Mastermind-like Domain-containing 1 (MAMLD1 or CXorf6) Transactivates the Hes3 Promoter, Augments Testosterone Production, and Contains the SF1 Target Sequence*

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Although chromosome X open reading frame 6 (CXorf6) has been shown to be a causative gene for hypospadias, its molecular function remains unknown. To clarify this, we first examined CXorf6 protein structure, identifying homology to mastermind-like 2 (MAML2) protein, which functions as a co-activator in canonical Notch signaling. Transactivation analysis for wild-type CXorf6 protein by luciferase assays showed that CXorf6 significantly transactivated the promoter of a noncanonical Notch target gene hairy/enhancer of split 3 (Hes3) without demonstrable DNA-binding capacity. Transactivation analysis was also performed for the previously described three apparently pathologic nonsense mutations, indicating that E124X and Q197X proteins had no transactivation function, whereas R653X protein retained a nearly normal transactivation function. Subcellular localization analysis revealed that wild-type and R653X proteins co-localized with MAML2 protein in nuclear bodies, whereas E124X and Q197X proteins were incapable of localizing to nuclear bodies. Thus, further studies were performed for R653X, revealing the occurrence of nonsense mediated mRNA decay in vivo. Next, transient knockdown of CXorf6 was performed using small interfering RNA, showing reduced testosterone production in mouse Leydig tumor cells. Furthermore, steroidogenic factor 1 (SF1) protein bound to a specific sequence in the upstream of the CXorf6 coding region and exerted a transactivation activity. These results suggest that CXorf6 transactivates the Hes3 promoter, augments testosterone production, and contains the SF1 target sequence, thereby providing the first clue to clarify the biological role of CXorf6. We designate CXorf6 as MAMLD1 (mastermind-like domain-containing 1) based on its characteristic structure.

Chromosome X open reading frame 6 (CXorf6) was identified by Laporte et al. (1, 2) and as a candidate gene for 46,XY disorders of sex development. It spans ~70 kb in genomic sequence and comprises at least seven exons. An open reading frame resides on exons 3–6 and produces two proteins of 701 and 660 amino acids because of in-frame alternative splicing with and without exon 4. PCR-based human cDNA library screening has revealed ubiquitous expression of both splice variants, with the exon 4 positive variant being the major form (3). To date, however, the products of CXorf6 have been poorly characterized, although glutamine- and proline-rich domains have been identified on exon 3 (1). We have recently shown that CXorf6 is a causative gene for hypospadias (3), a common male external genital anomaly defined by the urethral opening on the ventral side of the penis and classified into several types on the basis of the anatomical location of the urethral meatus (4). This notion is based primarily on the identification of nonsense mutations in two maternally related half-brothers (E124X) and in two sporadic boys (Q197X and R653X) with penoscrotal hypospadias (3). Because the mouse homolog (G630014P10Rik, NM_001081354) is transcribed in fetal Sertoli and Leydig cells around the critical period for sex development, it is likely that the CXorf6 mutations cause hypospadias primarily because of testicular dysfunction and the resultant compromised testosterone production around that period (3). Indeed, although various genetic and environmental factors have been implicated in the development of hypospadias, it has been widely accepted that hypospadias can be caused by impaired testosterone effects around the critical period for sex development (4). Furthermore, the mouse homolog is co-expressed with steroidogenic factor 1 (SF1; aliases, AD4BP and NR5A1) (3), which regulates the transcription of a vast array of genes involved in sex develop-

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2 The abbreviations used are: CXorf6, chromosome X open reading frame 6; SF1, steroidogenic factor 1; MAML, mastermind-like; N-ICD, Notch intracellular domain; RBP-J, recombination signal binding protein-J; STAT, signal transducer and activator of transcription; siRNA, small interfering RNA; RFP, red fluorescent protein; GFP, green fluorescent protein; MLT, mouse Leydig tumor; hCG, human chorionic gonadotropin; CR, conserved region; EMSA, electrophoretic mobility shift assay; NMD, nonsense-mediated mRNA decay; RT, reverse transcriptase; CHX, cycloheximide; MAMLD1, mastermind-like domain-containing 1.
opment (5–7), suggesting a possible interaction between SF1 and CXorf6.

Mastermind-like 2 (MAML2; alias, Mam-3) is a non-DNA-binding transcriptional co-activator in Notch signaling (8,9) that plays an important role in cell differentiation in multiple tissues by exerting either inductive or inhibiting effects according to the context of the cells (10). Upon ligand-receptor interaction, Notch intracellular domain (N-ICD) is translocated to the nucleus and interacts with a DNA-binding transcription factor, recombination signal binding protein-J (RBP-J), to activate target genes like hairy/enhancer of split 1 (Hes1) and Hes5 (11). In this canonical Notch signaling process, MAML2 forms a ternary complex with N-ICD and RBP-J at nuclear bodies, enhancing the transcription of the Notch target genes (8,9,12–14).

However, not all Hes genes are activated by the canonical Notch signaling pathway (11,15,16). Among such a distinct class of Hes genes, recent studies have shown that Hes3 can be induced by stimulation with a Notch ligand, via a STAT3 (signal transducer and activator of transcription 3)-mediated pathway (17). This finding, together with the lack of Hes3 induction by N-ICD (16), implies that Hes3 represents a target gene of a noncanonical Notch signaling.

Here, we report that CXorf6 produces a protein that has a structural homology with MAML2 and transactivates the Hes3 promoter activity and that CXorf6 is involved in testosterone production and harbors an SF1 target sequence.

EXPERIMENTAL PROCEDURES

Structural Analysis of CXorf6 Protein—We searched BLAST and TBLastn data bases using the CXorf6 protein sequence (NP_005482) as a bait. Protein sequences for the CXorf6 orthologs were compared by analyzing the human CXorf6 sequence with the genomic and transcribed sequences of different organisms using Clustal_X (18). The unrooted phylogenogram was generated by Clustal_X (18) from the sequence alignment of CXorf6 proteins and was visualized using TreeView 1.6.6 (19).

Primers, Probes, and Small Interfering RNAs (siRNAs)—The sequences of primers, probes, and siRNAs utilized in this study are summarized in supplemental Table 1.

Plasmid Vectors Utilized for CXorf6 Analyses—The cDNAs of the full-length CXorf6 (amino acids 1–701) and the minor splicing variant lacking exon 4 (ΔExon 4) were amplified from human fetal testis cDNA (Invitrogen) and subcloned into pEF-BOS vector (20) to construct the CXorf6 expression vector for the transactivation analysis. The expression vectors containing cDNAs of nonsense mutants and missense variants of CXorf6 were constructed by mutagenesis. For the subcellular localization analysis, cDNAs for the wild-type, mutant, and variant CXorf6 were designed to lose the start codon and fused to the C-terminal end of the gene encoding either red fluorescent protein (RFP) in pDsRED-monomer C1 vector or green fluorescent protein (GFP) in pAcGFP1-C1 vector (Clontech). For the Western blot analysis, cDNAs missing the start codon were subcloned into pCMV-Myc vector (Clontech).

We also utilized the following vectors reported in the literature: pEF-BOSneo-aNotch1 RAMIC with cDNA of mouse N1-ICD (21), pEF-BOSneo-aNotch2 with cDNA of mouse N2-ICD (22), pEF-BOS-Mam3 with cDNA of human MAML2 (8), pHes1-luc with the promoter sequence of mouse Hes1 (−467 + 46 bp), pHes5-luc with the promoter sequence of mouse Hes5 (−800 + 73 bp), pHes3-luc with the promoter sequence of mouse Hes3 (−2,715 + 261 bp) (16), pTP1-luc (pGa 981-6) with an iterated enhancer element with an RBP-J binding site (23), and pEF-BOS-Mam3-GFP with MAML2-GFP fusion gene (8).

Cell Culture—We primarily utilized mouse Leydig tumor (MLT) cells (ATCC, CRL-2065TM), which retain the capability to produce testosterone and the responsiveness to human chorionic gonadotropin (hCG) stimulation (24), because CXorf6 is a causative gene for hypospadias that is predicted to result from impairment of hCG-dependent testosterone production around the critical period for sex development (4,25) and because the mouse homolog for CXorf6 is expressed in testosterone-producing Leydig cells (3). We also utilized COS1 cells and HEK293 cells depending on the experimental purposes. These cells were maintained in RPMI 1640 or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

Transactivation Analysis of CXorf6—Transactivation function of CXorf6 was analyzed by the luciferase methods. MLT cells seeded in 6-well dishes (1.0–1.5 × 105 cells/well) were transiently transfected using Lipofectamine 2000 (Invitrogen) with 0.6 μg of luciferase reporter vector, 0.6 μg of expression vector for CXorf6 or MAML2, and/or 0.8 μg of expression vector for N1-ICD or N2-ICD, together with 20 ng of pRL-CMV vector used as an internal control. As controls for the expression vectors, empty counterpart vectors were transfected. Luciferase assays performed with a Lumat LB9507 (Berthold) at 48 h after transfection were repeated 4–5 times.

DNA Binding Analysis of CXorf6—We searched for conserved regions (CRs) between the mouse Hes3 promoter sequence in the pHes3-luc vector and the human AL031847 sequence containing HES3 and ~100.2 kb upstream and ~63.8 kb downstream regions using the BLAST data base and performed an electrophoretic mobility shift assay (EMSA) for the CRs using a Lightshift chemiluminescent EMSA kit (Pierce). The procedure was as described in the manufacturer’s instructions. In short, MLT cells or COS1 cells cultured in a plate with a diameter of 10 cm were transfected with 5 μg of empty or human CXorf6 cDNA positive vector, and nuclear extracts were obtained at 48 h after transfection. Then, a small amount of nuclear extracts was incubated with each of biotin-labeled 24–35-bp probes (20 fmol) covering the CRs, and the incubation mixture was subjected to gel electrophoresis. Subsequently, the biotin-labeled probe was detected by chemiluminescence on a nylon membrane.

Western Blot Analysis of CXorf6—Expression vectors for various Myc-tagged CXorf6 proteins (5 μg) were transfected into HEK293 cells in a plate with a diameter of 6 cm. Cell lysates obtained at 48 h after transfection were probed with antibodies for Myc and β-actin utilized as an internal control.

Subcellular Localization Analysis of CXorf6—Subcellular localization of CXorf6 proteins was studied by expressing fusion proteins with RFP or GFP. Vectors for fusion proteins (2
μg) were transfected into MLT cells in a glass dish with a diameter of 3.5 cm. The fluorescent signals were observed at 48–72 h after transfection using a laser-scanning microscope LSM510 (version 3.2; Carl Zeiss) shortly after nuclear staining with 4',6-diamidino-2-phenylindole.

Nonsense-mediated mRNA Decay (NMD)—Reverse transcriptase (RT)-PCR was performed for two regions of CXorf6 using lymphoblastoid cell lines of the patient with R653X and his heterozygous mother, with and without the treatment of an NMD inhibitor cycloheximide (CHX) (Sigma; 100 μg/ml, 8-h incubation) (26). The occurrence of NMD was assessed by the presence or absence of the PCR products on the agarose gel in the patient and by the heterozygosity or hemizygosity of the PCR products on the electrophorograms (CEQ 8000 Autosequencer, Beckman Coulter) in the mother after demonstrating a random X-inactivation pattern by the previously described method (27). Furthermore, maternal RT-PCR products were subcloned with a TOPO TA cloning kit (Invitrogen); 100 clones were subjected to sequencing to confirm the stabilization of mRNA with a nonsense mutation after CHX treatment.

Expression Analysis of HES3/Hes3—Human cDNA samples of penile and genital skin fibroblasts were prepared by RT-PCR using tissues obtained, after receiving permission, from a prepubertal boy with phimosis and from a prepubertal patient with ambiguous genitalia and mutant androgen receptor gene. Other human cDNA samples were purchased from Invitrogen or Clontech. For mouse Hes3, RT-PCR was performed for the MLT cells.

Knockdown Analysis for Mouse CXorf6 Homolog—We performed transient knockdown assay for the mouse CXorf6 homolog using two siRNAs (siRNA1 and siRNA2; final concentration 20 nM). The siRNAs were transfected into MLT cells seeded in 12-well dishes (5 × 10⁴ cells in each well with 1 ml of culture medium, using Lipofectamine RNAimax (Invitrogen). A nontargeting RNA (4611G Ambion) was similarly transfected as a negative control.

After 48 h of incubation, we examined mRNA quantity of mouse CXorf6 homolog in the harvested MLT cells and testosterone concentration in the culture medium using half of the wells. The relative amount of mRNA was determined by the Taqman real-time PCR method using the probe-primer mix for mouse CXorf6 homolog (assay No. mm01293665_m1, ABI) on an ABI PRISM 7000, using β2-microglobulin for an endogenous control. Testosterone concentration was measured by an electrochemiluminescence immunoassay. In addition, we further analyzed the testosterone production potential of the siRNA-transfected MLT cells in the remaining wells. After changing the old medium with a fresh medium containing hCG (Mochida Pharmaceutical; final concentration, 50 IU/liter), we cultured the cells for a further 1 h and measured testosterone concentration in the medium. These siRNA experiments were performed three times.

SF1 Target Sequence in CXorf6—We searched for a putative SF1 binding site in the genomic sequences of CXorf6 (AC109994) and the mouse homolog (NT_039706) and performed DNA binding and the transactivation analyses. For the DNA binding analysis, the 35S-labeled 30-bp probes containing the putative SF1 binding site in CXorf6 were incubated with nuclear extracts of COS1 cells transfected by an empty or human SF1 cDNA positive vector (pRK5) (Addgene) or with recombinant mouse SF1 protein and were subjected to gel electrophoresis. Similar analysis was also performed for a 32-bp probe harboring the known SF1 binding site of CYP11A1 (28) as a control. Furthermore, the biotin-labeled 30-bp probes containing the wild-type or the mutated SF1 binding site were incubated with nuclear extracts of COS1 cells transfected by a human SF1 cDNA positive vector (pCMX-PL2) and were subjected to gel electrophoresis.

For the transactivation analysis, a fragment (−1,924 to −1,690) containing a putative SF1 binding site of CXorf6 was PCR-amplified and inserted into the pGL3 basic luciferase reporter vector (Promega). Furthermore, a reporter vector carrying the mutation in the putative SF1 binding site was generated by mutagenesis. These reporter vectors (0.5 μg) were transfected into the MLT cells together with the empty or human SF1 cDNA positive expression vector (2.5 μg) as well as pRL-CMV vector (20 ng) used as an internal control. The luciferase assays were repeated three times.

Statistical Analysis—The results are expressed as the mean ± S.D. and statistical significance was determined by the t test. *p < 0.05 was considered significant.

RESULTS

Structural Analysis of CXorf6 Protein—We found that CXorf6 protein has a unique structure with homology to that of MAML2 protein (Fig. 1A). A unique amino acid sequence, which we designated the mastermind-like (MAML) motif, was inferred from sequence alignment with MAML1, MAML2, and MAML3 proteins (8, 9). The MAML motif was well conserved among CXorf6 orthologs identified in frog, bird, and mammals (Fig. 1B). In addition, a serine-rich domain was identified in CXorf6, as well as glutamine- and proline-rich domains.

Transcriptional Transactivation by the Wild-type CXorf6 Protein—We examined whether the wild-type CXorf6 (with exon 4) protein is involved in Notch signaling (Fig. 2A). Expression of CXorf6 alone slightly but significantly increased the luciferase activity in the absence of Hes promoters (pGL2 basic only), probably via some backbone vector sequence. This phenomenon was more evident for other vectors such as pGL3 basic and pGL4 basic (not shown). Thus, we utilized pGL2 basic-based luciferase reporter constructs with the promoter sequences of Hes1, Hes5, and Hes3 (16).

For the canonical Notch target genes Hes1 and Hes5 with the RBP-J binding site (16), CXorf6 was incapable of enhancing the promoter activities beyond those observed for the pGL2 basic only. MAML2 had no transactivating function, and N-ICDs activated the promoters. The N-ICD-induced promoter activities were further enhanced by CXorf6, probably because of additive or synergic effects via some backbone vector sequence, and by MAML2 because of its co-activator function. The results from the MAML2 and N-ICDs studies were consistent with those reported previously (8, 9, 16).

By contrast, for the noncanonical Notch target gene Hes3 without the RBP-J binding site (16), CXorf6 alone was capable of enhancing its promoter activity, whereas MAML2 and
Functional Analysis of MAMLD1 (CXorf6)

N-ICDs had no transactivating function. Consistent with this, co-expression of N-ICDs and CXorf6 or MAML2 exhibited no additive or synergic effects on the promoter activity.

These results argue that CXorf6 exerts its transactivation activity independently of the RBP-J binding sites. To confirm this, we performed similar analysis using pTP1-luc, which possesses an iterated enhancer element with an RBP-J binding site (23). As expected, CXorf6 was incapable of enhancing the N-ICD-induced transactivation, whereas MAML2 augmented the N-ICD-induced activities of this promoter (Fig. 2B).

DNA Binding Analysis of the Wild-type CXorf6 Protein—We attempted to examine whether the wild-type CXorf6 can bind to the Hes3 promoter sequence directly (supplemental Fig. 1). Comparison of the 2,976-bp mouse Hes3 promoter sequence with the human AL031847 sequence identified five CRs (CR1–CR5). Notably, the five CRs found in the human also resided in the upstream of the coding sequences of HES3, and the orientation of the CR1–CR5 was well conserved between human and mouse, whereas the mouse Hes3 promoter region was associated with repeat sequences between CR4 and CR5. EMSA was carried out using 24–35-bp overlapping biotin-labeled probes covering the CR1–CR5 (total, 25 probes), showing no evidence for the DNA-binding capacity of CXorf6.

Transactivation Function of Mutant and Variant CXorf6 Proteins—We next analyzed the transactivating activities of the previously identified three apparently pathologic nonsense mutants and three apparently non-pathologic missense variants of CXorf6 (3) (Fig. 3A) using the pHes3-luc vector. The E124X and Q197X proteins had no transactivation function, whereas the R653X protein as well as the three variant (P286S, Q507R, and N589S) proteins retained a nearly normal transactivating activity (Fig. 3B). In addition, the transactivation function was significantly reduced in the L103P protein (an artificially constructed variant affecting the MAML motif) and was normal in ΔExon 4. Transactivation analysis was also performed in the presence of N-ICDs, showing similar results (not shown). Western blot analysis for the three nonsense mutants verified the presence of proteins with expected molecular masses, whereas expression of the E124X protein appeared to be relatively reduced and that of Q197X protein was somewhat increased (Fig. 3C).

Subcellular Localization Analysis of Various CXorf6 Proteins—The RFP-CXorf6 (wild-type with exon 4) fusion protein were distributed in a speckled pattern and co-localized with the MAML2-GFP fusion protein (Fig. 4A). Furthermore, although the GFP-E124X and GFP-Q197X fusion proteins resided in the...
Nonsense-mediated mRNA Decay—The above results indicate that the artificially produced R653X protein retains an almost normal function. In this regard, we have shown that R653X, as well as E124X and Q197X, causes NMD in vivo, by RT-PCR analysis of leukocytes of the patients (3). Indeed, the positions of these mutations including R653X (1957C > T) satisfy the condition for the occurrence of NMD (29).

To further confirm this event in the R653X mutation, we examined two regions of CXorf6 mRNA obtained from lymphoblastoid cell lines of the patient and his heterozygous mother, using an NMD inhibitor, CHX (Fig. 5A). In the patient, RT-PCR amplification for regions 1 and 2 yielded no or very faint product without CHX treatment and a clear band with CHX treatment (Fig. 5B). In the mother, methylation pattern analysis of the androgen receptor gene (exon 1) indicated random X-inactivation (40-60%), and RT-PCR direct sequencing for a part of the region 2 encompassing the mutation delineated only the wild-type allele without CHX treatment and both wild-type and mutant alleles with CHX treatment (Fig. 5C). Furthermore, the analysis of 100 maternal RT-PCR clones showed only the wild-type sequence without CHX treatment and both wild-type and mutant sequences with a ratio of 56:44, which is similar to the X-inactivation ratio, after CHX treatment. These findings argue for the occurrence of NMD in the R653X mutation.

Expression Analysis of HES3/Hes3—PCR-based cDNA library screening for HES3 identified variable degrees of expression in a range of tissues including fetal testis and adult ovary (Fig. 6). In addition, Hes3 was expressed in the MLT cells (not shown).

Knockdown Analysis for Mouse CXorf6 Homolog—At 48 h after transfection, the mRNA level of the endogenous mouse CXorf6 homolog was severely reduced in the MLT cells (relative amount: 28% for siRNA1 and 29% for siRNA2), indicating the successful knockdown (Fig. 7A). At that time, testosterone concentration was also significantly decreased in the medium harboring the knockdown cells (relative concentration: 63% for siRNA1 and 81% for siRNA2) (Fig. 7B). Furthermore, hCG-stimulated testosterone production during a subsequent 1 h was also compromised in the knockdown MLT cells (relative concentration: 53% for siRNA1 and 55% for siRNA2) (Fig. 7C).

SF1 Target Sequence in CXorf6—A putative SF1 binding sequence, CCAAGGTCA, was identified at intron 2 upstream of the coding region of CXorf6 (~1,773 ~ 1,765) (Fig. 8A). This binding site was also found at intron 1 in the upstream coding region in the mouse homolog (~42,904 ~ ~42,896 and ~9,986 ~ 9,978) (not shown). Both human SF1 and mouse SF1 proteins were capable of binding to the putative SF1 binding site of CXorf6 as well as to the known SF1 binding site of CYP11A1 (Fig. 8B). This SF1 protein binding was drastically reduced when the putative SF1 binding site CCAAGGTCA was mutated as CCATTGTC (Fig. 8C). Consistent with this finding, although the SF1 protein transactivated the luciferase activity of the wild-type reporter, the transactivation function of SF1 protein was significantly reduced for the mutant reporter (Fig. 8C).

**DISCUSSION**

The wild-type CXorf6 co-localized with structurally related MAML2 in the nuclear bodies and transactivated the Hes3 promoter without demonstrable DNA-binding capacity. These findings are consistent with transcription usually occurring around nuclear bodies (30, 31) and suggest that CXorf6 may be recruited to the Hes3 promoter as a non-DNA-binding transcriptional co-activator, although the possibility that CXorf6...
CXorf6 alone was capable of enhancing the activity. This would be relevant to CXorf6 being devoid of the co-activator for the RBP-J dependent canonical Notch signal transactivation (32). Thus, further studies are required to clarify how CXorf6 transactivates Hes3 transcription. In addition, there may be other target gene(s) of CXorf6 besides Hes3.

Several domains were identified in the CXorf6 protein in addition to the previously reported glutamine- and proline-rich domains (1). In this regard, the evolutionally conserved MAML motif may play a critical role in the transactivating activity, because the L103P protein had a reduced transactivation function. The serine-rich domain may also be relevant to the transactivation (33). Furthermore, it is inferred that a nuclear localization signal resides on the N-terminal 123 amino acids and a nuclear body localization signal lies on amino acids 197–653, except for amino acids 567–607 encoded by exon 4. It might be possible that a nuclear body localization signal is preserved in the Q197X protein, and in the E124X protein as well, but could not function because of an aberrant protein folding.

Functional studies provided the molecular basis for the previously identified mutations and variations. The E124X and Q197X mutations previously have been shown to undergo NMD, such proteins would be non-functional. For the R653X mutation, although the artificially produced truncated protein had a normal transactivation function in vitro, the NMD analysis implies the occurrence of nearly complete NMD in vivo. The P286S, Q507R, and N589S proteins previously identified mutations and variations. The E124X and Q197X mutations previously have been shown to undergo NMD, such proteins would be non-functional. For the R653X mutation, although the artificially produced truncated protein had a normal transactivation function in vitro, the NMD analysis implies the occurrence of nearly complete NMD in vivo. The P286S, Q507R, and N589S proteins were confirmed to retain normal functions. These results are consistent with the previous genotype-phenotype correlations of the mutations and variations (3).

The siRNA experiments imply that CXorf6 is involved in testosterone production. In this context, it appears that testosterone production is not abolished in the absence of residual CXorf6 expression, because the degree of reduction was more obvious for the mRNA level than for the testosterone concentration. This would be consistent with the development of hypospadias in patients with CXorf6 nonsense mutations (3), because hypospadias is a phenotype caused by reduced, but not absent, testosterone effects around the critical period for sex development (4, 25). When testosterone effects are abolished,
is co-expressed with the mouse homolog for CXorf6 in fetal Sertoli cells and Leydig cells (3), and SF1 transactivated CXorf6. Thus, there may be an interaction among SF1, CXorf6, and HES3 in the fetal testis, which may play an important role in the testicular function including testosterone production. In this regard, although the mouse homolog for CXorf6 is not expressed in the adult testis, it is clearly expressed in the adult ovary (3) where SF1 (4, 5) and HES3 are also expressed. In addition, premature ovarian failure has been described in a female with heterozygosity for a microdeletion involving CXorf6 (34). Thus, CXorf6 and its possible interaction with SF1 and HES3 may also be relevant to adult ovarian function.

In summary, the present study implies that CXorf6 transactivates the Hes3 promoter, augments testosterone production, and contains the SF1 target sequence, thereby providing the first clue for the elucidation of the CXorf6 function. On the basis of the characteristic structure, we have therefore designated CXorf6 as MAMLD1.

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**FIGURE 7. Effects of siRNA on testosterone production in the MLT cells.** A, relative mouse CXorf6 mRNA level in MLT cells after 48 h of incubation with two siRNAs. B, testosterone concentration in the medium after 48 h of incubation with two siRNAs. C, testosterone concentration in the medium after one h of incubation with hCG, using MLT cells cultured with two siRNA for 48 h. N.C., negative control transfected with nontargeting RNA.

**FIGURE 8. Interaction between SF1 and CXorf6.** A, genomic sequence of CXorf6. The putative SF1 binding site is present at intron 2 in the upstream of the coding region. The black areas indicate exons E1–E7, with the start codon (ATG) on exon 3 and the stop codon (TGA) on exon 6. B, EMSA using the 32P-labeled probes containing the previously known SF1 binding site of CYP11A1 and the putative SF1 binding site of CXorf6. Lane 1, probe only; lane 2, mixture of the probe and nuclear extract of COS1 cells transfected with an empty vector; lane 3, mixture of the probe and nuclear extract of COS1 cells transfected with an expression vector with human SF1 cDNA; lane 4, mixture of the probe and recombinant mouse SF1 protein. Arrows indicate the bands retarded due to the binding of the SF1/SF1 protein. C, EMSA and transactivation analysis using wild-type (WT) and mutant sequences for the putative SF1 binding site. The binding of SF1 protein to biotin-labeled probes is drastically reduced for the mutant probe (shown by an arrow), and the relative luciferase activity is obviously decreased for the reporter with the mutant sequence.

this results in female external genitalia in genetic males (25). Thus, it is likely that CXorf6 augments testosterone production.

The present study has implications for the molecular network involving CXorf6. HES3 was expressed in the human fetal testis, and the Hes3 promoter was transactivated by CXorf6. SF1
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