Correlated in Testicular Leydig Cells—The overexpression of ARR19 inhibited the synthesis of testosterone in mouse testis, but the underlying mechanism was not elucidated. To explore the mechanism, we first correlated the developmental expression pattern of ARR19 with those of steroidogenic gene regulators such as Nur77 and SF-1, using primary Leydig cells isolated from testes at postnatal days 14, 21, 28, and 56 (Fig. 2*A*). ARR19 expression was high at day 14, gradually decreased to moderate levels at days 21 and 28, and was almost totally abrogated at day 56. On the other hand, Nur77 expression was detectable at day 21 and gradually increased thereafter. Interestingly, the expression of ARR19 and Nur77 proteins was overlapped during the prepubertal-early pubertal stage (34), opening a possibility of their functional interaction. In the case of SF-1, expression was evident at day 14 and thereafter increased a little to the adult stage. These results suggest that there is an inverse correlation between the expression of anti-steroidogenic factor ARR19 and steroidogenic gene regulators, especially Nur77, during the development of adult Leydig cells.

The serum level of LH is increased during puberty, and LH is a major stimulus for the biosynthesis of testosterone as well as the differentiation of Leydig cells in the testis (28, 35). Previous studies have shown that LH rapidly increases the expression of Nur77 in Leydig cells (28), whereas we have shown that it rapidly decreases the expression of ARR19 (26). Therefore, we compared the regulation of ARR19 expression by LH with those of Nur77 and SF-1 at the same time in MA-10 cells (Fig. 1*B*). Western blot analysis showed that ARR19 expression was significantly decreased in a time-dependent manner in LH-treated MA-10 Leydig cells (Fig. 2*B*). At 4 h of LH treatment, the ARR19 protein had almost vanished, and no protein was detected thereafter to 12 h. In contrast, Nur77 expression was dramatically increased by LH, as previously reported (28). Nur77 protein was detected at 2 h of LH treatment, and its level was gradually increased to 12 h. Meanwhile, the basal expression of SF-1 was found to be high in MA-10 cells, and LH treatment seemed to marginally increase the SF-1 protein level at 2 h of LH treatment, which remained constant thereafter. These results were clearly indicative of an inverse regulation of ARR19 and Nur77 expression by LH in Leydig cells.

To evaluate further this inverse regulation *in vivo*, 14-day-old mice were injected intraperitoneally with hCG/LH for up to 6 h, and whole testis extracts were analyzed for the expression of ARR19, Nur77, and SF-1 (Fig. 2*C*). Consistent with the results using MA-10 Leydig cells, the expression of ARR19 was markedly inhibited at 3 h of hCG treatment and was completely abrogated within 6 h. On the other hand, Nur77 expression was detected at 3 h of treatment and was increased at 6 h. There was no significant change in the expression of SF-1 with hCG injection. Altogether, these findings strongly suggest an inverse correlation between the regulations of ARR19 and Nur77 expression by LH/hCG in testicular Leydig cells.

ARR19 Inhibits Nur77 Transactivation—The gene regulation of ARR19 and Nur77 by LH was observed to be inversely correlated (Fig. 2). Although Nur77 induces the expression of steroidogenic enzyme genes in Leydig cells (3, 4, 5, 36), ARR19 was presently found to down-regulate the expression of the steroidogenic enzymes, P450c17, StAR, and 3β-HSD (Fig. 1). To determine whether ARR19 affects the transcriptional activity of Nur77, we performed transient transfection analyses with the reporter construct NurRE-luc and expression vectors of Nur77 and ARR19 in MA-10 cells. As shown in Fig. 3*A*, coexpression of increasing amounts of ARR19 with a constant amount of Nur77 expression vector caused the progressive and significant repression of Nur77 transactivation in a dose-dependent manner. ARR19 also inhibited the transactivation of SF-1 when tested with the reporter SF-1RE-luc, but ARR19 repression of SF-1 transactivation was less than that with Nur77 (Fig. 3*B*). These results suggest that ARR19 acts as a novel coregulator of Nur77, affecting the Nur77-mediated gene regulation of steroidogenic enzymes in Leydig cells.

ARR19 Suppresses Nur77-induced Promoter Activity of Steroidogenic Enzyme Genes—To confirm the significance of the repressive function of ARR19 on Nur77 transactivation, the inhibitory effect of ARR19 on Nur77 target promoters such as P450c17, StAR, and 3β-HSD was examined by transient transfection assays using reporter constructs in MA-10 cells (Fig. 3, *C–E*). Consistent with the previous results, ARR19 coexpression repressed Nur77-induced promoter activity of P450c17, 3β-HSD, and StAR genes in a dose-dependent manner. These results suggest that ARR19 suppresses the expression of steroidogenic enzyme genes through the repression of Nur77 transactivation on steroidogenic enzyme promoters in Leydig cells.

ARR19 Interacts with Nur77 in Vivo and in Vitro—To determine whether ARR19-mediated repression of Nur77 transactivation involves physical interaction of ARR19 with Nur77, we

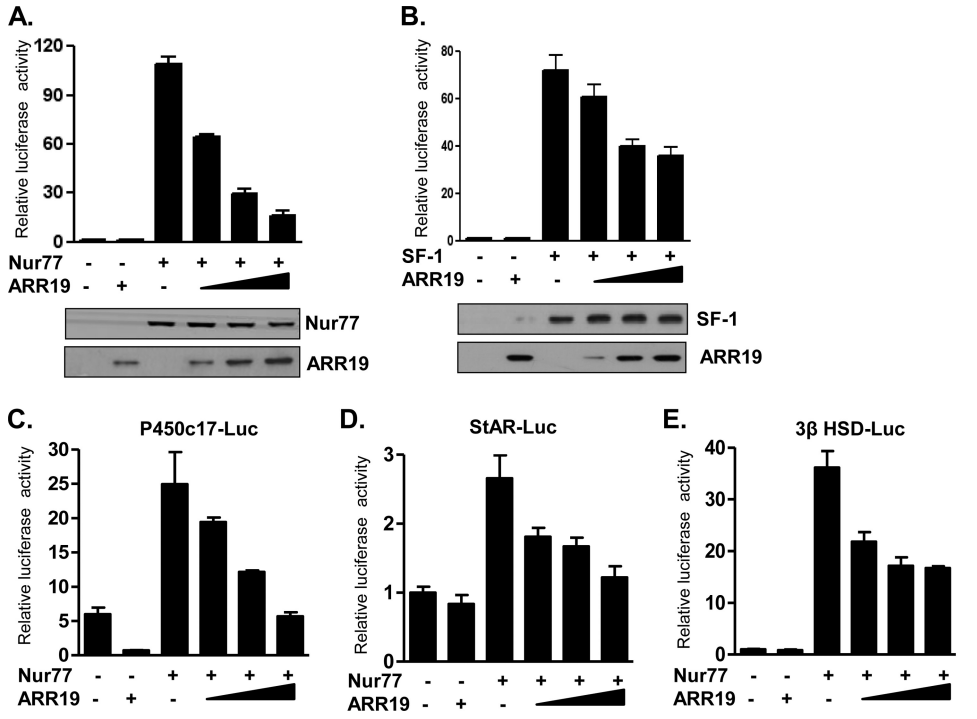

FIGURE 3. **Inhibition of Nur77 transactivation on the promoter of steroidogenic enzyme genes by ARR19.** *A*, *upper panel*, MA-10 cells were cultured on 24-well plates and transfected with 150 ng of NurRE-Luc reporter, 50 ng of pcDNA3-Nur77, 100 ng of pCMV-β-galactosidase, and increasing amounts (50, 100, and 200 ng) of pcDNA-HA-ARR19. After 36 h, the cells were harvested, and luciferase activities were determined and normalized with β-galactosidase activity for the transfection efficiency. *Lower panel*, expression levels of Nur77 and ARR19 protein were ascertained by Western blot analyses of 50 μg of cellular extracts from the transient transfections. *B*, *upper panel*, MA-10 cells were transfected with 150 ng of SF-1RE-Luc reporter, 50 ng of pcDNA3-SF-1, 100 ng of pCMV-β-galactosidase, and increasing amounts (50, 100, and 200 ng) of pcDNA-HA-ARR19. Luciferase activities were determined and normalized as in *A*. *Lower panel*, expression levels of SF-1 and ARR19 protein were ascertained by Western blot analyses as described in *A*. *C–E*, MA-10 cells were transiently transfected with 150 ng of the indicated reporter, StAR-Luc (*C*), P450c17-Luc (*D*), and 3β-HSD-Luc (*E*), together with pcDNA-Nur77 and an increasing amount of ARR19 expression vector (50, 100, and 200 ng). Luciferase activities were determined and normalized as described for *A*.

performed coimmunoprecipitations using MA-10 cells treated with cAMP for 2 h. As shown in Fig. 4*A*, ARR19 coimmunoprecipitated with Nur77 in cAMP-treated cells expressing both ARR19 and Nur77, but not in untreated cells expressing only ARR19.

To analyze further the physical interaction between ARR19 and Nur77 and the protein domains necessary for their interaction, we performed yeast two-hybrid assays using a series of deletion constructs of Nur77 and ARR19 (Fig. 4, *B* and *C*). The AF-1 domain of Nur77 (Nur77-AB; amino acids 1–254) or DNA-binding domain (Nur77-CD; amino acids 255–354) alone did not interact with ARR19, whereas Nur77-CDE (amino acids 255–601) containing the LBD and AF-2 domain of Nur77 fully interacted with ARR19 (Fig. 4*B*). However, Nur77-ΔAF2, the deletion construct of the Nur77 AF-2 domain, did not interact with ARR19, suggesting the essential role of the AF-2 domain of Nur77 in its interaction with ARR19. Similar yeast two-hybrid assays with ARR19 deletion constructs revealed that ARR19-LZ (amino acids 51–107) containing the leucine zipper domain was able to interact with Nur77 more avidly than the full-length ARR19, whereas other domains were not (Fig. 4*C*). Altogether, these results suggest that the AF2 domain of Nur77 and the leucine-zipper domain of ARR19 mediate the interaction between ARR19 and Nur77.

To confirm further the involvement of the AF-2 domain of Nur77 in the interaction with ARR19, we tested the ARR9-mediated repression with Nur77 full-length and Nur77-ΔAF2 (Fig. 4*D*). Both efficiently induced the expression of the reporter NurRE-Luc. However, the transcriptional activity of the full-length Nur77 was repressed by ARR19, whereas the activity of Nur77-ΔAF2 was not (Fig. 4*D*). A similar experiment conducted using GAL4-Nur77 and GAL4-Nur77-ΔAF2 together with GAL4-tk-Luc reporter also revealed the ARR19-mediated repression of the full-length Nur77, but not Nur77-ΔAF2 (Fig. 4*E*). Taken together, these results demonstrate that the AF-2 domain of Nur77 is required for the physical and functional interaction with ARR19.

ARR19 Colocalizes with Nur77 in the Nucleus—To examine the subcellular localization of ARR19 and Nur77, we performed transient transfection analyses using RFP-fused ARR19 (RFP-ARR19) and GFP-fused Nur77 (GFP-Nur77) proteins (Fig. 5*A*). Fluorescence of cells expressing RFP-ARR19 or GFP-Nur77 alone revealed ARR19 distribution in the cytoplasm as well as the nucleus, whereas the GFP-Nur77 protein was localized solely to the nucleus. Coexpression of RFP-ARR19 protein with GFP-Nur77 showed that the distributions of ARR19 and Nur77 in the nucleus overlapped extensively. ARR19-induced repression of Nur77 transactivation was also observed with GFP-Nur77 and RFP-ARR19 fusion proteins in MA-10 cells (Fig. 5*B*). These results suggest that ARR19 protein colocalizes with Nur77 and modulates the transcriptional activity of Nur77.

ARR19 Competes with SRC-1 for Binding to Nur77—It has been reported that SRC-1 and SRC-2 can directly bind to the AF-2 and AF-1 domains of Nur77, respectively, and enhance the transcriptional activity of Nur77 (11, 23). Because the AF-2 domain of Nur77 is the binding site for both SRC-1 and ARR19 (Fig. 4) despite their contrasting functions, we hypothesized that ARR19-mediated repression of Nur77 transactivation might be through a competition with SRC-1 for binding to Nur77. To investigate this hypothesis, we conducted transient transaction analyses with NurRE-Luc reporter together with expression vectors of Nur77, Nur77-ΔAF2, ARR19, SRC-1, and SRC-2. As shown in Fig. 6*A*, cotransfection of SRC-1 and SRC-2 enhanced Nur77 transactivation, and the expression of ARR19 protein repressed the SRC-1-enhanced, but not the SRC-2-enhanced, Nur77 transactivation in a dose-dependent manner.

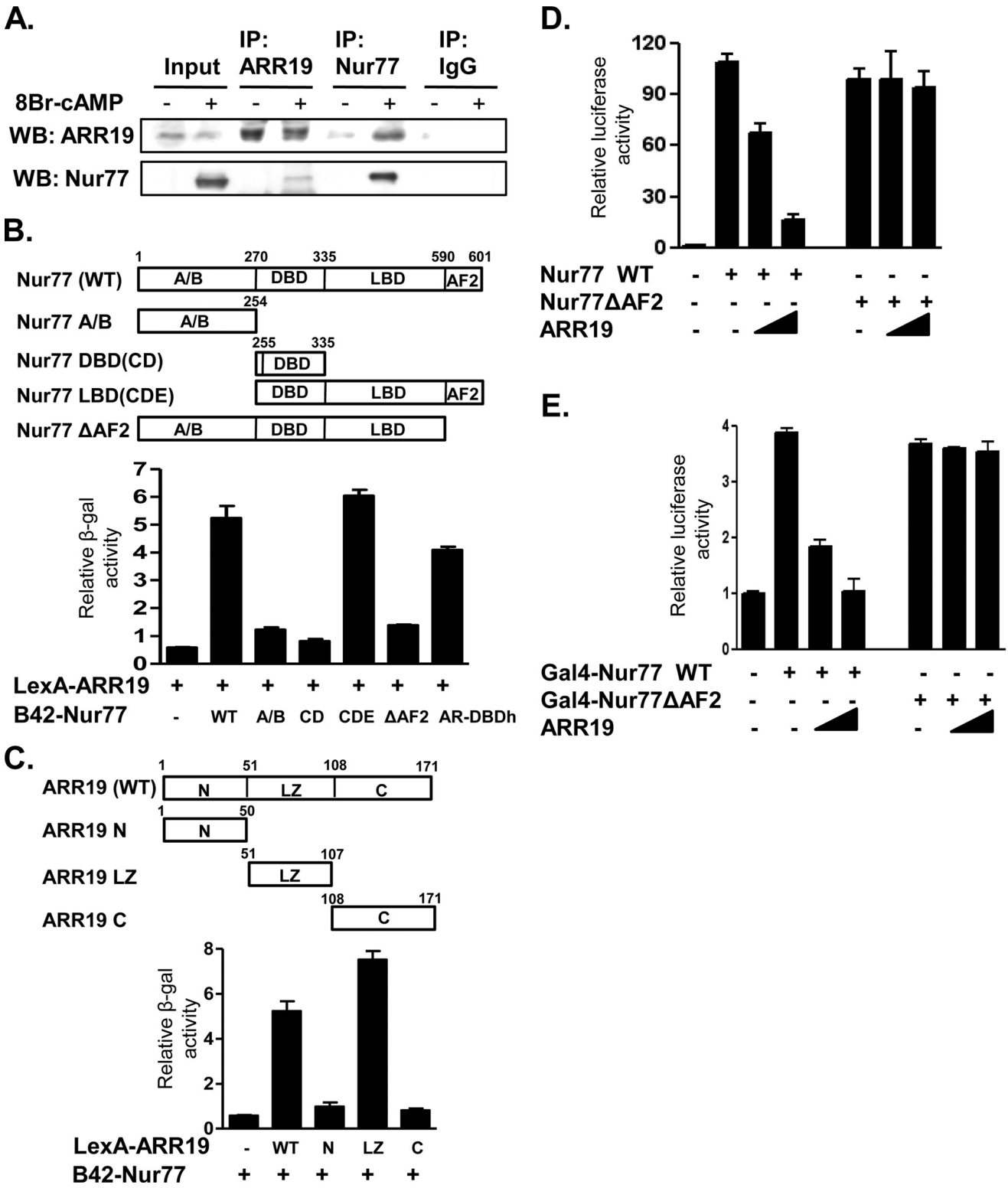

FIGURE 4. **Physical interaction between ARR19 and Nur77.** *A*, physical interaction of ARR19 with Nur77 in IP assays. Protein extracts were prepared from MA-10 cells treated with or without 300 μM of cAMP for 2 h, and immunoprecipitated with anti-Nur77 antibody (*IP: Nur77*), anti-ARR19 antibody (*IP: ARR19*), or IgG (*IP: IgG*) as a negative control. Immunoprecipitates were analyzed by Western blot analyses with anti-ARR19 and anti-Nur77 antibodies. *B*, mapping of the Nur77 domain responsible for its interaction with ARR19 using a yeast two-hybrid system. EGY48 yeast cells were transformed with LexA-ARR19 and B42-Nur77 full-length or deletion constructs (*upper panel*). Transformants were selected, and the β-galactosidase activity was measured. The values are the means $\pm$ S.D. from five independent transformants. B42-AR DNA-binding domain and hinge (DBDh) was used as a positive control for the interaction with ARR19. *C*, mapping of the ARR19 domain responsible for its interaction with Nur77. B42-Nur77 was coexpressed with LexA-ARR19 full-length or deletion constructs (*upper panel*), and yeast two-hybrid assays were performed as described in *B*. *D*, the AF-2 domain of Nur77 is essential for the interaction with ARR19. MA-10 cells were cotransfected with 200 ng of NurRE-Luc reporter, 50 ng of pcDNA3-Nur77 or pcDNA3-Nur77-ΔAF2, 100 ng of pCMV-β-galactosidase, and 100 or 300 ng of pcDNA-HA-ARR19. The luciferase activity was normalized with β-galactosidase activity. *E*, MA-10 cells were transfected with 250 ng of Gal4-tk-Luc reporter, 100 ng of pCMV-GAL4-Nur77 full-length or pCMV-GAL4-Nur77-ΔAF2, and 100 or 200 ng of pcDNA-HA-ARR19.

Further, the ARR19-mediated suppression of Nur77 transactivation was relieved by coexpression of SRC-1 in a dose-dependent manner (Fig. 6*B*). As expected, there was no significant enhancement or repression of Nur77-ΔAF2 transactivation by SRC-1 or ARR19 in contrast to the full-length Nur77 (Fig. 6*C*). Altogether, these results suggest that ARR19 represses Nur77 transactivation through competition with the Nur77 coactivator SRC-1.

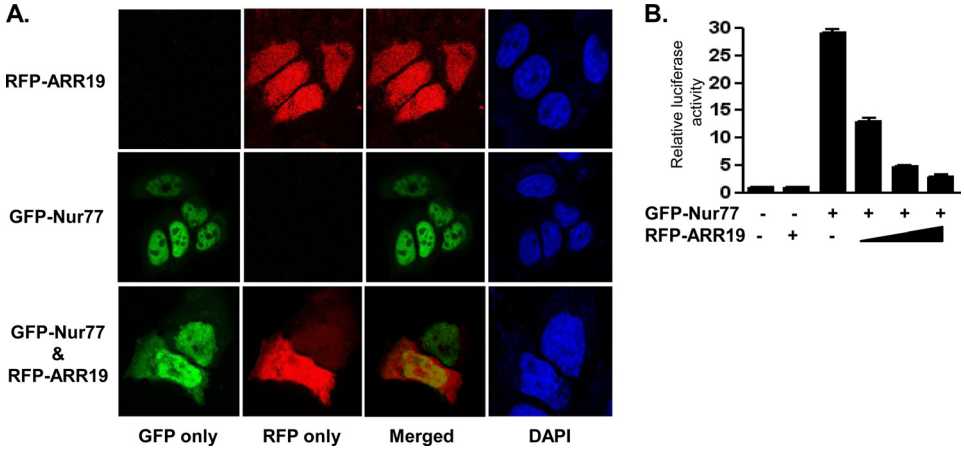

FIGURE 5. **Colocalization of ARR19 with Nur77 into the nucleus.** *A*, HeLa cells were transfected with RFP-ARR19, GFP-Nur77, or both simultaneously. RFP-fused ARR19 and GFP-fused Nur77 proteins were detected with red and green fluorescence, respectively, using confocal microscopy. The images were captured and merged using Leica TCS NT software. *B*, MA-10 cells were transfected with GFP-Nur77 alone or together with increasing amounts of RFP-ARR19 (50, 100, and 200 ng), and Nur77 transactivation was measured by the expression of the luciferase reporter gene.

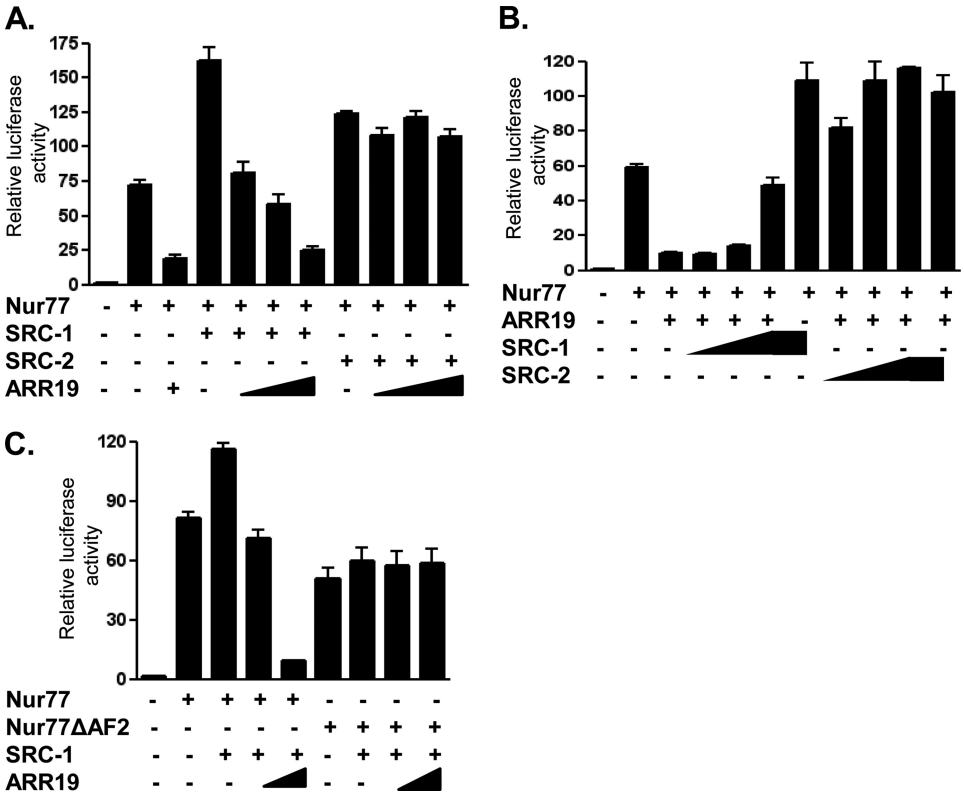

FIGURE 6. **Competition of ARR19 with SRC-1 for binding to the AF-2 domain of Nur77.** *A*, SRC-1-enhanced Nur77 transactivation is repressed by ARR19 expression. MA-10 cells were cotransfected with 200 ng of NurRE-Luc reporter, 50 ng of pcDNA3-Nur77, 100 ng of pcDNA-SRC-1 or pcDNA-SRC-2, 100 ng of pCMV-β-galactosidase, and increasing amounts (100, 200, and 300 ng) of pcDNA-HA-ARR19. After 36 h, the cells were harvested, and luciferase activities were determined and normalized with β-galactosidase activity. *B*, ARR19 suppression of Nur77 transactivation is relieved by SRRC-1 expression. MA-10 cells were cotransfected with 200 ng of NurRE-Luc reporter, 50 ng of pcDNA3-Nur77, 200 ng of pcDNA-HA-ARR19, 100 ng of pCMV-β-galactosidase, and increasing amounts (100, 200, and 300 ng) of pcDNA-SRC-1 or pcDNA-SRC-2. *C*, ARR19 and SRC-1 compete for the AF-2 domain of Nur77 for their action. MA-10 cells were cotransfected with 200 ng of NurRE-Luc reporter, 50 ng of pcDNA3-Nur77 or pcDNA-HA-Nur77ΔAF2, 100 ng of pCMV-β-galactosidase, 200 ng of pcDNA-SRC-1, and 200 or 300 ng of pcDNA-HA-ARR19.

ARR19 was previously reported to recruit histone deacetylase 4 to repress the transactivation of AR in the prostate (25). Thus, we also investigated the involvement of histone deacetylase in the ARR19-mediated repression of Nur77 transactivation using the histone deacetylase inhibitor trichostatin A. The results showed that the repressive effect of ARR19 on Nur77 transactivation remained unchanged in the presence of trichostatin A, suggesting no involvement of histone deacetylases (data not shown).

ARR19 Competes with SRC-1 for the Association with Nur77 on the Promoter of the P450c17 Gene—To examine whether ARR19 can inhibit the association of SRC-1 with Nur77 on the promoter of P450c17 gene, we performed ChIP assays with R2C Leydig cells and 6-week-old mouse testes, both of which were infected with Ad-ARR19 to overexpress ARR19 protein (Fig. 7*A*). In R2C cells infected with Ad-ARR19, the association of SRC-1 with the promoter of the P450c17 gene was significantly reduced in a time-dependent manner, being completely abolished at 6 h of viral infection, whereas the association of ARR19 was rapidly increased (Fig. 7*A*). The association of Nur77 on P450c17 promoter was hardly changed by Ad-ARR19 infection. In control cells infected with Ad-GFP, the association of SRC-1 as well as Nur77 showed no change.

To confirm such a competitive association between SRC-1 and ARR19 onto the P450c17 promoter in animals, we conducted ChIP assays with 6-week-old mouse testes infected with Ad-ARR19 or Ad-GFP as a control and dissected after 0, 6, and 12 h (Fig. 7*B*). Consistent with the results observed in R2C Leydig cells, the association of SRC-1 on the P450c17 promoter was reduced in a time-dependent manner,

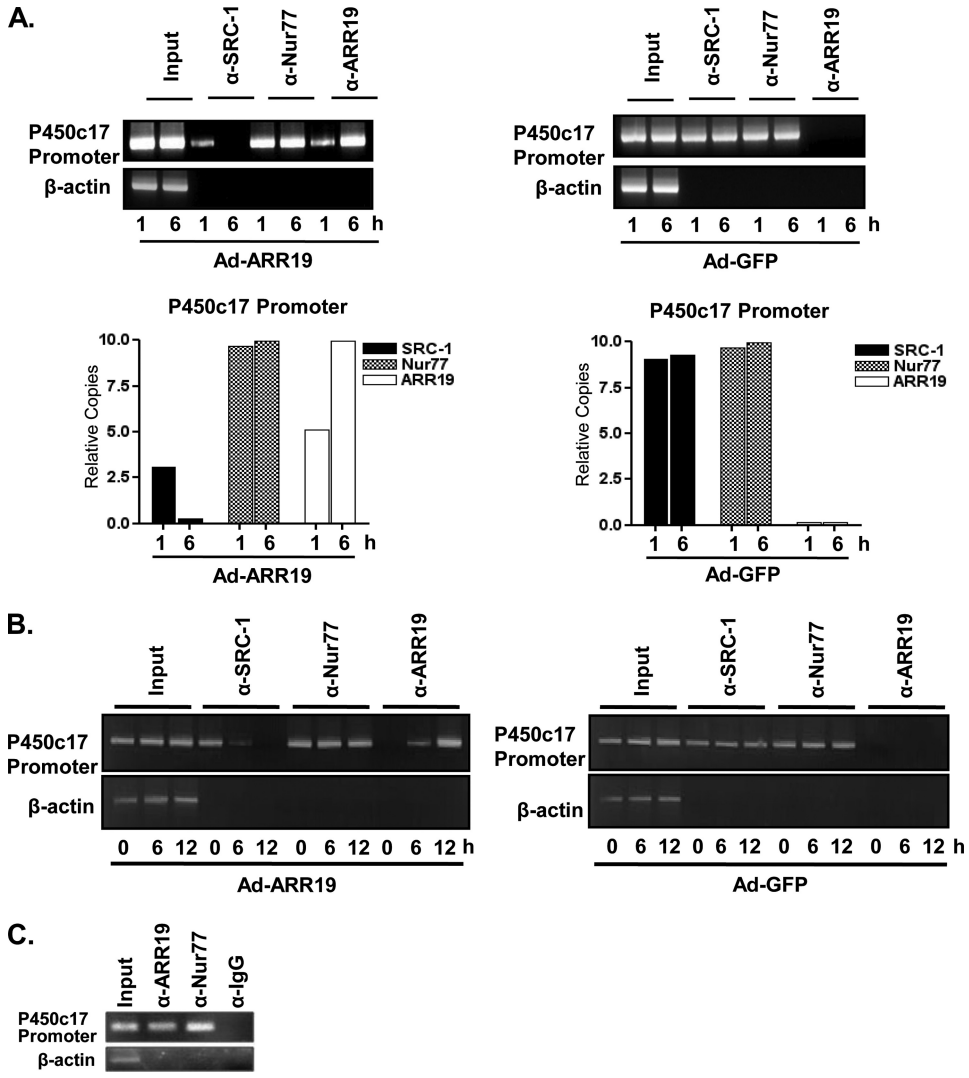

FIGURE 7. **Recruitment of ARR19, along with Nur77, onto the promoter of P450c17 gene competing with SRC-1.** *A*, ChIP assays were conducted with R2C cells infected with Ad-ARR19 or control Ad-GFP for 1 and 6 h. Anti-Nur77, anti-SRC-1, and anti-ARR19 antibodies were used for immunoprecipitations. *Upper panels*, chromatin immunoprecipitates were analyzed by PCR using a pair of specific primers spanning a region containing the Nur77-binding site of the promoter of P450c17 gene. A control PCR for nonspecific immunoprecipitation was done using primers specific for the β-actin coding region. *Lower panels*, real time PCR was performed with the same samples to quantify the relative amounts of SRC-1, Nur77, and ARR19 proteins associated with the P450c17 promoter. *B*, ChIP assays were done with 6-week-old mouse testes infected with Ad-ARR19 or control Ad-GFP at a concentration of 5×10^6 virus particles/testis for 0, 6, and 12 h. Chromatin immunoprecipitates were analyzed as described in *A*. *C*, ChIP assays were performed with primary Leydig cells isolated from 24-day-old mouse testes. Chromatin immunoprecipitates were analyzed as described for *A*.

whereas ARR19 association was increased. Their inverse associations with P450c17 promoter were completed within 12 h of Ad-GFP infection in the testis. The *in vivo* interaction of Nur77 with ARR19 under physiological conditions was confirmed by ChIP assays in primary Leydig cells isolated from 24-day-old testes, which coexpressed ARR19 and Nur77 endogenously (Fig. 2*A* and data not shown). As expected, the endogenous ARR19 and Nur77 proteins were together recruited onto the P450c17 promoter (Fig. 7*C*). Altogether, the results suggest that ARR19 competes with SRC-1 for binding with Nur77 on the promoter of steroidogenic enzyme genes when they are expressed together during the prepubertal-early pubertal stage, suppressing the expression of steroidogenic enzymes and consequently inhibiting testicular steroidogensis.

DISCUSSION

Regulation of testicular steroidogenesis in Leydig cells is mediated through multiple signaling pathways and nuclear factors that generate both positive and negative effects on steroidogenic response (8, 37–39). The cAMP-dependent protein kinase A signaling pathway is a major pathway for testicular steroidogenesis, which is primarily controlled by the pituitary gonadotropin LH in Leydig cells. LH stimulates the production of secondary intracellular messenger cAMP to regulate the expression of steroidogenic enzymes directly or indirectly for the synthesis of testosterone (1). In this study, we have demonstrated that ARR19 regulates the expression of steroidogenic enzymes in Leydig cells through interaction with Nur77. Nur77 is induced rapidly in response to LH/cAMP and is a major regulatory factor for the expression of steroidogenic enzyme genes. Other factors have been also implicated in the control of testicular steroidogenesis by regulating Nur77 transactivation. For example, DAX-1 and NF-κB activated by the proinflammatory cytokine tumor necrosis factor-α interact with Nur77 and suppress Nur77-induced expression of steroidogenic enzyme genes, resulting in the inhibition of steroidogenesis in Leydig cells (33, 13). Similarly to the case of ARR19, the expression of DAX-1 is down-regulated by LH signal in Leydig cells, whereas LH up-regulates Nur77 gene expression (28). DAX-1 and NF-κB bind to Nur77 competing with SRC-1 for Nur77 binding and block SRC-1-mediated enhancement of Nur77 transactivation (28, 29). The interference of SRC-1 recruitment is also the mechanism that suppresses the transcriptional activity of Nur77 by ARR19. A variety of Nur77 coregulators have been reported to date. However, the physiological roles of these coregulators are still poorly understood in animals. It will be interesting to investigate whether and when such Nur77 coregulators act in Leydig cells to modulate testicular steroidogenesis.

ARR19-mediated regulation of Nur77 transactivation occurred under the reciprocal regulation of ARR19 and Nur77 expression in Leydig cells during the prepubertal-early pubertal

stage. Leydig cells arise from undifferentiated stem cells and subsequently undergo phase transitions through the progenitor stage during prepuberty and the immature stage during puberty and ultimately to the terminally differentiated adult Leydig cell stage (40). The progenitor Leydig cells proliferate and also exhibit certain aspects of differentiated function (41), acquiring steroidogenic capacity with stimulation by LH (42). The steroidogenic capacity of progenitor cells is very low compared with the immature and mature Leydig cells (43). ARR19-mediated regulation of Nur77 activity, with increasing expression of Nur77 and decreasing expression of ARR19 by LH, in progenitor Leydig cells may contribute to fine-tuning the acquisition of steroidogenic capacity of Leydig cells at the intermediate differentiation stage.

The orphan nuclear receptor Nur77, which is induced rapidly in response to LH/cAMP, has received increasing consideration as an important regulator of steroidogenic enzyme genes such as P450c17, StAR, and 3β-HSD in steroidogenic cells including testicular Leydig cells (21, 25, 29). Another orphan nuclear receptor, SF-1, has an important role in the proliferation and differentiation of steroidogenic cells in embryonic gonads and also regulates the expression of steroidogenic enzyme genes in Leydig cells (4, 5, 36). The binding elements for SF-1 and Nur77 have been found in the promoter of steroidogenic enzyme genes. They are quite similar in their core sequence and even overlap in some cases such as with the p450c17 promoter (3, 6). Recently, it was shown that Nur77 and SF-1 bind to the same binding sites in the promoter of the mouse GnRH receptor gene (44). However, the relative importance of Nur77 and SF-1 as major regulators for the gene regulation of steroidogenic enzymes *in vivo* may depend on the developmental and physiological conditions of testicular Leydig cells, which will be reflected in the protein levels of coregulators such as ARR19 and SRC-1, as well as the Nur77 and SF-1 regulators themselves.

In the present study, we have outlined the molecular events underlying the suppression of Nur77-induced steroidogenesis by the anti-steroidogenic factor, ARR19, in Leydig cells. Adenovirus-mediated overexpression of ARR19 significantly reduced the production of testosterone in mouse testis as well as R2C Leydig cells, which was accompanied by reduced protein levels of steroidogenic enzymes such as P450c17, P450scc, 3β-HSD, and StAR (Fig. 1). In R2C Leydig cells, however, the protein levels of StAR and 3β-HSD began to recover 4–6 h after Ad-ARR19 infection and continued to increase to 24 h. These data suggest the possibility of the biphasic nature or an additional layer of regulation of steroidogenic enzyme gene expression. The biphasic nature of steroidogenic enzyme gene regulations by tumor necrosis factor-α was previously reported in testicular Leydig cells, which was due to the biphasic activation of NF-κB at the levels of both NF-κB nuclear translocation/DNA binding and modulation of Nur77 transactivation (45–47), although the biological significance of the biphasic profile of NF-κB activation remains poorly understood. Importantly, the protein levels of P450c17 and P450scc were gradually decreased and finally abrogated at 24 h of Ad-ARR19 infection, suggesting the direct regulation of P450c17 and P450scc expression by ARR19. Further studies are necessary to illuminate the biphasic nature or an additional layer of regulation of β-HSD and StAR regulation by ARR19.

A homologue of ARR19/mCklfsf2a, designated mCklfsf2b, shares a high degree of amino acid conservation with ARR19 and is commonly expressed in the testis, prostate, spleen, and thymus (18). Functionally, both can affect the transcription activity of the AR in PC-3 and HeLa cells, but ARR19/mCklfsf2a acts as a repressor, whereas mCklfsf2b acts as an enhancer (25, 24). Because ARR19/mCklfsf2a and mCklfsf2b are abundantly expressed in the testis, it would be interesting to investigate the functional role of mCklfsf2b in male reproduction as well as testicular steroidogenesis, which is unknown and currently under investigation.

In summary, ARR19 acts as a novel coregulator of Nur77 and inhibits the Nur77-induced expression of steroidogenic enzyme genes, resulting in the suppression of testicular steroidogenesis during the prepubertal-early pubertal stage. These phenomena may have a role in fine-tuning the acquisition of steroidogenic capacity of Leydig cells at the intermediate differentiation stage.

REFERENCES

1. Payne, A. H., and O'Shaughnessy, P. J. (1996) in *The Leydig Cell* (Payne, A. H., Hardy, M. P., and Russell, L. D., eds) 2nd Ed., pp. 259–285, Cache River Press, Vienna, IL
2. Song, K. H., Park, J. I., Lee, M. O., Soh, J., Lee, K., and Choi, H. S. (2001) *Endocrinology* **142,** 5116–5123
3. Mellon, S. H., Compagnone, N. A., and Zhang, P. (1998) *Endocr. Res.* **24,** 505–513
4. Parker, K. L., and Schimmer, B. P. (1997) *Endocr. Rev.* **18,** 361–377
5. Sadovsky, Y., and Dorn, C. (2000) *Rev. Reprod.* **5,** 136–142
6. Zhang, P., and Mellon, S. H. (1997) *Mol. Endocrinol.* **11,** 891–904
7. Wilson, T. E., Mouw, A. R., Weaver, C. A., Milbrandt, J., and Parker, K. L. (1993) *Mol. Cell. Biol.* **13,** 861–868
8. Stocco, C. O., Zhong, L., Sugimoto, Y., Ichikawa, A., Lau, L. F., and Gibori, G. (2000) *J. Biol. Chem.* **275,** 37202–37211
9. Giguère, V. (1999) *Endocr. Rev.* **20,** 689–725
10. Steinmetz, A. C., Renaud, J. P., and Moras, D. (2001) *Annu. Rev. Biophys. Biomol. Struct.* **30,** 329–359
11. Maira, M., Martens, C., Batsché, E., Gauthier, Y., and Drouin, J. (2003) *Mol. Cell. Biol.* **23,** 763–776
12. Wansa, K. D., Harris, J. M., and Muscat, G. E. (2002) *J. Biol. Chem.* **277,** 33001–33011
13. Wansa, K. D., Harris, J. M., Yan, G., Ordentlich, P., and Muscat, G. E. (2003) *J. Biol. Chem.* **278,** 24776–24790
14. Milbrandt, J. (1988) *Neuron* **1,** 183–188
15. Hazel, T. G., Nathans, D., and Lau, L. F. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85,** 8444–8448
16. Park, J. I., Park, H. J., Choi, H. S., Lee, K., Lee, W. K., and Chun, S. Y. (2001) *Endocrinology* **142,** 3051–3059
17. Kovalovsky, D., Refojo, D., Liberman, A. C., Hochbaum, D., Pereda, M. P., Coso, O. A., Stalla, G. K., Holsboer, F., and Arzt, E. (2002) *Mol. Endocrinol.* **16,** 1638–1651
18. Fahrner, T. J., Carroll, S. L., and Milbrandt, J. (1990) *Mol. Cell. Biol.* **10,** 6454–6459
19. Hazel, T. G., Misra, R., Davis, I. J., Greenberg, M. E., and Lau, L. F. (1991) *Mol. Cell. Biol.* **11,** 3239–3246
20. Davis, I. J., Hazel, T. G., Chen, R. H., Blenis, J., and Lau, L. F. (1993) *Mol. Endocrinol.* **7,** 953–964
21. Hirata, Y., Kiuchi, K., Chen, H. C., Milbrandt, J., and Guroff, G. (1993) *J. Biol. Chem.* **268,** 24808–24812
22. Pekarsky, Y., Hallas, C., Palamarchuk, A., Koval, A., Bullrich, F., Hirata, Y., Bichi, R., Letofsky, J., and Croce, C. M. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 3690–3694

23. Sohn, Y. C., Kwak, E., Na, Y., Lee, J. W., and Lee, S. K. (2001) *J. Biol. Chem.* **276,** 43734–43739
24. Li, T., Han, W., Yang, T., Ding, P., Rui, M., Liu, D., Wang, Y., and Ma, D. (2006) *Int. J. Biochem. Cell Biol.* **38,** 420–429
25. Jeong, B. C., Hong, C. Y., Chattopadhyay, S., Park, J. H., Gong, E. Y., Kim, H. J., Chun, S. Y., and Lee, K. (2004) *Mol. Endocrinol.* **18,** 13–25
26. Qamar, I., Park, E., Gong, E. Y., Lee, H. J., and Lee, K. (2009) *J. Biol. Chem.* **284,** 18021–18032
27. Ge, R. S., Dong, Q., Sottas, C. M., Papadopoulos, V., Zirkin, B. R., and Hardy, M. P. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 2719–2724
28. Song, K. H., Park, Y. Y., Park, K. C., Hong, C. Y., Park, J. H., Shong, M., Lee, K., and Choi, H. S. (2004) *Mol. Endocrinol.* **18,** 1929–1940
29. Hong, C. Y., Park, J. H., Ahn, R. S., Im, S. Y., Choi, H. S., Soh, J., Mellon, S. H., and Lee, K. (2004) *Mol. Cell. Biol.* **24,** 2593–2604
30. Moraga, P. F., Llanos, M. N., and Ronco, A. M. (1997) *J. Endocrinol.* **154,** 201–209
31. Kim, J. Y., Kim, H. J., Kim, K. T., Park, Y. Y., Seong, H. A., Park, K. C., Lee, I. K., Ha, H., Shong, M., Park, S. C., and Choi, H. S. (2004) *Mol. Endocrinol.* **18,** 2880–2894
32. Wells, J., and Farnham, P. J. (2002) *Methods* **26,** 48–56
33. Fan, W., Yanase, T., Morinaga, H., Okabe, T., Nomura, M., Daitoku, H., Fukamizu, A., Kato, S., Takayanagi, R., and Nawata, H. (2007) *J. Biol. Chem.* **282,** 7329–7338
34. Mohan, S., Richman, C., Guo, R., Amaar, Y., Donahue, L. R., Wergedal, J., and Baylink, D. J. (2003) *Endocrinology.* **144,** 929–936
35. Teerds, K. J., Rijntjes, E., Veldhuizen-Tsoerkan, M. B., Rommerts, F. F., and de Boer-Brouwer, M. (2007) *J. Endocrinol.* **194,** 579–593
36. Zhang, P., and Mellon, S. H. (1996) *Mol. Endocrinol.* **10,** 147–158
37. Manna, P. R., and Stocco, D. M. (2005) *Curr. Drug Targets Immune Endocr. Metabol. Disord.* **5,** 93–108
38. Cooke, B. A. (1999) *Mol. Cell. Endocrinol.* **151,** 25–35
39. Ascoli, M., Fanelli, F., and Segaloff, D. L. (2002) *Endocr. Rev.* **23,** 141–174
40. Ge, R. S., Dong, Q., Sottas, C. M., Chen, H., Zirkin, B. R., and Hardy, M. P. (2005) *Biol. Reprod.* **72,** 1405–1415
41. Haider, S., and Rai, U. (1986) *Gen. Comp. Endocrinol.* **64,** 321–329
42. Cooke, B. A. (1996) *In The Leydig Cell*, 1st Ed., pp. 352–363, Cache River Press, Vienna, IL
43. Mendis-Handagama, S. M., and Ariyaratne, H. B., (2001) *Biol. Reprod.* **65,** 660–671
44. Sadie, H., Styger, G., and Hapgood, J. (2003) *Endocrinology* **144,** 1958–1971
45. Haddad, J. J. (2002) *Cytokine* **17,** 301–310
46. Han, Y., and Brasier, A. R. (1997) *J. Biol. Chem.* **272,** 9825–9832
47. Ladner, K. J., Caligiuri, M. A., and Guttridge, D. C. (2003) *J. Biol. Chem.* **278,** 2294–2303