THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 28, pp. 23657–23666, July 6, 2012

Transcription Factors ER71/ETV2 and SOX9 Participate in a Positive Feedback Loop in Fetal and Adult Mouse Testis*

Received for publication, November 2, 2011, and in revised form, May 20, 2012 Published, JBC Papers in Press, May 21, 2012, DOI 10.1074/jbc.M111.320101

Luciano DiTacchio[‡], **Josephine Bowles**[§], **Sook Shin**[¶], **Dae-Sik Lim**[‖], **Peter Koopman**[§1], **and Ralf Janknecht**[¶2]

From the [‡]*Salk Institute, La Jolla, California 92037, the* [§]*University of Queensland, Brisbane, QLD 4072, Australia, the* [¶]*University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, and the* [‖]*Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea*

Background: Male sex determination is dependent on the SRY and SOX9 transcription factors.
Results: SRY activates *Er71* transcription. Then, ER71 and SOX9 may sustain each other's expression.
Conclusion: The transcription factor ER71 may be part of the SRY-SOX9 axis.
Significance: Understanding how male gonadal development is induced explains how the Y chromosome leads to a male phenotype.

ER71, also known as ETV2, is an ETS transcription factor that is expressed during embryogenesis and in adult testes. We show that *Er71* transcription can be up-regulated by SRY, the key determinant of male differentiation. Accordingly, SRY bound to and activated the *Er71* promoter, and mutation of a putative SRY binding site abolished this promoter activation. In turn, ER71 was able to bind to the promoter of *Sox9*, the primary target of SRY and a critical transcription factor for maintenance of the Sertoli cell phenotype. Mutation of the ER71 binding site in the *Sox9* promoter suppressed ER71-dependent up-regulation of *Sox9* transcription, and a dominant-negative ER71 molecule severely reduced *Sox9* transcription in a Sertoli cell line. Conversely, SOX9 bound the *Er71* promoter *in vivo* and *Sox9* down-regulation reduced *Er71* transcript levels. Together, these data suggest a mechanism by which SRY induces *Sox9* and *Er71* transcription early in testis differentiation, whereas ER71 and SOX9 participate in an autoregulatory loop to sustain each other's expression after *Sry* expression has subsided in mice. Thereby, ER71 and SOX9 may affect late testis development as well as the function of the adult male gonad.

In most mammals, including humans and mice, development of testes is dependent on the Y-chromosomal *Sry* (sex-determining region Y)[3] gene. Initially, gonads emerge as bipotential structures that develop by default into ovaries, but the correct timing of *Sry* expression triggers the path to testis differentiation (1, 2). Accordingly, transgenic expression of *Sry* in XX mice leads to male development (3), indicating that no other gene on the Y chromosome is needed for testis determination.

Sry encodes a protein whose high mobility group (HMG) domain binds to the minor groove of DNA and induces strong DNA bending. Aside from the HMG domain, SRY is surprisingly unconserved during evolution. For instance, whereas mouse SRY consists of 395 amino acids with a glutamine-rich repeat, the 204 amino acid human SRY protein lacks this repeat but possesses 57 amino acids preceding the HMG domain that are absent from mouse SRY (4). Therefore, it is commonly concluded that the conserved HMG domain is crucial for SRY action in sex determination and, consistently, most mutations in human SRY that cause male to female sex reversal are located within the HMG domain (5).

One downstream effector of SRY in testis development is SOX9 (SRY-type HMG box protein 9). Mutation of one *SOX9* allele leads to campomelic dysplasia (6, 7), a skeletal dysmorphology syndrome that is commonly characterized by bowing and angulation of long bones, hypoplastic scapulae, and a missing pair of ribs. Most patients die soon after birth, probably due to the hypoplasia of the tracheobronchial cartilage and small thoracic cage causing respiratory distress. Analysis of SOX9 expression and knock-out models revealed that SOX9 is essential for the proper differentiation of chondrocytes, which explains how *SOX9* haploinsufficiency leads to defects in skeletal elements derived from cartilage (8–10). However, another feature of campomelic dysplasia is that about 75% of XY patients develop as phenotypic females or intersexes (11), indicating that SOX9 is crucial for male development.

Consistently, SOX9 overexpression causes XX mice to develop as males (12–14), indicating that SOX9 in itself is sufficient for the male sex determination process in mice. Similarly, XX sex reversal has been observed in a human upon duplication of the *SOX9* gene (15). Moreover, conditional knock-out models revealed that the absence of *Sox9* in the developing gonads of XY mice leads to their differentiation into ovaries (16, 17), clearly demonstrating that SOX9 is not only sufficient but also necessary for male gonad development. Like SRY, SOX9 binds and bends DNA by virtue of its HMG domain. In addition, SOX9 contains two transactivation domains allowing it to stimulate gene transcription (4). The discovery that the anti-Müllerian hormone gene, which is required for male duct

* This work was funded in part by the National Health and Medical Research Council of Australia.
[1] Federation Fellow of the Australian Research Council.
[2] To whom correspondence should be addressed: University of Oklahoma Health Sciences Center, Department of Cell Biology, 975 NE 10th Street, BRC-1464, Oklahoma City, OK 73104. Tel.: 405-2712377; Fax: 405-2713548; E-mail: ralf-janknecht@ouhsc.edu.
[3] The abbreviations used are: SRY, sex-determining region Y; Cbln4, cerebellin 4 precursor; dpc, days *post coitum*; ER71, ETS related 71; ETV2, ETS variant 2; HMG, high mobility group; MLTC-1, mouse Leydig tumor cell 1; SF1, steroidogenic factor 1; SOX9, SRY-type HMG box protein 9.

development, is a target of SOX9 was the first revelation of how SOX9 induces the development of the male sexual phenotype (18, 19), and subsequently more SOX9 target genes relevant for sex determination have been uncovered (1, 2).

An obvious hypothesis would be that SRY directly activates the transcription of the *Sox9* gene and thereby exerts its sex determining power. In fact, a recent study identified an enhancer located ~10 kb upstream of the *Sox9* transcription start site that stimulates *Sox9* transcription upon binding of steroidogenic factor 1 (SF1) and SRY (20). However, this does not exclude the presence of other regulatory elements in the *Sox9* promoter, or the involvement of other factors in *Sox9* gene expression. Further, this does not explain how *Sox9* transcription remains elevated in the developing testes after the transient, short burst of *Sry* expression at the beginning of male gonadal differentiation. Notably, *Sox9* transcription is also elevated in the adult testes, where SRY is not expressed (4).

We speculated that the transcription factor ER71 (ETS related 71; also called ETV2 for ETS variant 2) might be a candidate regulator of *Sox9* transcription, as *Er71* is testis-specifically expressed in the adult mouse and also present in mouse embryos at the onset of gonadal differentiation (21, 22). ER71 belongs to the ETS class of transcription factors characterized by a conserved, ~85 amino acids long DNA binding domain (23–25). It is a constitutively nuclear protein and is capable of activating gene transcription (26). In addition, ER71 is an essential protein whose absence leads to hematopoietic, vascular and endocardial defects (27–29). Here, we present evidence that the *Er71* gene is a target of SRY. Further, the *Er71* and *Sox9* gene products up-regulate each other's transcription. This implicates ER71 in the formation of testes in the embryo as well as in testicular function in adults.

EXPERIMENTAL PROCEDURES

Luciferase Assay—Cells were grown in 6-cm dishes in DMEM supplemented with 10% fetal bovine serum (30) and transiently transfected with a total of 9 μg of DNA by the calcium phosphate coprecipitation method (31, 32). 36 h after transfection, cells were washed once with phosphate-buffered saline, and lysed in 25 mM Tris-HCl (pH 7.8), 2 mM EDTA, 10% glycerol, 1% Triton X-100, and 2 mM DTT (33). Luciferase activity of the cleared supernatant was then determined in a luminometer (34, 35). Shown are averages (with standard error) of triplicate experiments.

Electrophoretic Mobility Shift Assay—GST (glutathione *S*-transferase) and GST-SRY$_{1-130}$ protein were produced in *Escherichia coli* and purified on glutathione-agarose according to standard procedures (36, 37). ER71 was produced by *in vitro* transcription and translation using Promega's TNT kit (26). Proteins were incubated with ^{32}P-labeled, double-stranded oligonucleotides and separated on 5% polyacrylamide gels as previously described (38, 39). Gels were dried and respective autoradiograms obtained by exposure to film (40).

^{32}P-labeled probes were obtained by annealing complementary oligonucleotides, whose ends were filled in with Klenow enzyme in the presence of [α-^{32}P]dATP (41, 42). Probes were purified on NucTrap columns (Stratagene) before utilization in binding reactions. The following oligonucleotides were employed: SRY site (sense): 5′-GCCGATTGTGACGTAGGCT. SRY site (antisense): 5′-CATCAGCCTACGTCACAATCGGC. −5/−8 (sense): 5′-AGCTATTTATTAGAGACCCTGAGCTGGAAGT-CGG. −5/−8 (antisense): 5′-AGCTCCGACTTCCAGCTC-AGGGTCTCTAATAAAT. −111/−114&−118/−121 (sense): 5′-AGTCCCCTTCCAAAATCCGGTCCAATCAGC. −111/−114&−118/−121 (antisense): 5′-AGTCGCTGATTGGAC-CGGATTTTGGAAGGG. −135/−138 (sense): 5′-AGCT-CACCCAACCCGGAGCCACAATCCTCCCC. −135/−138 (antisense): 5′-AGCTGGGGAGGATTGTGGCTCCGGGTT-GGGTG. −204/−207 (sense): 5′-AGTCCCCTCACCCCACC-ATCCACCCTCTGG. −204/−207 (antisense): 5′-AGCTCC-AGAGGGTGGATGGTGGGGTGAGG. −292/−295&−308/−311 (sense): 5′-GCTCGGAGACCGTTCCAAAACTGTGA-CATTCCGAG. −292/−295&−308/−311 (antisense): 5′-AGCTCTCGGAATGTCACAGTTTTGGAACGGTCTCCG.

RT-PCR Analysis of Transfected Cells—Cells were grown in 12-well plates and transfected utilizing 2 μg of DNA and 2 μl of Lipofectamine 2000 reagent (Invitrogen). Total RNA was isolated from cells employing Trizol (Invitrogen). Approximately 0.5 μg of RNA was utilized to generate and amplify cDNA with the Access RT-PCR kit (Promega) (43, 44). Briefly, after 45 min of reverse transcription at 48 °C, DNA was amplified for 36 cycles (*Er71*) or 30 cycles (*Sox9*) at 98 °C for 1 min, 60 °C for 1 min and 68 °C for 2 min. Amplification for *Gapdh* with respective primers was done as described before (22). DNA was finally separated on 1.5% agarose gels and visualized by ethidium bromide staining (45). *Er71* primers yielding a 348 bp product and *Sox9* primers yielding a 256 bp product were: Er71: 5′-CAAG-AGGACACACCGATCACACC/5′-CACAGAACAGTCCCA-GCTGGTAGTG. Sox9: 5′-AGGAAGCTGGCAGACCA-GTA/5′-CCTTGAAGATAGCATTAGGAGAGATGTG.

Mouse Embryos—Embryos were collected from timed matings, with noon of the day on which the mating plug was observed designated 0.5 days *post coitum* (dpc). CD1 mice were used for *in situ* hybridization and W^e/W^e mice (46) for quantitative RT-PCR analysis. When necessary, embryo sexing was carried out by analysis of tail tissue for the presence of *Zfy* as described (47). All animal work was conducted according to protocols approved by the University of Queensland Animal Ethics Committee.

In Situ Hybridization, Microarray Analysis, and RT-PCR on Embryos—Whole-mount *in situ* hybridization was performed as described (48). Section *in situ* hybridization was performed on 10 μm sections of paraffin-embedded embryos as described previously (49). The *Er71* probe used spans nucleotides 641–937 of the mouse *Er71* transcript (L10427), encoding most of the 3′-UTR (untranslated region). Expression of mouse *Sox9* was detected using the probe *Sox9.5a* (8).

A genome-wide Affymetrix microarray analysis of gene expression in mouse fetal gonads, with a focus on time points 11.5 and 12.0 dpc, was conducted and described previously (50). Data are shown for *Sry* (1450578_at), *Sox9* (1433889_at), *Cbln4* (1433607_at), and *Er71* (1421773_at).

Relative cDNA levels were analyzed by the comparative cycle time method of quantitative RT-PCR with reactions including Taqman PCR master mix (ABI) and Taqman gene expression sets as described (51). Endogenous control, used to normalize

gene expression levels, was *Tbp* (encoding TATA box binding protein). Taqman gene expression sets were as follows: Mm0044973_m1 (*Tbp*), Mm00448840_m1 (*Sox9*), and Mm00468389_m1 (*Er71*).

Chromatin Immunoprecipitation—Where indicated, cells were transfected utilizing Lipofectamine 2000 reagent (Invitrogen). 36 h after transfection, chromatin immunoprecipitation (ChIP) assays were performed essentially as described (52, 53). To ascertain that equal amounts of chromatin were present in each sample, we split cell extracts after formaldehyde treatment and sonication into identical portions. *Sox9* promoter DNA was amplified utilizing nested PCR with the ChIP-Sox9-forward/ChIP-Sox9-reverse-new primer pair in the first PCR reaction (20 repeats) and ChIP-Sox9-forward-new/ChIP-Sox9-reverse primer pair in the second PCR reaction (22 repeats). The PCR program was: 98 °C for 2 min, 8 cycles of 98 °C for 30 s, 64 °C for 30 s (minus 1 °C per cycle), 72 °C for 25 s followed by the indicated number of repeats for the cycle 98 °C for 30 s, 56 °C for 30 s, 72 °C for 25 s (plus 1 s per cycle), and finally by a 4 min extension at 72 °C. The expected 286 bp DNA product was visualized by ethidium bromide staining after electrophoresis on a 1.5% agarose gel (54).

Similarly, a 309 bp *Flk1* promoter fragment was amplified by utilizing ChIP-Flk-forward and ChIP-Flk-reverse primers. Moreover, *Sox9* 3′-UTR sequences were amplified with 2646-for/3044-rev or 3268-for/3599-rev primers in the first PCR reaction followed by 2664-for/3023-rev or 3293-for/3572-rev primers, respectively, in the second PCR reaction. To amplify a 362 bp *Er71* sequence around the SRY binding-site, a first amplification with ChIP-Er71-for-1/ChIP-Er71-rev1 primers was followed by a second amplification with ChIP-Er71-for-2/ChIP-Er71-rev2 primers. As a control, a 306 bp *Er71* fragment spanning intron 6 and parts of exons 6 and 7 was amplified with ChIP-Er71-end-for-1/ChIP-Er71-end-rev1 primers followed by a second amplification with ChIP-Er71-end-for-3/ChIP-Er71-end-rev2 primers. Lastly, a 329 bp fragment from the beginning of the *Sry* coding sequence was amplified with SRY-for-1/SRY-rev-1 primers in the first PCR reaction followed by SRY-for-2/SRY-rev-2 primers in the second PCR reaction. The primers used were: ChIP-Sox9-forward: 5′-TCGGTTCACACGGAGACCGTTCC. ChIP-Sox9-forward-new: 5′-CCGTTCCAAAACTGTGACATTCCGAG. ChIP-Sox9-reverse: 5′-TACTCCTCCTTCACGTTAGATACC. ChIP-Sox9-reverse-new: 5′-TCGGCTCTCCGACTTCCAGCTCAG. ChIP-Flk-forward: 5′-AGGGCTAATCAGGTAACTTCGGAC. ChIP-Flk-reverse: 5′-CTCATTAGGAGCCTGCAAGTGCAG. 2646-for: 5′-GCTTTGATTAATTCCCCAGGCTCTTG. 2664-for: 5′-GGCTCTTGGATTTCAAGAGTAGCTG. 3023-rev: 5′-GTGCTTGGGCACTTAGGGCTGCGTG. 3044-rev: 5′-CAGGAGGCAACCAGGGAAAATGTGC. 3268-for: 5′-TCTTTAAGGTAGATTGTTGGCGCCTTC. 3293-for: 5′-TCCTCAAAGGGTATGGTCATCTGTTG. 3572-rev: 5′-GCTCCTCACTGCATCTGAAACCTCTC. 3599-rev: 5′-GGCTGAAAACATTGCAAAGGACTCAG. ChIP-Er71-for-1: 5′-CTCGAAATTCTCCAATGGTTCGGGAC. ChIP-Er71-for-2: 5′-GCTATAGCCCAGAAAGCCCAGACAG. ChIP-Er71-rev-1: 5′-CTGGGCAGCTCTGGGCTTATCTGCAAC. ChIP-Er71-rev-2: 5′-CAACAGGAAGAGGGATTTCCGGCCAG. ChIP-Er71-end-for-1: 5′-CATTCAGCTGTGGCAATTCCTCCTG. ChIP-Er71-end-for-3: 5′-GGCAATAGCCGCGAGTTCCAGCTG. ChIP-Er71-end-rev-1: 5′-CATCCTGATAGGCGAGGACAGGCACAC. ChIP-Er71-end-rev-2: 5′-CGCCCACCACTCTTGAGCACGATGTC. SRY-for-1: 5′-ATGGAGGGCCATGTCAAGCG. SRY-for-2: 5′-CCCATGAATGCATTTATGGTGTGG. SRY-rev-1: 5′-CAGTGGGGATATCAACAGGCTG. SRY-rev-2: 5′-CAGTCTTGCCTGTATGTGATGGC.

RNA Interference—The sequence 5′-GGACAGACCUUAAUUCUUA in the 3′-UTR of mouse *Sox9* mRNA was targeted with shRNA, which was cloned into pSIREN-RetroQ (Clontech). Respective retrovirus was then produced in 293T cells according to standard procedures (55). 15P-1 cells were then twice infected with control or *Sox9* shRNA expressing retrovirus (56), selected for 5 days with 1 μg/μl puromycin and then grown for another 2 days before preparation of RNA utilizing Trizol (Invitrogen). RNA was then amplified with mSox9-for-604/627 (5′- GTCAGCCAGGTGCTGAAGGGCTAC) and mSox9-rev-993/969 (5′-CGCCTTGAAGATAGCATTAGGAGAG) primers in a MiniOpticon (Bio-Rad) real-time PCR machine utilizing the iScript One-Step RT-PCR kit with SYBR green (Bio-Rad) and the following PCR program: 50 °C for 15 min; 95 °C for 5 min; 35 cycles of 95 °C for 15 s, 58 °C for 15 s, 65 °C for 45 s. Likewise, *Gapdh* was amplified with previously described primers (22). *Er71* mRNA was first amplified with the Access RT-PCR kit (Promega) and then with the iQ SYBR Green Supermix (Bio-Rad) using nested primer pairs published before (22). *Sox9* and *Er71* mRNA levels were normalized to *Gapdh* by the comparative cycle time method.

RESULTS

SRY Activates Transcription of Er71—Previously, we cloned the mouse *Er71* gene promoter (22), allowing us to set out to analyze whether SRY activates *Er71* transcription and may thus contribute to the embryonic expression of ER71. To this end, successively smaller *Er71* promoter fragments were cloned in front of luciferase cDNA and their activation by mouse SRY investigated. As shown in Fig. 1*A*, SRY activated all three *Er71* promoter fragments utilized, and the degree of activation was basically unchanged from the longest (−460 to +51) to the shortest (−85 to +51) promoter fragment. Moreover, we expressed SRY in the mouse Leydig tumor cell 1 (MLTC-1) line that expresses no significant amounts of *Sry* or *Er71* (22) and found that endogenous *Er71* transcription was activated by ectopic SRY expression (Fig. 1*B*). In addition, we performed ChIP assays in MLTC-1 cells transfected with a FLAG-tagged SRY expression construct. We thereby found that mouse SRY bound to the *Er71* promoter within the region −334 to +28 (Fig. 1*C*), whereas SRY did not interact with a downstream region of the *Er71* gene or the *Sry* coding region. These data strongly indicate that the *Er71* gene is a potential target of SRY.

SRY Binds to the Er71 Promoter—The smallest *Er71* promoter fragment identified above to be responsive for activation by SRY spans the *Er71* promoter sequences from −85 to +51. If SRY directly up-regulates *Er71* transcription, SRY should bind within this region of the *Er71* promoter. Mouse SRY binds preferentially to sites of the sequence 5′-NACAAT-3′ (or reverse: 5′-ATTGTN-3′) (57) and inspection of the *Er71* promoter revealed one such site from −34 to −29 (Fig. 2*A*). Thus, we

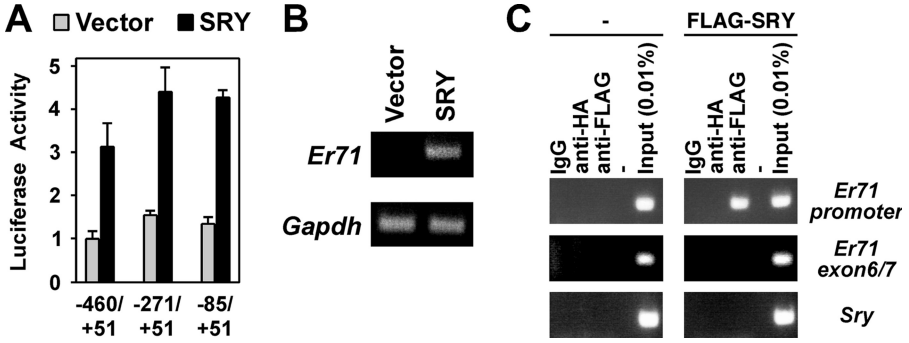

FIGURE 1. **Activation of the *Er71* promoter by SRY.** *A*, indicated *Er71* promoter fragments were cloned into the pGL2-Basic luciferase reporter plasmid and cotransfected with 1.5 μg of empty vector or mouse SRY expression vector into MLTC-1 cells. Resultant luciferase activities were measured. *B*, RT-PCR revealing *Er71* and *Gapdh* mRNA levels in MLTC-1 cells transfected with empty vector or 0.4 μg of mouse SRY expression vector. *C*, untransfected MLTC-1 cells or transfected with FLAG-SRY_{2-130} were subjected to ChIP assays. Immunoprecipitations were performed with control mouse IgG or mouse monoclonal anti-HA or anti-FLAG antibodies. DNA from the *Er71* promoter (−334 to +28), an *Er71* region ranging from exon 6 to exon 7, or the coding region of *Sry* was amplified by PCR. IgG and anti-HA immunoprecipitations served as negative controls.

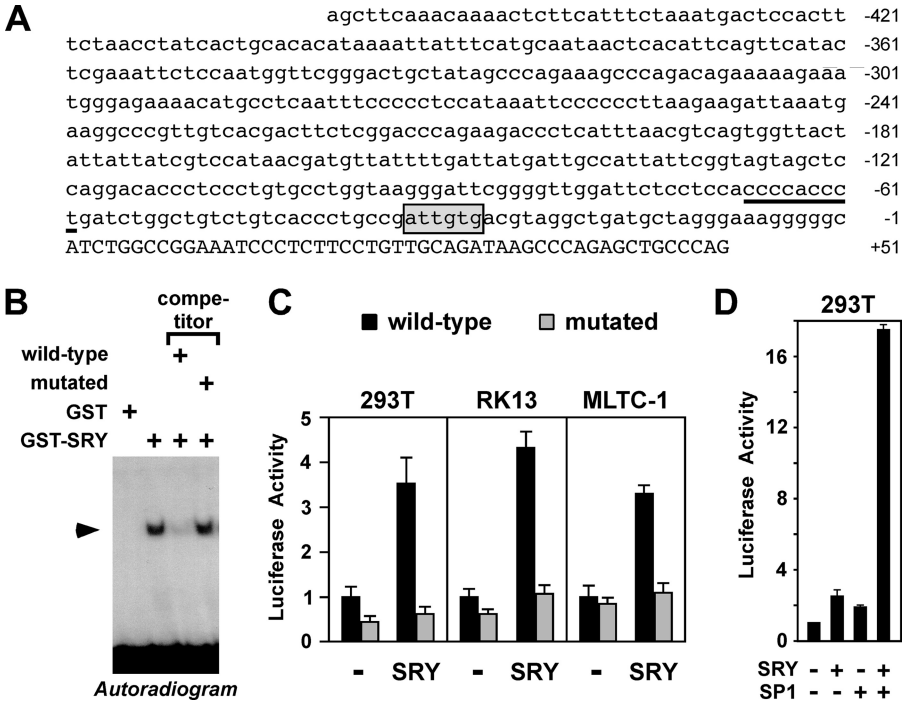

FIGURE 2. **Binding of SRY to the *Er71* promoter.** *A*, sequence of the mouse *Er71* promoter from −460 to +51. A potential SRY binding site (−34 to −29) is *boxed* and a previously identified SP1 binding site (−68 to −60) *underlined*. *B*, electrophoretic mobility shift assays with a probe spanning the putative SRY site (−29/−34) in the *Er71* promoter. GST or GST-SRY_{1-130} protein were incubated with ^{32}P-labeled probe and resultant DNA:protein complexes resolved by native polyacrylamide gel electrophoresis. Where indicated, a 20-fold excess of unlabeled wild-type probe or mutated at the SRY binding site (5′-ATTGTG-3′ to 5′-ATTACG-3′ at −34/-29) was utilized for competition. *C*, activation of wild-type or binding site-mutated −460/+51 *Er71* luciferase reporter by SRY in 293T, RK13 or MLTC-1 cells. Either 1 μg of wild-type or mutated (5′-ATTGTG-3′ to 5′-ATTACG-3′ at −34/−29) reporter plasmid was cotransfected with 1.5 μg of SRY plasmid. *D*, synergistic activation of the −460/+51 *Er71* luciferase reporter by SRY (375 ng expression vector) and SP1 (625 ng expression vector) in 293T cells.

tested in electrophoretic mobility shift assays whether SRY binds to this site. Indeed, bacterially expressed, mouse SRY formed a complex with a radiolabeled oligonucleotide spanning the −34/−29 site (Fig. 2*B*). This binding was specific, as this complex formation was suppressed by an excess of unlabeled oligonucleotide, but not by an unlabeled oligonucleotide in which the −34/−29 site was mutated. Thus, SRY can bind to the *Er71* promoter and may thereby regulate its activity.

To further support this hypothesis, we analyzed how mutating the −34/−29 site would affect the ability of SRY to activate the *Er71* promoter. In three different cell lines, mutation of the putative SRY site in the *Er71* promoter abolished its responsiveness to SRY (Fig. 2*C*), thus underscoring the importance of this SRY site for *Er71* promoter activity.

Previously, the *Er71* promoter was shown to be regulated by the ubiquitously expressed transcription factor SP1 (22). Thus, we also assessed whether SP1 and SRY might cooperate in inducing *Er71* transcription. To this end, we cotransfected suboptimal amounts of SP1 and SRY expression plasmids and indeed observed a synergistic stimulation of the *Er71* promoter by these two transcription factors (Fig. 2*D*).

Expression of Er71 in Developing Gonads—Sry transcription in the genital ridge of XY mice starts at 10.5 dpc, peaks at around 11.5 dpc and vanishes by 13 dpc (58–60). If *Er71* is a

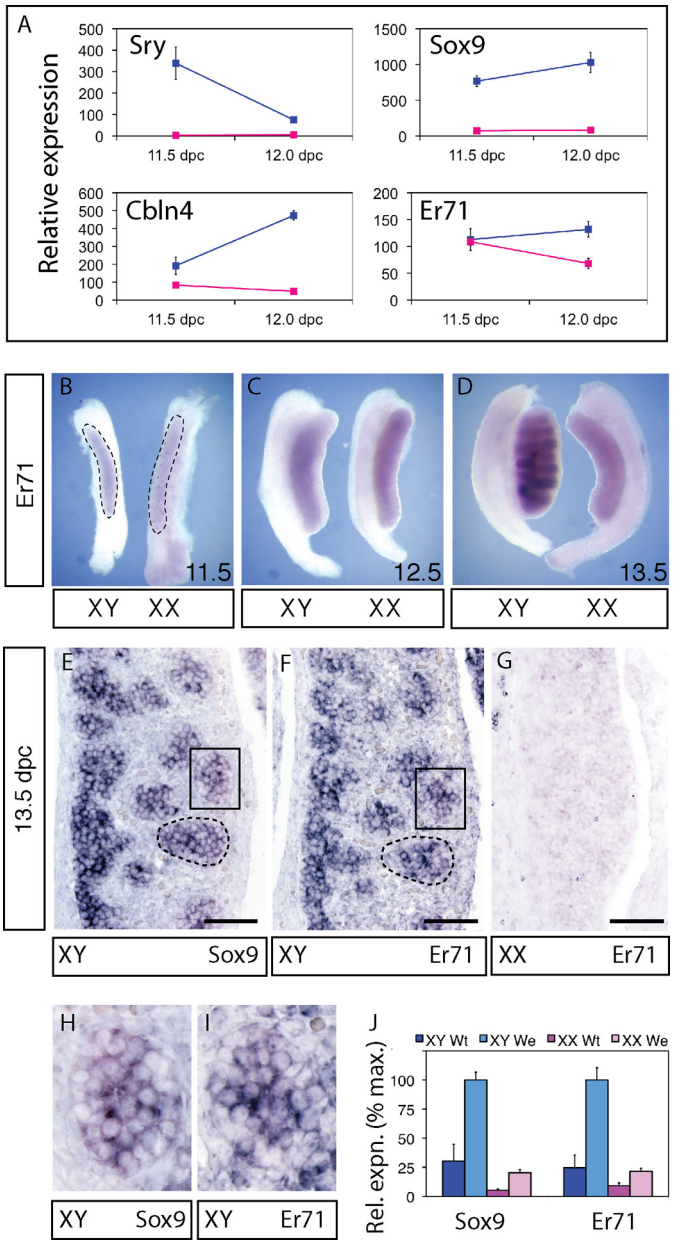

FIGURE 3. **Expression of *Er71* in 11. 5–13.5 dpc mouse gonads.** *A*, microarray expression data for *Sry*, *Sox9*, *Cbln4*, and *Er71* in gonad samples at 11.5 dpc and 12.0 dpc. XY samples are shown in *blue*, XX samples in *red*. Whole-mount *in situ* hybridization for *Er71* in developing testes and ovaries at (*B*) 11.5 dpc, (*C*) 12.5 dpc, and (*D*) 13.5 dpc. The extent of the gonad tissue within the gonad/mesonephros complex is outlined in *B*. Section *in situ* hybridization for *Sox9* (*E*) and *Er71* (*F*) in XY 13.5 dpc gonads and for *Er71* in XX 13.5 dpc gonads (*G*). Testis cords are outlined (*E*, *F*); scale bar, 100 μm. Areas marked with *rectangles* are shown at higher magnification (*H*, *I*). *J*, quantitative RT-PCR analysis of *Sox9* and *Er71* expression in wild-type (*wt*) and W^e gonads at 13.5 dpc.

true SRY target gene, its expression should closely follow that of *Sry*. We assessed the dynamics of *Er71* expression in fetal mouse gonads, in comparison to expression patterns of *Sry*, *Sox9* and *Cbln4* (*cerebellin 4 precursor*), a known target of both SRY and SOX9 (61). As expected, *Sry* expression was high at 11.5 dpc and was diminishing by 12.0 dpc in XY embryos (Fig. 3*A*). In contrast, expression of known SRY targets, *Sox9* and *Cbln4*, along with *Er71* increased from 11.5 dpc to 12.0 dpc. The similar profiles of *Er71*, *Sox9*, and *Cbln4* expression are consistent with all three genes being direct targets of SRY.

Next, we assessed the expression of *Er71* mRNA by *in situ* hybridization in genital ridges of 11.5–13.5 dpc embryos. *Er71* expression was present at low amounts in 11.5 dpc genital ridges of both XX and XY animals (Fig. 3*B*). By 12.5 dpc, the expression level of *Er71* had increased in both XX and XY animals, but we noted that *Er71* expression appeared to be more interior (*i.e.* within the developing gonads) in XY than in XX animals (Fig. 3*C*). By 13.5 dpc, there was a clear distinction between XY and XX animals, with *Er71* expression being much higher in XY than XX gonads (Fig. 3*D*). This result was corroborated by RT-PCR of 13.5 dpc male and female gonads (see wild-type in Fig. 3*J*). Section *in situ* hybridization at 13.5 dpc confirmed that *Er71* was male-specifically expressed and that, as was also the case for *Sox9*, expression was localized to testicular cords (Fig. 3, *E–I*). To confirm that *Er71* is expressed in somatic (Sertoli) cells of the cords rather than in the germ cells, we analyzed expression in W^e/W^e mutant gonads, which are devoid of germ cells (46). Like *Sox9*, *Er71* was more highly expressed in XY wild-type gonads than in XX wild-type gonads at 13.5 dpc (Fig. 3*J*). Further, *Er71* expression was increased rather than diminished in XY W^e/W^e mutants compared with XY wild-type samples, consistent with *Er71* expression being restricted to Sertoli cells. Together these data show that *Er71* is up-regulated in the Sertoli lineage shortly after SRY is expressed in XY gonads, consistent with *Er71* being an *in vivo* target gene of SRY.

Binding of ER71 to the Sox9 Promoter—*Sox9* expression is induced shortly after *Sry* in the genital ridges and remains high even after *Sry* transcription has ceased (62, 63). Since our studies identified *Er71* to have a similar expression profile as *Sox9*, we wondered whether ER71 could be involved in the regulation of the *Sox9* promoter. Indeed, inspection of the *Sox9* promoter revealed seven putative ETS core binding sites that might be bound by ER71 (Fig. 4*A*). Therefore, we systematically investigated by gel electrophoretic mobility shift assays whether any of these sites could be bound by mouse ER71 *in vitro*. As shown in Fig. 4*B*, an oligonucleotide spanning both ETS sites at −111/−114 and −118/−121 was bound by ER71, but oligonucleotides spanning the other five putative ETS sites at −5/−8, −135/−138, −204/−207, −292/−295, or −308/−311 did not interact with ER71. Binding to the −111/−114&−118/−121 oligonucleotide was specific, since (i) simultaneous incubation with anti-ER71 antibodies but not control anti-hTERT antibodies resulted in supershifts (Fig. 4*C*) and (ii) competition with unlabeled −111/−114&−118/−121 oligonucleotide but not when both ETS sites were mutated resulted in suppression of mobility shift. Moreover, when mutating either the ETS site −111/−114 or the ETS site −118/−121 alone (Fig. 4*C*), we observed that only unlabeled oligonucleotide mutated at the −111/−114 site lost the ability to suppress mobility shift, indicating that ER71 binds to the −111/−114 site.

Activation of Sox9 Transcription by ER71—The fact that ER71 can bind to the *Sox9* promoter prompted us to test whether ER71 might stimulate *Sox9* transcription. To this end, we first assessed whether mouse ER71 activates a *Sox9* promoter fragment spanning sequences from −453 to +13.

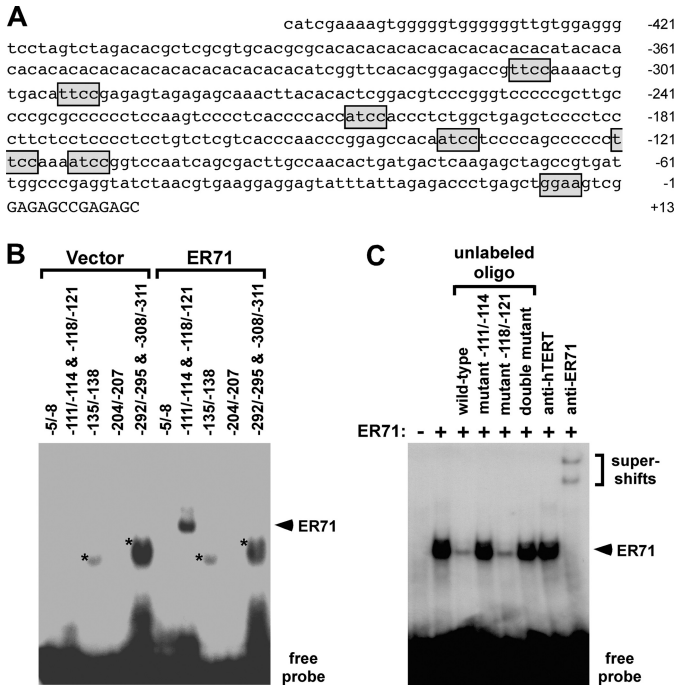

FIGURE 4. **Interaction of ER71 with the *Sox9* promoter.** *A*, sequence of the mouse *Sox9* promoter from −453 to +13. Sequences matching the ETS core sequence (5′-GGA$^{A}/_{T}$-3′, or 5′-$^{T}/_{A}$TCC-3′ in reverse) are *boxed*. *B*, electrophoretic mobility shift assays with indicated ^{32}P-labeled probes. Either *in vitro* transcribed/translated ER71 or vector control was employed for the binding reactions. *Asterisks* mark unspecific complexes, whereas the *arrowhead* points to an ER71:DNA complex. *C*, −111/−114&−118/−121 radiolabeled probe was incubated with ER71 protein and, as indicated, with a 20-fold excess of unlabeled oligonucleotide, in which the ETS sites at −111/−114 and/or −118/−121 were mutated. In the last two lanes, either antibodies directed against hTERT or ER71 were included in the binding reaction.

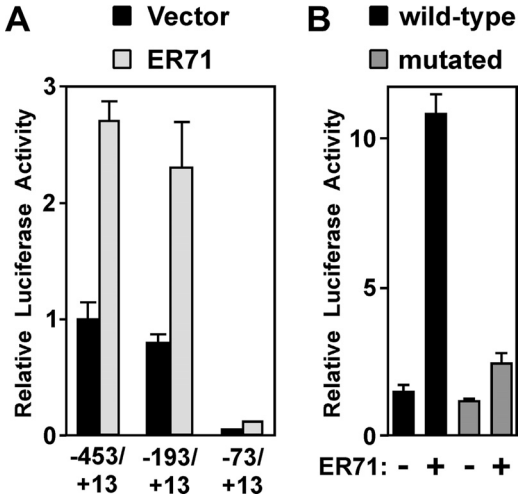

FIGURE 5. **Stimulation of the *Sox9* promoter by ER71.** *A*, indicated *Sox9* promoter-luciferase constructs were cotransfected with 4 μg of ER71 expression construct or control vector into MLTC-1 cells. *B*, −453/+13 *Sox9* luciferase reporter plasmid (wild-type or mutated at the ETS site −111/−114) was cotransfected with ER71 expression vector into Mv1Lu cells. Resultant luciferase activities are shown.

Indeed, ER71 did so in two different cell lines tested (MLTC-1 and Mv1Lu; Fig. 5, *A* and *B*). Moreover, deletion of 260 bp of 5′-terminal base pairs (−193/+13 construct) did not compromise the ability of ER71 to stimulate the *Sox9* promoter, whereas further deletion of 120 base pairs (−73/+13 construct) resulted in transcriptional inactivity (Fig. 5*A*). This is consistent with a previous report suggesting that *Sox9* promoter activity in the gonads is dependent on sequences between −193 and −73 (64), and accordingly we have above identified ER71 to bind an ETS site (−111/−114) within this region (see Fig. 4). Next, we mutated the −111/−114 ETS site to determine whether this would compromise the ability of ER71 to activate the *Sox9* promoter (Fig. 5*B*). Indeed, mutation of the −111/−114 ETS site greatly diminished the ability of ER71 to activate the −453/+13 *Sox9* promoter, proving the functionality of the −111/−114 ETS site and corroborating that *Sox9* is a potential target gene of ER71.

ER71 Is Involved in Endogenous Sox9 Regulation—Next, we asked whether ER71 may also regulate the endogenous *Sox9* gene promoter. First, we determined whether ER71 binds to the *Sox9* promoter. To this end, we employed a mouse Sertoli cell line (15P-1) that robustly expresses *Sox9*. We then transfected 15P-1 cells with either the empty control vector (pEV3S) or a FLAG-tagged ER71 expression vector. Thereafter, cells were subjected to ChIP assays. When the control vector pEV3S was transfected, no *Sox9* promoter DNA was immunoprecipitated with anti-FLAG antibodies (Fig. 6*A*, *left panel*). However, when equivalent amounts, as evidenced by comparable input levels (see Fig. 6*A*, *right panel*), of chromatin were immunoprecipitated from $FLAG_3$-ER71-transfected cells, *Sox9* promoter DNA was immunoprecipitated with anti-FLAG antibodies but not with anti-HA or no antibody (Fig. 6*A*, *middle panel*). These results indicate that ER71 is capable of binding to the *Sox9* promoter.

As a positive control for our ChIP assay, we also assessed whether ER71 would bind to another previously reported target gene, *Flk1* (27). Indeed, ER71 expectedly interacted with the *Flk1* promoter (Fig. 6*B*). Furthermore, we performed negative controls by assessing whether ER71 would bind to two different regions in the *Sox9* 3′-UTR or within the coding region of *Sry*. No binding of ER71 to these genomic regions was observable (Fig. 6*C*), attesting to the specificity of our ChIP assay.

Thereafter, we assessed if ER71 can stimulate *Sox9* transcription in 15P-1 Sertoli cells. Overexpression of ER71 did not result in changes of *Sox9* mRNA (data not shown), possibly because *Sox9* transcription may already be maximally induced in 15P-1 cells by endogenous ER71. Therefore, we took a dominant-negative approach expressing ER71-C, a molecule that consists of the last 129 amino acids of ER71 encompassing its DNA binding domain but lacking any transactivation domain. As previously shown (26), ER71-C can still bind to ER71 target sites and thereby prevent any productive transcriptional activation mediated by full-length endogenous ER71. Accordingly, we observed that ER71-C led to a down-regulation of *Sox9* transcription in 15P-1 cells (Fig. 6*D*), corroborating that ER71 can target the *Sox9* promoter.

SOX9 as a Regulator of Er71 Transcription—SOX9 and SRY have similar DNA binding preferences (1), prompting us to investigate whether SOX9 may regulate *Er71* expression similar to SRY. To test this, we first performed ChIP assays in 15P-1 Sertoli cells (Fig. 7*A*). Indeed, SOX9 occupied the *Er71* promoter *in vivo*, but not a region ~2.5 kbp downstream ranging

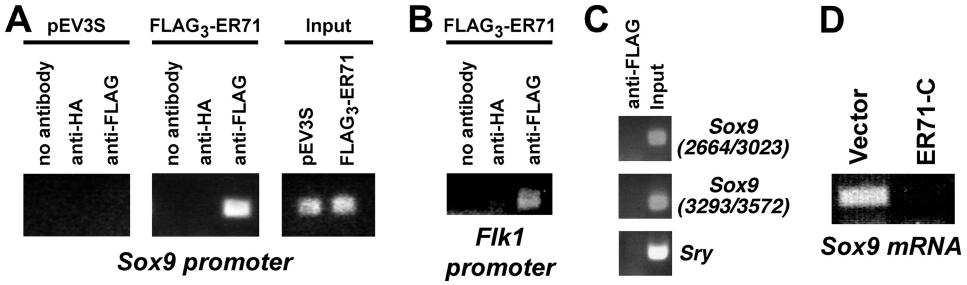

FIGURE 6. **ER71 targets the endogenous *Sox9* promoter.** *A*, ChIP assays testing the binding of ER71 to the *Sox9* promoter. As indicated, the vector control pEV3S or $FLAG_3$-ER71 expression vector were transfected into 15P-1 cells and immunoprecipitations performed with no, anti-HA, or anti-FLAG antibodies. 0.04% of the input for each reaction was analyzed (*right panel*) as a control. *B*, corresponding ChIP assay to assess binding of ER71 to the *Flk1* promoter. *C*, negative controls showing absence of ER71 binding to two regions (2664–3023 and 3293–3572) within the 3′-UTR of *Sox9* and the coding region of *Sry*. *D*, equal amounts of RNA from 15P-1 cells transfected with vector control or ER71-C were subjected to RT-PCR to determine *Sox9* mRNA levels.

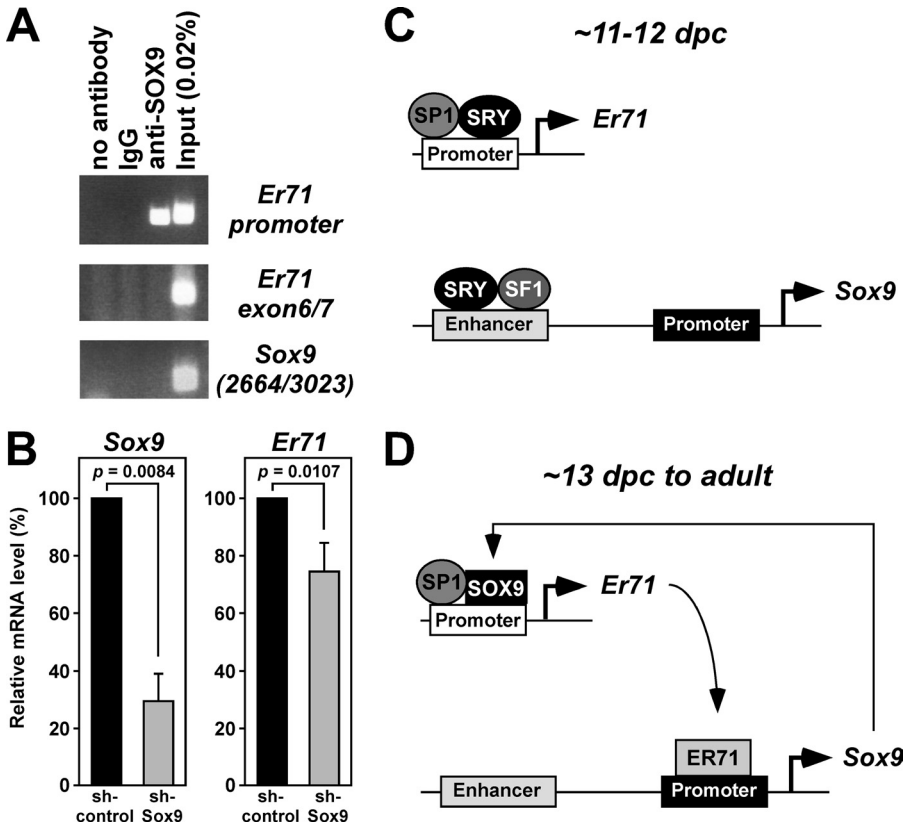

FIGURE 7. **SOX9-mediated regulation of *Er71* transcription and models for the role of ER71 in male gonads.** *A*, ChIP assay in 15P-1 cells. Immunoprecipitations were performed with anti-SOX9 antibodies (Abcam ab3697), control rabbit IgG or no antibody. DNA from the *Er71* promoter (−334 to +28), an *Er71* region ranging from exon 6 to exon 7, or the 3′-UTR of *Sox9* (2664–3023) was PCR amplified. *B*, down-regulation of *Sox9* with shRNA in 15P-1 cells. Shown are levels of *Sox9* and *Er71* mRNA. Statistical significance was determined with Student's *t* test. *C*, during gonadal differentiation, *Er71* transcription is up-regulated by SRY in parallel to *Sox9* gene expression. *D*, after SRY expression has subsided, ER71 and SOX9 mutually sustain their expression.

from *Er71* exon 6 to exon 7; neither did SOX9 bind to the *Sox9* 3′-UTR.

Finally, we tested whether SOX9 may not only bind to the *Er71* promoter, but also regulate its activity. To this end, we down-regulated *Sox9* with shRNA in 15P-1 cells and found as a consequence a 70% reduction of *Sox9* mRNA (*left panel*, Fig. 7*B*). Simultaneously, a small, yet significant reduction of *Er71* mRNA levels was observed (*right panel*, Fig. 7*B*). These data suggest that SOX9 is capable of activating *Er71* transcription.

DISCUSSION

Our present data suggest that *Er71* is a *bona fide* SRY target gene as evidenced by the following facts: (i) SRY can bind to the *Er71* promoter *in vitro* and *in vivo*; (ii) SRY activates the *Er71* promoter and mutation of the −29 to −34 SRY binding site compromises this activation; (iii) ectopic expression of SRY leads to the up-regulation of endogenous *Er71* transcription; and (iv) *Er71* mRNA levels are enhanced in male gonads shortly after *Sry* is up-regulated to trigger male differentiation of the bipotential gonads. Thus, our study has not only identified a novel direct SRY target gene but also for the first time implicates ER71 as a potential player in sex determination and gonadal development.

The fact that *Er71* is also expressed in the absence of any SRY in the XX gonads, albeit at eventually lower levels than in XY gonads, indicates that SRY is not absolutely required for *Er71*

transcription. Possibly SP1, a ubiquitously expressed zinc finger transcription factor (65, 66) that we have previously shown to bind to and activate the *Er71* promoter (22), may be responsible for the low levels of ER71 expression in XX gonads. Interestingly, SP1 is also required for efficient SRY expression during sexual differentiation (67, 68), emphasizing the important function of this transcription factor in the developing gonad. Therefore, SP1 and SRY may indeed collaborate to induce common target genes, and our data indicate that *Er71* is such a target gene since its promoter was synergistically activated by SP1 and SRY.

Er71 mRNA levels are slightly up-regulated between 11.5 and 12.0 dpc in XY genital ridges and drastically increased at 13.5 dpc, whereas *Sry* transcription starts at 10.5 dpc, peaks at around 11.5 dpc and has subsided again by 12.5 dpc (58–60), indicating that *Er71* transcription trails behind *Sry* transcription by about 1 day. This expression pattern of *Er71* mRNA is consistent with *Er71* being a target gene of SRY *in vivo*. Moreover, the *Er71* expression pattern is reminiscent of that of *Sox9*: before overt sexual differentiation at 10.5 dpc, *Sox9* expression is indiscriminate between XX and XY animals, but becomes elevated in XY and reduced in XX gonads after *Sry* induction (62, 63). This may suggest that *Er71* and *Sox9* are jointly up-regulated by SRY (Fig. 7*C*), with SRY cooperating with SP1 in upregulating the *Er71* promoter and SRY and SF1 synergistically activating a gonad-specific *Sox9* enhancer located ~10 kb upstream of the transcription start site (20).

Sox9 expression persists in the absence of SRY past 13 dpc in the mouse embryo and is present in Sertoli cells of the adult testes (1, 4). Similarly, *Er71* is also expressed in Sertoli cells (22), suggesting that *Er71* and *Sox9* might be coregulated. It has been hypothesized that the continued expression of *Sox9* is due to an autoregulatory loop (2, 5). Alternatively, ER71 and SOX9 may act mutually in a positive feedback loop, with ER71 activating the *Sox9* promoter, and SOX9 in turn activating the *Er71* gene (Fig. 7*D*). In support of this model, our data show that ER71 can interact with the *Sox9* promoter *in vitro* and *in vivo* and stimulate its activity, whereas the dominant-negative ER71-C molecule caused down-regulation of *Sox9* transcription. Conversely, SOX9 occupied the *Er71* promoter *in vivo* and *Sox9* down-regulation resulted in a concomitant reduction of *Er71* transcription. Possibly like SRY, SOX9 might cooperate with the ubiquitously expressed SP1 transcription factor in stimulating the *Er71* promoter.

Clearly, such models are in need of testing, and the construction of transgenic mice overexpressing ER71 in the genital ridge or appropriate tissue-specific knock-outs will provide us with a more definite answer to ER71's role in male sex determination. Similarly, the role of ER71 in Sertoli cells of adult testes should be investigated utilizing mouse models. Notably, JMJD1A/TSGA/JHDM2A, a histone 3 lysine 9 demethylase (69), interacts with ER71 (70) and knocking-out this cofactor leads to defective spermatogenesis and infertility in male mice (71, 72), suggesting that ER71 and JMJD1A/TSGA/JHDM2A may cooperate in the physiological function of adult testes.

Apart from *Sox9* transcriptional regulation, it is possible that ER71 affects male embryonic gonads and testicular function in adults by other mechanisms. Previously, we showed that ER71 activates transcription from the matrix metalloproteinase-1 promoter (26). It is likely that other matrix metalloproteinase genes are also induced by ER71, since many proteinase genes are transcriptionally controlled by ETS binding sites (24). Matrix metalloproteinases play an important role in early testicular development, including mesonephric cell migration (1), and also during spermatogenesis (73), indicating that ER71 may pleiotropically affect these processes through regulating the expression of one or more matrix metalloproteinases.

In conclusion, our study provides evidence that *Er71* is a novel SRY target gene in the male developing gonad and that ER71 and SOX9 regulate each other's gene transcription. Thus, ER71 is strongly implicated in SRY-triggered male sex determination and to be a key regulator of adult testicular function in conjunction with SOX9.

Acknowledgment—We thank Chun-Wei Feng for technical assistance.

REFERENCES

1. Wilhelm, D., Palmer, S., and Koopman, P. (2007) Sex determination and gonadal development in mammals. *Physiol. Rev.* **87,** 1–28
2. Sekido, R., and Lovell-Badge, R. (2009) Sex determination and SRY: down to a wink and a nudge? *Trends Genet.* **25,** 19–29
3. Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1991) Male development of chromosomally female mice transgenic for Sry. *Nature* **351,** 117–121
4. Harley, V. R., Clarkson, M. J., and Argentaro, A. (2003) The molecular action and regulation of the testis-determining factors, SRY (sex-determining region on the Y chromosome) and SOX9 (SRY-related high-mobility group (HMG) box 9). *Endocr. Rev.* **24,** 466–487
5. Polanco, J. C., and Koopman, P. (2007) Sry and the hesitant beginnings of male development. *Dev. Biol.* **302,** 13–24
6. Foster, J. W., Dominguez-Steglich, M. A., Guioli, S., Kwok, G., Weller, P. A., Stevanovi, M., Weissenbach, J., Mansour, S., Young, I. D., Goodfellow, P. N., Brook, J. D., and Schafer, A. J. (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* **372,** 525–530
7. Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., Pasantes, J., Bricarelli, F. D., Keutel, J., Hustert, E., Wolf, U., Tommerup, N., Schempp, W., and Scherer, G. (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* **79,** 1111–1120
8. Wright, E., Hargrave, M. R., Christiansen, J., Cooper, L., Kun, J., Evans, T., Gangadharan, U., Greenfield, A., and Koopman, P. (1995) The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. *Nat. Genet.* **9,** 15–20
9. Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., and de Crombrugghe, B. (1999) Sox9 is required for cartilage formation. *Nat. Genet.* **22,** 85–89
10. Bi, W., Huang, W., Whitworth, D. J., Deng, J. M., Zhang, Z., Behringer, R. R., and de Crombrugghe, B. (2001) Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 6698–6703
11. Schafer, A. J., Foster, J. W., Kwok, C., Weller, P. A., Guioli, S., and Goodfellow, P. N. (1996) Campomelic dysplasia with XY sex reversal: diverse phenotypes resulting from mutations in a single gene. *Ann. N.Y. Acad. Sci.* **785,** 137–149
12. Bishop, C. E., Whitworth, D. J., Qin, Y., Agoulnik, A. I., Agoulnik, I. U., Harrison, W. R., Behringer, R. R., and Overbeek, P. A. (2000) A transgenic insertion upstream of sox9 is associated with dominant XX sex reversal in the mouse. *Nat. Genet.* **26,** 490–494
13. Vidal, V. P., Chaboissier, M. C., de Rooij, D. G., and Schedl, A. (2001) Sox9 induces testis development in XX transgenic mice. *Nat. Genet.* **28,** 216–217

14. Qin, Y., and Bishop, C. E. (2005) Sox9 is sufficient for functional testis development producing fertile male mice in the absence of Sry. *Hum. Mol. Genet.* **14,** 1221–1229
15. Huang, B., Wang, S., Ning, Y., Lamb, A. N., and Bartley, J. (1999) Autosomal XX sex reversal caused by duplication of SOX9. *Am. J. Med. Genet.* **87,** 349–353
16. Chaboissier, M. C., Kobayashi, A., Vidal, V. I., Lützkendorf, S., van de Kant, H. J., Wegner, M., de Rooij, D. G., Behringer, R. R., and Schedl, A. (2004) Functional analysis of Sox8 and Sox9 during sex determination in the mouse. *Development* **131,** 1891–1901
17. Barrionuevo, F., Bagheri-Fam, S., Klattig, J., Kist, R., Taketo, M. M., Englert, C., and Scherer, G. (2006) Homozygous inactivation of Sox9 causes complete XY sex reversal in mice. *Biol. Reprod.* **74,** 195–201
18. De Santa Barbara, P., Bonneaud, N., Boizet, B., Desclozeaux, M., Moniot, B., Sudbeck, P., Scherer, G., Poulat, F., and Berta, P. (1998) Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Müllerian hormone gene. *Mol. Cell. Biol.* **18,** 6653–6665
19. Arango, N. A., Lovell-Badge, R., and Behringer, R. R. (1999) Targeted mutagenesis of the endogenous mouse Mis gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. *Cell* **99,** 409–419
20. Sekido, R., and Lovell-Badge, R. (2008) Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. *Nature* **453,** 930–934
21. Brown, T. A., and McKnight, S. L. (1992) Specificities of protein-protein and protein-DNA interaction of GABPα and two newly defined Ets-related proteins. *Genes Dev.* **6,** 2502–2512
22. De Haro, L., and Janknecht, R. (2005) Cloning of the murine ER71 gene (Etsrp71) and initial characterization of its promoter. *Genomics* **85,** 493–502
23. Janknecht, R., and Nordheim, A. (1993) Gene regulation by Ets proteins. *Biochim. Biophys. Acta* **1155,** 346–356
24. Oikawa, T., and Yamada, T. (2003) Molecular biology of the Ets family of transcription factors. *Gene* **303,** 11–34
25. Hsu, T., Trojanowska, M., and Watson, D. K. (2004) Ets proteins in biological control and cancer. *J. Cell. Biochem.* **91,** 896–903
26. De Haro, L., and Janknecht, R. (2002) Functional analysis of the transcription factor ER71 and its activation of the matrix metalloproteinase-1 promoter. *Nucleic Acids Res.* **30,** 2972–2979
27. Lee, D., Park, C., Lee, H., Lugus, J. J., Kim, S. H., Arentson, E., Chung, Y. S., Gomez, G., Kyba, M., Lin, S., Janknecht, R., Lim, D. S., and Choi, K. (2008) ER71 acts downstream of BMP, Notch, and Wnt signaling in blood and vessel progenitor specification. *Cell Stem Cell* **2,** 497–507
28. Sumanas, S., Gomez, G., Zhao, Y., Park, C., Choi, K., and Lin, S. (2008) Interplay among Etsrp/ER71, Scl, and Alk8 signaling controls endothelial and myeloid cell formation. *Blood* **111,** 4500–4510
29. Ferdous, A., Caprioli, A., Iacovino, M., Martin, C. M., Morris, J., Richardson, J. A., Latif, S., Hammer, R. E., Harvey, R. P., Olson, E. N., Kyba, M., and Garry, D. J. (2009) Nkx2–5 transactivates the Ets-related protein 71 gene and specifies an endothelial/endocardial fate in the developing embryo. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 814–819
30. Rossow, K. L., and Janknecht, R. (2001) The Ewing's sarcoma gene product functions as a transcriptional activator. *Cancer Res.* **61,** 2690–2695
31. Shin, S., and Janknecht, R. (2007) Diversity within the JMJD2 histone demethylase family. *Biochem. Biophys. Res. Commun.* **353,** 973–977
32. Mooney, S. M., Grande, J. P., Salisbury, J. L., and Janknecht, R. (2010) Sumoylation of p68 and p72 RNA helicases affects protein stability and transactivation potential. *Biochemistry* **49,** 1–10
33. Janknecht, R. (2003) Regulation of the ER81 transcription factor and its coactivators by mitogen- and stress-activated protein kinase 1 (MSK1). *Oncogene* **22,** 746–755
34. Shin, S., Rossow, K. L., Grande, J. P., and Janknecht, R. (2007) Involvement of RNA helicases p68 and p72 in colon cancer. *Cancer Res.* **67,** 7572–7578
35. Kim, T. D., Shin, S., and Janknecht, R. (2008) Repression of Smad3 activity by histone demethylase SMCX/JARID1C. *Biochem. Biophys. Res. Commun.* **366,** 563–567
36. Mooney, S. M., Goel, A., D'Assoro, A. B., Salisbury, J. L., and Janknecht, R. (2010) Pleiotropic effects of p300-mediated acetylation on p68 and p72 RNA helicase. *J. Biol. Chem.* **285,** 30443–30452
37. Kim, J., Shin, S., Subramaniam, M., Bruinsma, E., Kim, T. D., Hawse, J. R., Spelsberg, T. C., and Janknecht, R. (2010) Histone demethylase JARID1B/KDM5B is a corepressor of TIEG1/KLF10. *Biochem. Biophys. Res. Commun.* **401,** 412–416
38. Bosc, D. G., Goueli, B. S., and Janknecht, R. (2001) HER2/Neu-mediated activation of the ETS transcription factor ER81 and its target gene MMP-1. *Oncogene* **20,** 6215–6224
39. Wu, J., and Janknecht, R. (2002) Regulation of the ETS transcription factor ER81 by the 90-kDa ribosomal S6 kinase 1 and protein kinase A. *J. Biol. Chem.* **277,** 42669–42679
40. Dowdy, S. C., Mariani, A., and Janknecht, R. (2003) HER2/Neu- and TAK1-mediated up-regulation of the transforming growth factor β inhibitor Smad7 via the ETS protein ER81. *J. Biol. Chem.* **278,** 44377–44384
41. Papoutsopoulou, S., and Janknecht, R. (2000) Phosphorylation of ETS transcription factor ER81 in a complex with its coactivators CREB-binding protein and p300. *Mol. Cell. Biol.* **20,** 7300–7310
42. Shin, S., Kim, T. D., Jin, F., van Deursen, J. M., Dehm, S. M., Tindall, D. J., Grande, J. P., Munz, J. M., Vasmatzis, G., and Janknecht, R. (2009) Induction of prostatic intraepithelial neoplasia and modulation of androgen receptor by ETS variant 1/ETS-related protein 81. *Cancer Res.* **69,** 8102–8110
43. Goel, A., and Janknecht, R. (2004) Concerted activation of ETS protein ER81 by p160 coactivators, the acetyltransferase p300 and the receptor tyrosine kinase HER2/Neu. *J. Biol. Chem.* **279,** 14909–14916
44. Shin, S., and Janknecht, R. (2007) Activation of androgen receptor by histone demethylases JMJD2A and JMJD2D. *Biochem. Biophys. Res. Commun.* **359,** 742–746
45. Goel, A., and Janknecht, R. (2003) Acetylation-mediated transcriptional activation of the ETS protein ER81 by p300, P/CAF, and HER2/Neu. *Mol. Cell. Biol.* **23,** 6243–6254
46. Buehr, M., and McLaren, A. (1993) Isolation and culture of primordial germ cells. *Methods Enzymol.* **225,** 58–77
47. Koopman, P., Gubbay, J., Collignon, J., and Lovell-Badge, R. (1989) Zfy gene expression patterns are not compatible with a primary role in mouse sex determination. *Nature* **342,** 940–942
48. Hargrave, M., Bowles, J., and Koopman, P. (2006) *In situ* hybridization of whole-mount embryos. *Methods Mol. Biol.* **326,** 103–113
49. Wilkinson, D. G., and Nieto, M. A. (1993) Detection of messenger RNA by *in situ* hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225,** 361–373
50. Holt, J. E., Jackson, A., Roman, S. D., Aitken, R. J., Koopman, P., and McLaughlin, E. A. (2006) CXCR4/SDF1 interaction inhibits the primordial to primary follicle transition in the neonatal mouse ovary. *Dev. Biol.* **293,** 449–460
51. Bowles, J., Feng, C. W., Knight, D., Smith, C. A., Roeszler, K. N., Bagheri-Fam, S., Harley, V. R., Sinclair, A. H., and Koopman, P. (2009) Male-specific expression of Aldh1a1 in mouse and chicken fetal testes: implications for retinoid balance in gonad development. *Dev. Dyn.* **238,** 2073–2080
52. Goueli, B. S., and Janknecht, R. (2003) Regulation of telomerase reverse transcriptase gene activity by upstream stimulatory factor. *Oncogene* **22,** 8042–8047
53. Shin, S., and Janknecht, R. (2007) Concerted activation of the Mdm2 promoter by p72 RNA helicase and the coactivators p300 and P/CAF. *J. Cell. Biochem.* **101,** 1252–1265
54. Goueli, B. S., and Janknecht, R. (2004) Upregulation of the catalytic telomerase subunit by the transcription factor ER81 and oncogenic HER2/Neu, Ras, or Raf. *Mol. Cell. Biol.* **24,** 25–35
55. Kim, T. D., Shin, S., Berry, W. L., Oh, S., and Janknecht, R. (2012) The JMJD2A demethylase regulates apoptosis and proliferation in colon cancer cells. *J. Cell. Biochem.* **113,** 1368–1376
56. Shin, S., Bosc, D. G., Ingle, J. N., Spelsberg, T. C., and Janknecht, R. (2008) Rcl is a novel ETV1/ER81 target gene upregulated in breast tumors. *J. Cell. Biochem.* **105,** 866–874
57. Dubin, R. A., and Ostrer, H. (1994) Sry is a transcriptional activator. *Mol. Endocrinol.* **8,** 1182–1192

58. Koopman, P., Münsterberg, A., Capel, B., Vivian, N., and Lovell-Badge, R. (1990) Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature* **348,** 450–452
59. Hacker, A., Capel, B., Goodfellow, P., and Lovell-Badge, R. (1995) Expression of Sry, the mouse sex determining gene. *Development* **121,** 1603–1614
60. Jeske, Y. W., Mishina, Y., Cohen, D. R., Behringer, R. R., and Koopman, P. (1996) Analysis of the role of Amh and Fra1 in the Sry regulatory pathway. *Mol. Reprod. Dev.* **44,** 153–158
61. Bradford, S. T., Wilhelm, D., Bandiera, R., Vidal, V., Schedl, A., and Koopman, P. (2009) A cell-autonomous role for WT1 in regulating Sry *in vivo*. *Hum. Mol. Genet.* **18,** 3429–3438
62. Kent, J., Wheatley, S. C., Andrews, J. E., Sinclair, A. H., and Koopman, P. (1996) A male-specific role for SOX9 in vertebrate sex determination. *Development* **122,** 2813–2822
63. Morais da Silva, S., Hacker, A., Harley, V., Goodfellow, P., Swain, A., and Lovell-Badge, R. (1996) Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. *Nat. Genet.* **14,** 62–68
64. Kanai, Y., and Koopman, P. (1999) Structural and functional characterization of the mouse Sox9 promoter: implications for campomelic dysplasia. *Hum. Mol. Genet.* **8,** 691–696
65. Bouwman, P., and Philipsen, S. (2002) Regulation of the activity of Sp1-related transcription factors. *Mol. Cell. Endocrinol.* **195,** 27–38
66. Safe, S., and Abdelrahim, M. (2005) Sp transcription factor family and its role in cancer. *Eur. J. Cancer* **41,** 2438–2448
67. Desclozeaux, M., Poulat, F., de Santa Barbara, P., Soullier, S., Jay, P., Berta, P., and Boizet-Bonhoure, B. (1998) Characterization of two Sp1 binding sites of the human sex determining SRY promoter. *Biochim. Biophys. Acta* **1397,** 247–252
68. Assumpção, J. G., Ferraz, L. F., Benedetti, C. E., Maciel-Guerra, A. T., Guerra, G., Jr., Marques-de-Faria, A. P., Baptista, M. T., and de Mello, M. P. (2005) A naturally occurring deletion in the SRY promoter region affecting the Sp1 binding site is associated with sex reversal. *J. Endocrinol. Invest.* **28,** 651–656
69. Yamane, K., Toumazou, C., Tsukada, Y., Erdjument-Bromage, H., Tempst, P., Wong, J., and Zhang, Y. (2006) JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. *Cell* **125,** 483–495
70. Knebel, J., De Haro, L., and Janknecht, R. (2006) Repression of transcription by TSGA/Jmjd1a, a novel interaction partner of the ETS protein ER71. *J. Cell. Biochem.* **99,** 319–329
71. Okada, Y., Scott, G., Ray, M. K., Mishina, Y., and Zhang, Y. (2007) Histone demethylase JHDM2A is critical for Tnp1 and Prm1 transcription and spermatogenesis. *Nature* **450,** 119–123
72. Liu, Z., Zhou, S., Liao, L., Chen, X., Meistrich, M., and Xu, J. (2010) Jmjd1a demethylase-regulated histone modification is essential for cAMP-response element modulator-regulated gene expression and spermatogenesis. *J. Biol. Chem.* **285,** 2758–2770
73. Monsees, T. K., Schill, W. B., and Miska, W. (1997) Protease-protease inhibitor interactions in Sertoli cell-germ cell crosstalk. *Adv. Exp. Med. Biol.* **424,** 111–123