Estrogen Down-regulation of the *Scx* Gene Is Mediated by the Opposing Strand-overlapping Gene *Bop1*[*][S]

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Recent genome-wide transcriptome studies suggest the presence of numerous bidirectional overlapping coding gene pairs in mammalian genomes. Various antisense RNAs are reported as non-coding RNAs that regulate the expression of sense RNA. However, it is still unclear whether the expression of bidirectional overlapping coding genes are regulated by the opposite strand gene transcript acting as a non-coding RNA. *Bop1* and *Scx* are a pair of bidirectional overlapping coding genes related to cellular proliferation and differentiation, respectively. *Scx* gene is localized in the intron 3 region of the *Bop1* gene. The expression of these genes is reciprocally regulated by estrogen (E2) in the mouse uterus. *In situ* hybridization indicated that both genes are expressed in the uterine endometrial epithelial cells and that the antisense RNA of *Scx* (*Bop1* intronic RNA) accumulates as a stable RNA in these cells. The existence of *Bop1* intronic RNA was confirmed by reverse transcription-PCR and was increased after E2 treatment, coinciding with a decrease in *Scx* mRNA. Murine myoblasts expressing doxycycline-inducible endogenous *Bop1* gene showed an increase in *Bop1* intronic RNA and a simultaneous decrease in *Scx* mRNA. Murine fibroblasts expressing *Scx* mRNA from an exogenous *Scx* mini-gene indicated that the accumulation of *Bop1* intronic RNA impairs the *Scx* gene expression in a trans-acting manner, which resulted in the reduction of the *Scx* mRNA level. This study demonstrates a novel example of hormone-stimulated intronic non-coding RNA down-regulating the expression of an opposing strand-overlapping coding gene.

Genome-wide transcriptome studies suggest that about 15–25% of mammalian genes are arranged such that they overlap one another on opposite DNA strands, giving rise to pairs of sense and antisense RNAs (1–6). Several groups have classified the bidirectional overlapping gene pairs (1, 5), although a precise mechanism of the regulation is not well understood. For instance, Numata *et al.* (5) suggested six structural categories as follows: category 1, one transcription unit is completely overlapped within an exon of the transcription unit on the opposite strand; category 2, exonic regions overlap in convergent orientation; category 3, exonic regions overlap in divergent orientation; category 4, one transcript unit is completely overlapped within an intron of the transcription unit on the opposite strand; category 5, transcription units overlap in convergent orientation, but exonic regions do not overlap; category 6, transcription units overlap in divergent orientation, but exonic regions do not overlap. Studies focused on the exonic overlapping gene pairs (categories 1, 2 and 3) have suggested that double strand RNA can be derived from sense-antisense mRNAs (7, 8); however, little is known regarding intronic overlapping gene pairs that are included in categories 4, 5, and 6.

Antisense non-coding RNAs have the ability to regulate the expression of sense RNA through an epigenetic mechanism, as exemplified by X chromosome inactivation by *Xist* (9–11) or genome imprinting by *Air* (12–14). Recently, genome-wide studies suggest the presence of numerous bidirectional overlapping coding gene pairs (1). Whether the expressions of bidirectional overlapping coding genes can be regulated by an opposite strand gene transcript acting like a non-coding RNA is still unclear.

Bop1 and *Scx* are a pair of bidirectional overlapping coding genes located on mouse chromosome 15. The *Scx* gene consists of two exons and is embedded in the intron 3 region of the *Bop1* gene (Fig. 1); therefore, based on the categorization defined above, this gene pair falls into category 4. Bop1 is a component of the nucleolar ribonucleoprotein complex, is involved in 5 S and 28 S ribosomal RNA maturation (15), and is necessary for the biogenesis of 60 S ribosomal subunit (16). It is also involved in cell proliferation because reports show that a dominant negative mutation of Bop1 prevents cell proliferation (17, 18). On the other hand, Scx is a basic helix-loop-helix type transcription factor that regulates the function of differentiated cells, such as osteoblastic cells (19), chondrogenic cells (20), tendon (21), and Sertoli cells (22). Thus, this chromosome locus is occupied by genes involved in both cell proliferation and differentiation. Studies using the *Scx* transgenic or knock-out mice predicted that the expression of *Scx* and *Bop1* genes may affect each other (21, 23, 24); however, any correlation of regulation between *Bop1* and *Scx* gene expression has not been described.

Estrogen (E2)[2] triggers the initiation of the proliferation of endometrial epithelial cells in the mammalian uterus (25, 26).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures" and Figs. S1 and S2.
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[2] The abbreviations used are: E2, estrogen; ER, estrogen receptor; ICI, ICI182,780; OVX, ovariectomized; 18 S, 18 S ribosomal RNA; ISH, *in situ* hybridization; Tet, tetracycline; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CMV, cytomegalovirus; RT-PCR, reverse transcription-PCR; +RT, with reverse transcriptase; −RT, without reverse transcriptase.
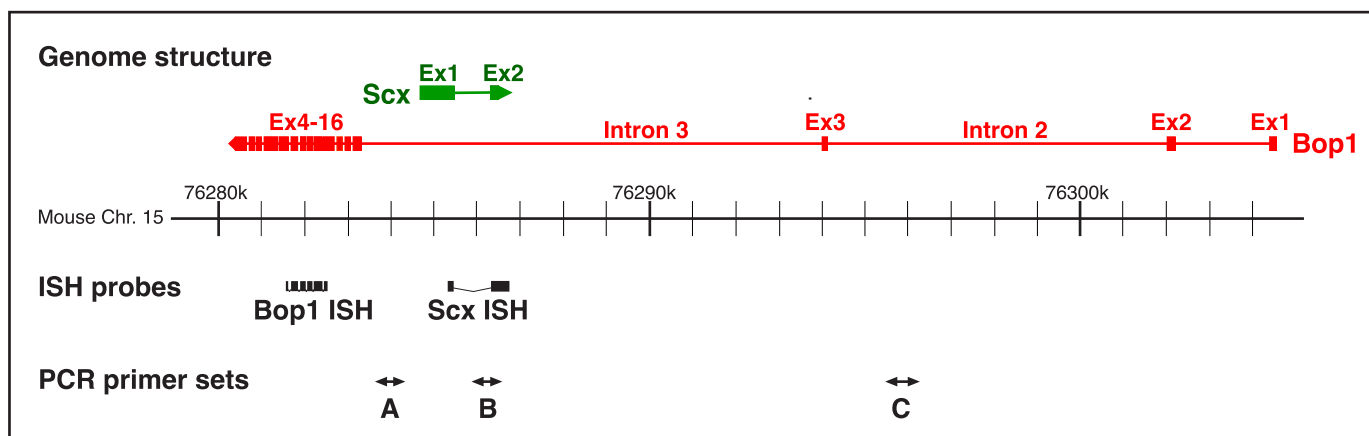
FIGURE 1. **Schematic illustration of the *Bop1* and *Scx* genes.** The location of the *Bop1* (*red*) and *Scx* (*green*) genes on mouse chromosome 15 is illustrated with their exon-intron structure. The *boxes* and *lines* indicate exonic and intronic regions, respectively. The *arrowheads* depict the direction of gene transcription. The positions of the probes for ISH are indicated as *black boxes* (Bop1 ISH and Scx ISH). The positions of PCR-amplified element are indicated as *A*, *B*, and *C*. Primer sets A and B are located on *Bop1* intron 3. Set A does not overlap, and set B overlaps the *Scx* gene. Primer set C is located on *Bop1* intron 2. *Ex1*, *Ex2*, *Ex3*, and *Ex4–16* indicate exons 1, 2, 3, and 4–16, respectively.

Our laboratory has examined the effect of E2 responsiveness in the mouse uterus (27–29). Previously, we reported that E2 induces the expression of cell proliferation-related genes similar to different growth factors in the ovariectomized (OVX) mouse uterus (29). In our microarray gene profile obtained from the E2-treated OVX mouse uterus, we found that the *Bop1* mRNA, a proliferation-related factor, is induced, whereas the level of *Scx* mRNA, a differentiating cell factor, is decreased simultaneously. In this report, we demonstrate a mechanism of reciprocal regulation of *Bop1* and *Scx* gene expression.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The pTRE-c-Myc plasmid was constructed as follows. pCMV-SPORT6-mouse 3962047 plasmid that contains full-length mouse c-*Myc* cDNA (ATCC, Manassas, VA) was cut with EcoRI and XhoI, and the excised fragment (1.4 kb) was cloned into pCR2.1 vector (Invitrogen) at EcoRI and XhoI sites (pCR2.1-c-Myc). The XbaI fragment (1.4 kb) from pCR2.1-c-Myc plasmid was cloned into pTRE vector (Clontech) at the XbaI site. The direction of the inserted fragment was confirmed by digestion with EcoRI and EcoRV. pTRE-mBop1-cDNA plasmid was constructed as follows. pCMV-SPORT6-mouse 4193123 plasmid that contains full-length mouse *Bop1* cDNA (ATCC) was cut with SacII and XbaI, and the excised fragment (2.5 kb) was cloned into pTRE vector at the SacII and XbaI site. CMV-Scx-cDNA plasmid was constructed as follows. pCMV-SPORT6.1-mouse 30138970 plasmid that contains full-length mouse *Scx* cDNA (ATCC) was cut with EcoRV and NotI, and the excised fragment (1.1 kb) was cloned into pcDNA3 vector (Invitrogen) at the EcoRV and NotI site. To construct the CMV-Scx-gDNA(+1) and CMV-Scx-gDNA(−250) plasmids, SKA3SH8Z11NA1 plasmid (30) that contains ~11 kb of mouse *Scx* genomic DNA was used (kindly given by Dr. A. Perez, Center for Functional Genomics University at Albany). The DNA fragment (+1 to +2392) and DNA fragment (−250 to +2392), respectively, of *Scx* gene were cloned into the pcDNA3 vector. All plasmids were confirmed by sequencing (NIEHS sequencing core).

Animals and Treatments—All animals were handled according to National Institutes of Health guidelines and in compliance with an NIEHS, National Institutes of Health-approved animal protocol. OVX C57BL/6 mice were purchased from Charles River Laboratories (Raleigh, NC). Groups of animals (six mice per group) were treated with sesame oil vehicle (Sigma-Aldrich) or with 1 μg of E2 (Steraloids, Newport, RI), either dissolved in 100 μl of sesame oil and injected subcutaneously (6-, 12-, and 24-h groups) or dissolved in 100 μl of normal saline and injected intraperitoneally (0.5-, 1-, and 2-h groups) before necropsy. In some cases, animals (four mice per group) were treated with 45 μg of ICI 182,780 (ICI; Zeneca Pharmaceuticals, Cheshire, UK) that was dissolved in 50 μl of dimethyl sulfoxide and injected intraperitoneally, 30 min before E2 injection. Animals were euthanized at the indicated times using CO_2, and uteri were collected and stored at −80 °C until use.

In Situ Hybridization—Uterine tissue was fixed in 4% paraformaldehyde and then embedded in paraffin before sectioning. Probes that corresponded to the mouse *Bop1* and *Scx* genes were amplified by PCR using the following primers: *Bop1* gene (AK079627), 5′-CCA TGC CGA GTC TTA CAA CCC ACC-3′ (nucleotides 913–936) and 5′-AGC AGC AAC ACG GCA TCA TCC ATG GC-3′ (reverse complement to nucleotides 1431–1406); *Scx* gene (BC062161), 5′-ACA CCC AGC CCA AAC AGA TCT GCA C-3′ (nucleotides 615–639) and 5′-CCA GGT AGA GAG CCA GCA TGG AAA GTC-3′ (reverse complement to nucleotides 960–934). A 570-bp length of plant-derived sequence (57.9% of guanine-cytosine content) was used for negative control (GENOSTAFF, Tokyo Japan). Probes (cRNA) labeled with digoxigenin were hybridized to the uterus tissue sections, and this procedure was followed by treatment with anti-digoxigenin alkaline phosphatase-conjugated antibodies and visualization by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (GENOSTAFF).

Establishment of Stable Transformants—C2C12 mouse myoblasts (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS; Gemini Bio-products, West Sacramento, CA), 1000

TABLE 1

The primer sets used for real-time PCR and conventional PCR

Name of primer set	Sequence of primer (forward)	Sequence of primer (reverse)
ScxQ	5′–CGTCTTTCTGTCACGGTCTTTGCTC–3′	5′–CTTTCTTCCACAGCGGTCGTGC–3′
Bop1Q	5′–CAGCTCTGATGAGGAGGACATTCGGAAC–3′	5′–CAACCTGCTCATCAGTTAGCCG–3′
Bop1 intron A	5′–CATTTCTGGGCTGGTGTGGACAG–3′	5′–GTGGGTAGTTTACAAGGCAGGAACCAT–3′
Bop1 intron B	5′–CGTCTTTCTGTCACGGTCTTTGCTC–3′	5′–AAATACAACGGCTTTCCCCAATCC–3′
Bop1 intron C	5′–TCCGTCCCTCGTCACACATTTG–3′	5′–CCTGCTTTTTCTGTGAACATCCTC–3′
c-Myc	5′–ACCCCTCAGTGGTCTTTCCCTACCC–3′	5′–GTTTGCCTCTTCTCCACAGACACCACATC–3′
Scx	5′–CTTCACTGCGCTGCGCACACTCATCC–3′	5′–GCTCTCCGTGACTCTTCAGTGGCATCC–3′
Bop1	5′–CAGCTCTGATGAGGAGGACATTCGGAAC–3′	5′–TCACAGGGTGGATCATGATGTCACCGC–3′

units/ml penicillin, and 1000 μg/ml streptomycin. Cells were cultured at 37 °C under a 5% CO_2, 95% air atmosphere. Tet-On-C2C12 was established as follows. C2C12 cells were seeded in a 60-mm diameter dish for transfection. Transfection was performed by using FuGENE 6 (Roche Diagnostics) according to the manufacturer's protocol. pTet-On vector (Clontech) was transfected into the C2C12 cells. The cells were switched to fresh medium with 400 μg/ml G418 at 24 h after transfection. The medium was changed every 4 days for 3 weeks. To establish the Tet-On-Myc-C2C12 and Tet-On-mBop1cDNA-C2C12, Tet-On-C2C12 cells were transfected with pTK-Hyg (Clontech) and pTRE-c-Myc or pTRE-mBop1-cDNA plasmids. To select for stable cell lines, the cells were cultured with 300 μg/ml G418 and 300 μg/ml hygromycin for 2 weeks. BALB/3T3 clone A31 mouse fibroblasts (ATCC) were maintained in DMEM with 10% newborn calf serum (Invitrogen), penicillin, and streptomycin. To establish the CMV-Scx-gDNA-BALB/3T3 and CMV-Scx-cDNA-BALB/3T3 cells, CMV-Scx-gDNA(+1), CMV-Scx-gDNA(−250), or CMV-Scx-cDNA plasmids, respectively, were transfected to BALB/3T3 cells. The cells were cultured with 400 μg/ml G418 for 3 weeks to select the stable cell line.

Cell Culture and Treatments—Tet-On-Myc-C2C12 and Tet-On-mBop1cDNA-C2C12 cells were maintained in DMEM with 10% FBS, penicillin, streptomycin, 300 μg/ml G418, and 200 μg/ml hygromycin. Tet-On-Myc-C2C12 cells (1.2×10^6 cells) were plated into 100-mm diameter dishes. Two days after, cells (90–95% confluent) were switched into DMEM with 1% FBS, penicillin, streptomycin, G418, and hygromycin that was subsequently changed every 2 days. Five days after (time 0), the cells were switched to DMEM with 10% FBS, 1 mg/ml doxycyclin, penicillin, streptomycin, G418, and hygromycin. The cells were harvested to collect RNA at 2 or 6 h after changing the medium. Transformed BALB/3T3 cell lines were maintained in DMEM with 10% newborn calf serum, penicillin, streptomycin, and G418. The cells (1.7×10^5 cells/well for CMV-Scx-gDNA(+250), 1.7×10^5 cells/well for CMV-Scx-gDNA(+1), and 1.9×10^5 cells/well for CMV-Scx-cDNA) were plated in a 6-well plate. The cells were cultured in the DMEM with 10% newborn calf serum for 24 h and then switched into the DMEM with 1% horse serum (Invitrogen) for 3 days (low serum medium condition) to maintain quiescent cells. To induce the proliferation of the cells, three wells were switched to DMEM with 10% FBS (high serum medium). The other three wells were replaced with fresh low serum medium. The cells were harvested 6 h after changing the medium for RNA extraction.

RNA Extraction and Reverse Transcription—Frozen uterine tissue was pulverized, and total RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA of the cells was extracted by using a Qiagen RNeasy kit (Qiagen, Valencia CA) according to the manufacturer's instructions. Extracted RNA was treated with TURBO DNase (Ambion) to remove the genomic DNA contamination. To make the cDNA samples, total RNA (1 μg) was incubated in 20-μl reaction mixes containing 2 μl of 10× RT buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl) (Invitrogen), 100 ng of random hexamer, 0.5 mM dNTPs, 5 mM $MgCl_2$, 10 mM dithiothreitol, 40 units of RNaseOUT (Invitrogen), 50 units of SuperScript II reverse transcriptase (Invitrogen) at 42 °C for 50 min.

Quantitative PCR—Real-time PCR was performed by using the primer sets as described in Table 1. To amplify the mRNA, forward and reverse primers were designed on the different exonic sequences except the primers for *Bop1* introns. The primers of ScxQ-F and ScxQ-R are localized on the exons 1 and 2, respectively, of the *Scx* gene. The Bop1Q-F primer is localized on the junction of exons 3 and 4 of the *Bop1* gene, and the Bop1Q-R primer is localized on the *Bop1* exon 5. The primer mix of Classic II 18 S Standards (Ambion) was used for 18 S ribosomal RNA (18 S). For cDNA amplification, 1–10 ng of cDNA were combined with 25 μl of a mixture containing Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and 160 nM forward and reverse primers for *Scx* (ScxQ), *Bop1* (Bop1Q), c-*Myc*, and *Bop1* introns A, B, and C or 26 nM primer mix for 18 S. Samples were analyzed in triplicate. Amplification was carried out as follows: 50 °C for 1 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1min. To determine the cycle time for each gene, the ABI PRISM 7900 sequence detection system (Applied Biosystems) and SDS 2.1 analysis software (Applied Biosystems) were used. We used the mathematical model described by Pfaffl (31) to get the relative expression levels. 18 S was used as an internal control for all reactions.

PCR—PCR was performed using the primer sets as described in Table 1. The PCR of *Scx* was run for 26 or 32 cycles (95 °C for 30 s, 66 °C for 50 s, and 72 °C for 2 min), the PCR of *Bop1* was run for 22 cycles (95 °C for 30 s, 66 °C for 50 s, and 72 °C for 2 min), the PCR of 18 S rRNA was run for 14 cycles (95 °C for 30 s, 64 °C for 50 s, and 72 °C for 4 min), and the PCR of *Bop1* introns (A, B, and C) was run for 28 cycles (95 °C for 30 s, 64 °C for 50 s and 72 °C for 2 min) using Platinum *Taq* DNA polymerase (Invitrogen). Aliquots (6 μl) of PCR products were resolved on 2% (w/v) agarose gels stained with ethidium bromide and viewed using the UVP, LLC bioimaging system.
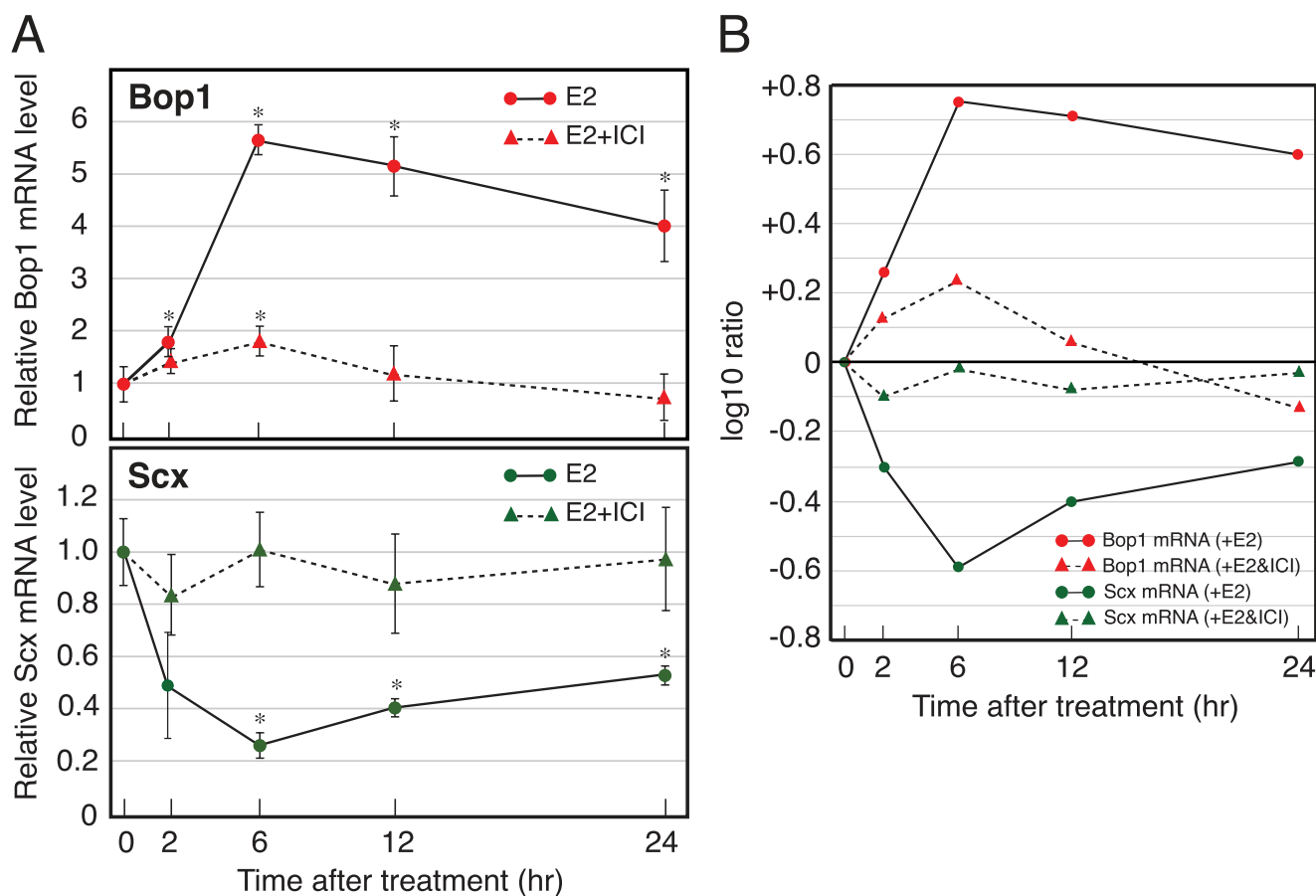
FIGURE 2. **Expression of the *Bop1* and *Scx* genes in the E2-treated OVX mouse uterus.** Animals (six or four mice per group) were administered E2 with (*dashed line*) or without (*solid line*) ICI, and uterine total RNA was prepared at the times indicated. *A*, the mRNA levels of *Bop1* (*upper panel*) and *Scx* (*lower panel*) gene were quantified by real-time PCR as described under "Experimental Procedures." Results were normalized to the level of 18 S in each sample. The values are the means ± S.D. *, $p < 0.01$ compared with time 0. *B*, the result expressed as the log 10 ratio for changes relative to time 0 to compare the changes between *Bop1* and *Scx* mRNA levels.

Statistical Analysis—Statistical analysis was performed with two-tailed paired *t* tests, and $p < 0.05$ was considered statistically significant.

RESULTS

The Expression of Bop1 and Scx Genes Is Reciprocally Regulated—OVX adult female mice were treated with E2 at time 0, and total RNA was isolated at the times indicated (Fig. 2). Relative amounts of *Bop1* and *Scx* mRNAs were quantified by real-time PCR and normalized with the amount of 18 S as an internal control. As shown in Fig. 2 by the *solid lines*, E2 stimulated a 6-fold peak of increase (+0.64 as log 10 ratio) in *Bop1* mRNA following a 6-h exposure, and the mRNA level was gradually reduced until 24 h. In contrast, the level of *Scx* mRNA was decreased ~80% (−0.59 as log 10 ratio) at 6 h after E2 treatment and gradually recovered by 24 h. These results indicated that the expression of *Bop1* and *Scx* mRNAs was reciprocally regulated by E2 in the OVX mouse uterus. Additionally, we determined the effect of E2-mediated transcriptional activation on the *Bop1* and *Scx* gene expression using ICI, an estrogen receptor (ER) antagonist that prevents ER-E2-mediated transcription (32). As shown in Fig. 2 by the *dashed lines*, ICI attenuated the *Bop1* gene activation while also attenuating the down-regulation of the *Scx* gene expression. These results suggested that

E2-activated transcription of the *Bop1* gene may be coordinately related to the *Scx* gene down-regulation.

Antisense RNA for the Scx Gene Is Present in the OVX Mouse Uterus—In situ hybridization (ISH) was performed using the OVX mouse uterus to localize which uterine cells express the *Scx* and *Bop1* transcripts. The signals for the antisense probes of *Scx* and *Bop1* transcripts were detected in the endometrial epithelial cells (Fig. 3*A*, *panels a*, *b*, *e*, and *f*). Interestingly, the signal for the sense probe of the *Scx* transcript was also detected in the endometrial epithelial cells (Fig. 3*A*, *panels c* and *d*). These results suggest that the antisense RNA for the *Scx* gene is present in the uterine cells that express *Scx* and *Bop1* mRNAs. To confirm the existence of the antisense RNA for the *Scx* gene, RT-PCR was performed using RNA extracted from OVX mouse uteri. As depicted in Fig. 1, primer set A amplifies a region of intron 3 of the *Bop1* gene that does not overlap with the *Scx* gene. Primer set B amplifies a region of intron 3 that overlaps with the *Scx* gene, and primer set C amplifies the intron 2 region of the *Bop1* gene. We used templates that were synthesized with or without reverse transcriptase (Fig. 3*B*, +*RT* and −*RT*, respectively) to verify negligible amounts of genomic DNA contamination. No appreciable signal was detected in the −RT samples, demonstrating that the amplified fragments from the +RT samples were derived from
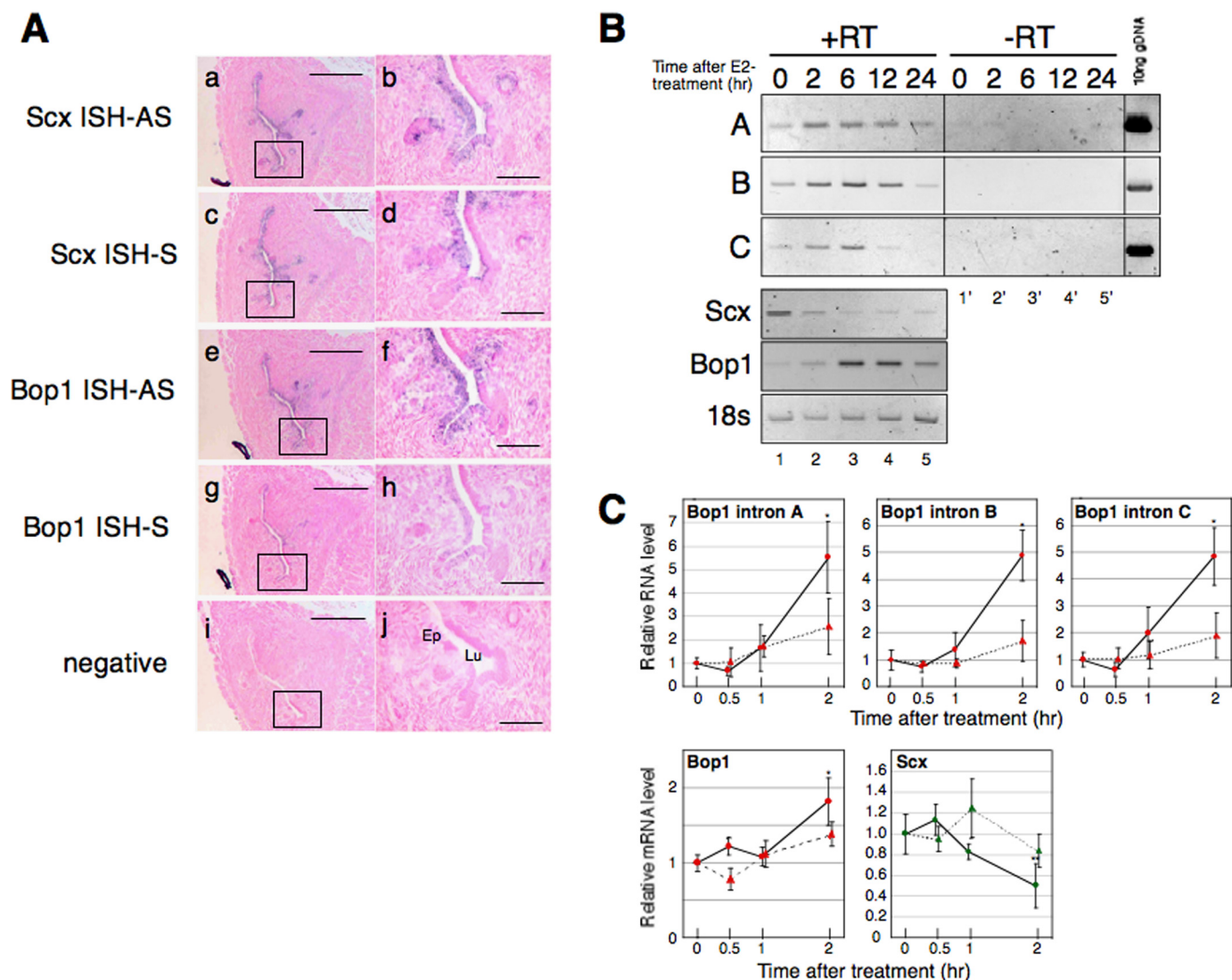
FIGURE 3. **Antisense RNA for the *Scx* gene transcript exists in the OVX mouse uterus.** *A,* ISH results from serial sections of the OVX mouse uterus. ISH was performed using an antisense probe (*AS*) for *Scx* cDNA (*panels a* and *b*), a sense probe (*S*) for *Scx* cDNA (*panels c* and *d*), an antisense probe for *Bop1* cDNA (*panels e* and *f*), and a sense probe for *Bop1* cDNA (*panels g* and *h*). The position of the probes is indicated in Fig. 1. A nonspecific plant sequence was used as the negative control (*panels i* and *j*) as described under "Experimental Procedures." The *purple signal* shows specific hybridization of the indicated probes. The *boxes* in *panels a, c, e, g,* and *i* are increased in magnification correspond to *panels b, d, f, h,* and *j,* respectively. *Ep* indicates endometrial epithelial cells, and *Lu* indicates lumen. *Scale bars* = 200 μm in *panels a, c, e, g,* and *i* and 50 μm in *b, d, f, h,* and *j. B,* total uterine RNA was prepared from the E2-treated mice at the indicated times. A representative RT-PCR result of various primer sets (A, B, C, Scx, Bop1, and 18 S) using the identical samples is shown. *A, B,* and *C* indicate the *Bop1* intronic elements in Fig. 1. The samples of *lanes 1–5* were reverse-transcribed from RNA extracted from E2-treated uteri at the indicated times (+*RT*). The samples of *lanes 1'–5'* were not reverse-transcribed using the same RNA of 1–5 (−*RT*). Ten ng of genomic DNA were used as the positive control for the intronic RNA. *C,* E2 was administered to mice (six mice per group) with (*dashed line*) or without (*solid line*) ICI, and uterine total RNA was prepared at the times indicated. Real-time PCR was performed, and the results were normalized to the level of 18 S in each sample. The values are the means ± S.D. *, $p < 0.01$ when compared with time 0, **, $p < 0.02$ when compared with time 0.

RNA and not genomic DNA contamination (Fig. 3*B*). The size of the amplified fragment is identical to the amplicon from mouse genomic DNA, suggesting that the antisense RNA for the *Scx* gene, which is derived from the *Bop1* intron, is present in the mouse uterus. The amount of *Bop1* intron 3- and 2-derived RNA was increased at 2 h after E2 treatment, which is prior to the accumulation of *Bop1* mRNA (Fig. 3, *B* and *C*). *Scx* mRNA was decreased at 2 h after E2 treatment, which coincides with the accumulation of *Bop1* intron-derived RNA.

The Induction of the Endogenous Bop1 Gene Correlates with the Down-regulation of the Scx mRNA Level in Tet-On-Myc-C2C12 Cells—To examine the effect of *Bop1* gene activation on the *Scx* gene down-regulation in an estrogen-independent

manner, we established an *in vitro* model using C2C12 myoblasts, a murine cell line reported to express endogenous *Scx* mRNA (33). Several reports indicate that the *Bop1* gene expression is induced by c-Myc (34). To activate endogenous *Bop1* gene expression, we established a Tet-On-Myc C2C12 cell line that contains a tetracycline (Tet)-mediated transcription activator (TetR) expression vector and a TetR binding element fused to a c-Myc expression vector. Cells were stimulated by a Tet derivative compound, doxycycline, and harvested 6 h after stimulation. Fig. 4*A* shows that *c-Myc* mRNA was stimulated by the treatment (3.4-fold) and that *Bop1* mRNA was also increased by 1.6-fold (+0.21 as log 10 ratio). In contrast, *Scx* mRNA was decreased 30% (−0.12 as log 10 ratio) 6 h after
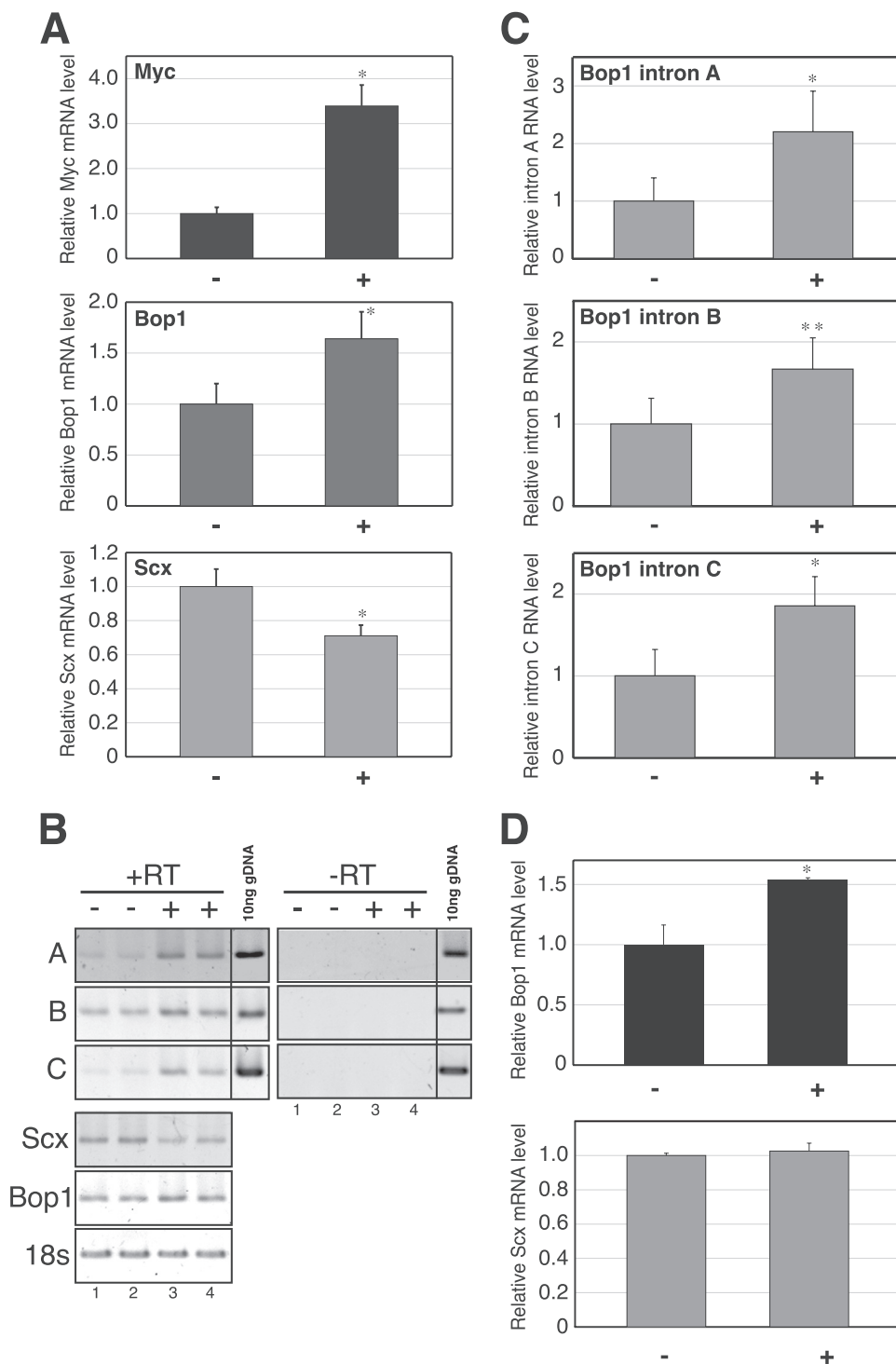
FIGURE 4. **The down-regulation of *Scx* mRNA is correlated with the induction of endogenous *Bop1* gene expression.** *A*, Tet-On-Myc-C2C12 cells were treated with (+) or without (−) doxycycline, and total RNA was prepared 6 h after treatment. Real-time PCR was performed, and the results were normalized to the level of 18 S in each sample. The values are the means ± S.D. for four independent replicates. *, $p < 0.01$ when compared with (−). *B*, a representative RT-PCR result of various primer sets using the identical samples is shown. The positions of PCR-amplified *Bop1* intronic elements A, B, and C are indicated in Fig. 1. The samples of *lanes 1–4* were reverse-transcribed (+*RT*) from the RNA that was extracted from cells treated with (+) doxycycline (2 h) without (−) doxycycline. The samples of *lanes 1'–4'* were not reverse-transcribed (−*RT*) using the same RNA as *lanes 1–4*. Ten ng of genomic DNA were used for positive control of intronic RNA. *C*, total RNA was prepared at 2 h after with (+) or without (−) doxycycline treatment. *D*, Tet-On-mBop1cDNA-C2C12 cells were treated with (+) or without (−) doxycycline, and total RNA was prepared 6 h after treatment. Real-time PCR was performed, and the results were normalized to the level of 18 S in each sample. The values are the means ± S.D. for four independent replicates. *, $p < 0.01$ when compared with (−), **, $p < 0.05$ when compared with (−).

treatment. Samples were harvested 2 h after treatment to evaluate the expression level of *Bop1* intron-derived RNA in this system. As shown in Fig. 4, *B* and *C*, *Bop1* intron-derived RNA is increased by 2 h after treatment.

Additionally, a Tet-On-mBop1-cDNA C2C12 cell line that expresses the mature *Bop1* mRNA, which does not contain intronic sequences, was established to evaluate the involvement of *Bop1* intron-derived RNA in the down-regulation of the *Scx* mRNA level. Fig. 4*D* shows that *Bop1* mRNA was induced by doxycycline treatment after 6 h (1.5-fold); however, the level of *Scx* mRNA did not change after *Bop1* mRNA accumulation, suggesting the need for *Bop1* intronic RNA and not mRNA in *Scx* gene down-regulation.

Down-regulation of Scx mRNA Occurs via a Trans-acting Mechanism—The findings above suggested that the accumulation of *Bop1* intron-derived RNA is related to the reduction of the *Scx* mRNA level. Therefore, we examined whether the *Bop1* intronic RNA leads to the down-regulation of the *Scx* gene in a trans-acting manner. The murine fibroblast BALB/3T3 cells were used for this experiment because these cells do not express the endogenous *Scx* mRNA and can be induced to express endogenous *Bop1* by serum stimulation (15). We established the BALB/3T3 cell lines that express *Scx* mRNA from the CMV promoter fused to the *Scx* genomic DNA that contains +1 to +2309 sequence of the *Scx* gene (CMV-Scx-gDNA(+1)), −250 to +2039 sequence of the *Scx* gene (CMV-Scx-gDNA(−250)), or full-length *Scx* cDNA (CMV-Scx-cDNA) (Fig. 5*A*). Initially, we examined the transcripts expressed from the *Scx* genomic DNA and *Scx* cDNA by RT-PCR. The 409-bp amplicon corresponding to mature *Scx* mRNA was detected as the major transcript in these cell lines. The amplicon corresponding to premature *Scx* mRNA (1304 bp) was undetectable in the CMV-Scx-gDNA cell lines.
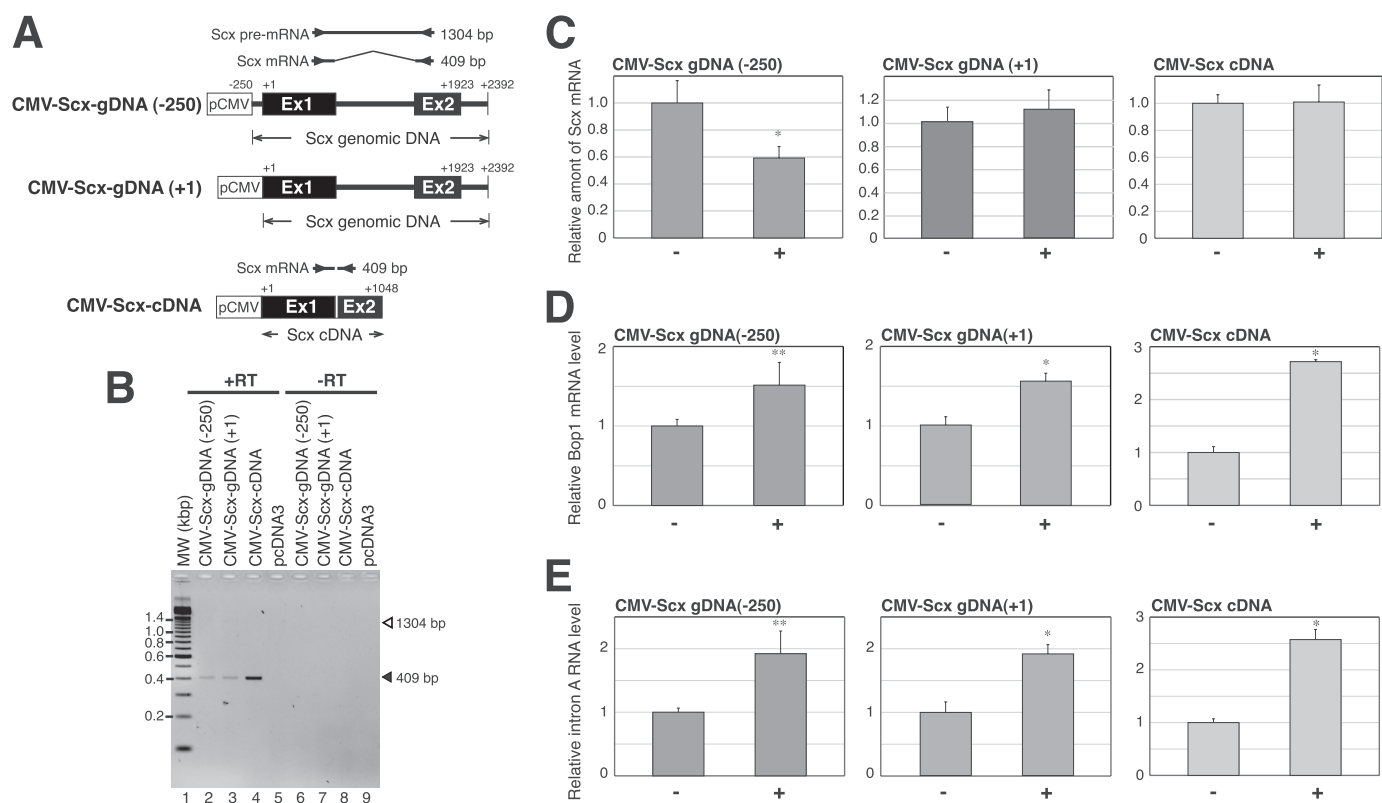
FIGURE 5. **Scx mRNA expressed from the exogenous *Scx* genomic DNA is reduced by increasing the endogenous *Bop1* gene expression.** *A*, schematic representation of CMV-Scx-gDNA(+1), CMV-Scx-gDNA(−250), and CMV-Scx-cDNA is shown. The position of PCR primers used for the amplification of *Scx* mRNAs is indicated. The expected size of the PCR amplicons is shown to the *right* of each primer pair. *Ex1* and *Ex2* indicate exons 1 and 2, respectively. *B*, a representative RT-PCR result of *Scx* transcript is shown. The samples were prepared from BALB/3T3 cell lines containing CMV-Scx-gDNA(+1), CMV-Scx-gDNA(−250), CMV-Scx-cDNA, or pcDNA3 (as a negative control). The samples in *lanes 2–5* were reverse-transcribed (+*RT*) from the RNA, and the samples of *lanes 6–9* were not reverse-transcribed (−*RT*) using the same RNA of 2–5. *MW*, molecular weight markers. *C*, total RNA was prepared from treated (high serum medium; +) or untreated (low serum medium; −) CMV-Scx-gDNA(+1), CMV-Scx-gDNA(−250), and CMV-Scx-cDNA BALB/3T3 cells as described under "Experimental Procedures." A representative real-time PCR result of *Scx* mRNA is shown. *D*, a representative real-time PCR result of *Bop1* mRNA is shown. *E*, a representative real-time PCR result of *Bop1* intronic RNA (primer set A) is shown. The RNA level was normalized to the level of 18 S in each sample. The values are the means ± S.D. for three independent replicates. *, $p < 0.01$ when compared with (−), **, $p < 0.05$ when compared with (−).

No amplicon was detected in the empty pcDNA3 transfected cell line or in the −RT samples, suggesting that this transcript is expressed from the transfected gene (Fig. 5*B*). The cells were cultured in low serum medium for 3 days to reduce the endogenous *Bop1* expression level. *Bop1* was induced in these cells by changing to high serum medium, and the cells were harvested 6 h after changing the medium for RNA extraction. As shown in Fig. 5*C*, *Scx* mRNA decreased 40% 6 h after treatment in the CMV-Scx-gDNA(−250) cell line, whereas the level of *Scx* mRNA did not change in the CMV-Scx-gDNA(+1) and CMV-Scx-cDNA cell lines. The premature *Scx* mRNA was not detected in these cells with or without treatment (data not shown). The levels of *Bop1* mRNA and *Bop1* intron-derived RNA (primer set A) increased 6 h after changing to high serum medium in this system (Fig. 5, *D* and *E*). Taken together, these results suggest that *Bop1* intronic RNA impairs the *Scx* gene transcription and acts in a trans-acting manner.

DISCUSSION

Although gene profiling studies both *in vivo* and *in vitro* show that hormones stimulate and repress gene expression, the mechanisms for estrogen-mediated gene down-regulation are not extensively studied and are poorly understood. Down-regulation of gene expression related to the arrangement of overlapping genes on opposing strands, as a potential mechanism, is thought to be due to transcriptional interference, RNA masking, and/or RNA interference (35, 36). In the transcriptional interference model, because transcription by RNA polymerase II involves both large protein complexes and the unwinding of duplex DNA, it is unlikely that two overlapping transcriptional units could be transcribed concomitantly. Thus, for example, the transcript from the initiator element, which is in the first intron of the *eIF2α* gene on the opposing strand, decreases *eIF2α* transcription (37, 38). In the RNA-masking mechanism, formation of RNA duplexes between sense and antisense transcripts occurs and then inhibits the binding of trans-acting factors to the regulatory features of the transcripts. This form of steric inhibition could affect steps involved in mRNA splicing, transport, polyadenylation, translation, and degradation. As an example of this mechanism, the *TRα* gene encodes *TRα1* as well as an alternatively spliced product, *TRα2*. The *RevErbAα* gene, which is transcribed from the opposite direction from the same locus, overlaps the *TRα2* coding region. The ratio of *TRα1* and *TRα2* expression level is modified by the *RevErbAα* gene posttranscriptionally (39). In the RNA interference mechanism, formation of RNA duplexes leads to gene silencing via RNA interference pathways. Double strand RNA is potentially cleaved by the Dicer enzyme into 21–23-nucleotide duplexes (small inter-

fering RNA). These fragments may target the specific destruction of homologous RNA (40) or silence the gene expression epigenetically (41). In this report, we show that the level of *Scx* mRNA expressed from the exogenous *Scx* genomic DNA, which contains the *Scx* gene promoter (CMV-Scx-gDNA($-$250)), reduced as *Bop1* intron-derived RNA accumulated. The *Scx* mRNA levels expressed from genomic DNA and cDNA without the *Scx* gene promoter (CMV-Scx-gDNA($+$1) and CMV-Scx-cDNA) were not affected by *Bop1* intronic RNA accumulation. This *Scx* gene promoter region, between -250 to $+1$, harbors two TATA boxes that are involved in *Scx* gene transcription initiation (supplemental Fig. S1). This result suggests that the reduction of the *Scx* mRNA level is due at least in part to the inhibition of *Scx* gene transcription. Additional detail of the antisense RNA-mediated *Scx* gene transcription repression will need to be assessed in future studies.

We attempted to identify a negative estrogen-responsive element in the 5′-flanking region (-7 kb) of *Scx*. Examination by reporter analysis did not reveal negative regulatory elements in this region; however, a potential ER-E2-mediated positive regulatory element was found (supplemental Fig. S2). ER-E2-mediated enhancement of the initiation of *Scx* gene transcription might be inhibited by *Bop1* intronic RNA, although further investigation is needed to confirm this hypothesis.

Two different *Scx* knock-out mouse lines are reported, and both indicate the importance of intron 3 of the *Bop1* gene with regard to *Scx* gene expression (21, 23). Both knock-out mice were targeted in a similar genomic position, namely a Neo cassette was inserted into exon 1 of the *Scx* gene to disrupt the expression of *Scx* mRNA (23) or an FRT-Neo cassette was inserted into the intron of the *Scx* gene flanking the first exon, allowing removal of the *Scx* exon 1 using the cre-loxP system (21). Retention of the Neo cassette in this genomic region in both mice lead to embryonic lethality due to the inhibition of cell proliferation between embryonic day 6.0 and embryonic day 6.5 (23), which indicates that this genomic region may be important for correct *Bop1* expression. On the other hand, the deletion of *Scx* exon 1 (about 500 bp) is not embryonic lethal (21). Our ISH and RT-PCR analyses showed that the *Bop1* intronic RNA corresponding to this region exists stably, and our *in vitro* studies showed that the accumulation of *Bop1* intronic RNA impairs the *Scx* gene expression in a trans-acting manner, causing the reduction of the mRNA level. *Bop1* intronic RNA, overlapping with the *Scx* gene, may be a coordinate means by which a mitogenic stimulus such as estrogen invokes an increase in a proliferation gene (*Bop1*) while simultaneously suppressing a differentiation gene (*Scx*) to facilitate the biological action. Further evaluation of other gene pairs will be needed to determine the broadness of this type of regulation and possible application to the hormonal responsiveness in this and other tissues.

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