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Transcriptional regulation of the human establishment of cohesion 1 homolog 2 gene

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ABSTRACT

Transcriptional regulation of human establishment of cohesion 1 homolog 2 (*ESCO2*), the causative gene of Roberts syndrome, was investigated. Deletion and mutation analyses of the *ESCO2* promoter indicated that the selenocysteine tRNA-activating factor (Staf) binding site (SBS) is an essential element for transcriptional activation of *ESCO2*. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay revealed that the zinc finger protein 143 (ZNF143), a human homolog of *Xenopus* Staf, bound to the *ESCO2* promoter. The ACTACAN submotif, adjacent to SBS, also contributed to transcriptional activation of *ESCO2*. EMSA indicated that the ACTACAN submotif was not involved in binding of ZNF143 to SBS. S phase-specific expression of the *ESCO2* gene was confirmed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR), but EMSA revealed binding of ZNF143 to SBS in G1/S and G2/M phases. These results demonstrated that SBS functioned as the basal transcriptional activator of the S phase-specific gene *ESCO2*, but other mechanisms are required for cell cycle-dependent expression.

Introduction

Establishment of cohesion 1 homolog 2 (*ESCO2*) is the causative gene of Roberts syndrome, an autosomal recessive disorder characterized by craniofacial anomalies, tetraphocomelia, and loss of cohesion at heterochromatic regions of centromeres and the Y chromosome [1]. The ESCO2 protein is a member of a conserved protein family involved in establishment of sister chromatid cohesion during S phase and has acetyltransferase activity [2–4]. Recent studies have shown that establishment of cohesion protein 1 (ECO1), the yeast (*Saccharomyces cerevisiae*) homolog of human ESCO2, acetylates structural maintenance of chromosomes 3 (SMC3), a component of the cohesion complex, at two conserved lysine residues [5–7]. Furthermore, ESCO2 is cell cycle-regulated at both the mRNA and protein levels. Transcription of *ESCO2* peaks in S phase [8], while the protein level of ESCO2 increases in early S phase and decreases sharply when cells enter G2/M phase, probably as a result of protein degradation [4].

Selenocysteine tRNA-activating factor (Staf) is a vertebrate transcription factor originally identified in *Xenopus laevis* [9]. There are seven C_2H_2 zinc finger repeats for DNA binding in the middle of the Staf molecule. However, not all seven zinc fingers are involved in the binding of Staf to Staf binding site (SBS) [10–12]. Zinc finger protein 143 (ZNF143) and its paralog zinc finger protein 76 (ZNF76) are two human homologs of Staf [13]. It is interesting that the expression of the ZNF143 gene is not cell cycle-dependent [8], while many cell cycle-regulated promoters contain the potential SBS [14]. Both ZNF143 and ZNF76 upregulate the transcription of the *TCP1* gene [15], and *TCP1* expression is maximum at G1/S transition to S phase [16]. ZNF143 also activates *BUB1B* gene transcription; in addition, G2/M phase-specific transcription of *BUB1B* is mainly regulated through repression by the cell cycle-dependent element (CDE) and cell cycle gene homology region (CHR) element [17].

Here we report that SBS was the essential element for transcriptional activation of *ESCO2*, and ZNF143 bound to the *ESCO2* promoter.

Materials and methods

Reporter and effector constructs. In this study, promoter numbering started from transcription start site of one clone, GenBank ID: AY882862 [Ref. 1; Suppl. Fig. 2].

The human *ESCO2* promoter fragment −1037/+67 was polymerase chain reaction (PCR)-amplified from human genomic DNA. The amplified product was directly cloned at the 5′-end of the firefly luciferase reporter gene into the pGL4.10 vector (Promega). The 5′-end truncated derivatives of the −1037/+67 fragment were PCR-amplified from the construct −1037/+67 and ligated to the pGL4.10 vector. Nucleotide substitutions and deletions were intro-

Abbreviations: ESCO2, establishment of cohesion 1 homolog 2; Staf, selenocysteine tRNA-activating factor; SBS, Staf binding site; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; ZNF143, zinc finger protein 143; RT, reverse transcriptase; PCR, polymerase chain reaction; ZNF76, zinc finger protein 76; CDE, cell cycle-dependent element; CHR, cell cycle gene homology region; DBD, DNA-binding domain.

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duced into the pGL4.10 constructs −750/+67 or −265/+67 using the QuikChange site-directed mutagenesis kit (Stratagene).

Human ZNF143 (GenBank ID: NM_003442) and ZNF76 (GenBank ID: NM_003427) cDNAs were amplified from human thymus (Clontech) and small intestine (Takara) cDNA libraries, respectively, with the following primers:

ZNF143 forward, 5′-TTTCTCGGAGGTAGAAGATGTTG-3′;
ZNF143 reverse, 5′-ATTGCTCCATTGTTCTGAGGA-3′;
ZNF76 forward, 5′-AGTTTGCCATGGAGAGCTTG-3′;
ZNF76 reverse, 5′-CATGCAGATGGCACTTCCTC-3′.

For the *in vitro* translation experiment, ZNF143 and ZNF76 cDNAs were amplified and subcloned into the TnT luciferase T7 control DNA vector (Promega).

For mammalian expression experiments, the EcoRI/BglII sites of the pCAGGS vector [18] were converted into fragments containing a coding sequence of Flag tag, 5′-GAATTCGAGCTCGCGGCCGCTCGAGAGCTGATTACAAGGATGACGATGACAAGTAGACTAG-3′, by the linker ligation method. ZNF143 and ZNF76 cDNAs were amplified and subcloned into pCAGGS/Flag vector. Furthermore, in spite of full-length ZNF143, the DNA-binding domain (DBD) of ZNF143 was introduced into construct pCAGGS/Flag–ZNF143–DBD with the following primers: forward, 5′-CCGGAATTCGCCGCCACCATGGAGAAGGCATTTCGATGTG-3′; and reverse, 5′-CGAGCGGCCGCCGGGCTCAGTGTCGTTGTGGGC-3′.

For stable transformant assays, the PCR-amplified ZNF143 cDNA was subcloned into the NotI/BamHI-digested p3×Flag–CMV-14 vector (Sigma). The Kozak consensus sequence (GCCGCCACCATG) was incorporated immediately upstream of the BamHI site in the p3×Flag–CMV-14 vector using the QuikChange site-directed mutagenesis kit (Stratagene) to establish mock-transfected HeLa cell lines.

Cell culture, transfections, and luciferase reporter assays. HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (streptomycin and penicillin at usual concentrations).

For transfection experiments, HeLa cells were grown at a density of 0.5×10^4 cells/well. Subsequently, 200 ng of each pGL4.10-based reporter construct was transfected into HeLa cells with 4 ng of the phRL-TK vector (Promega) as an internal control using FuGENE 6 (Roche Applied Science) reagent according to the manufacturer's instructions. The transfected cells were maintained for 24 h in serum-supplemented medium before harvesting. At the end of the culture period, the luciferase activities were measured using a dual luciferase reporter system (Promega).

Cloning of stable transfectants. Two micrograms of p3×Flag–CMV-14–ZNF143 or p3×Flag–CMV-14–Mock were transfected into 1×10^5 HeLa cells using FuGENE 6. After transfection, the cells were maintained in medium containing 1 mg/ml G418 (Nacalai Tesque). The cellular expression level of the ZNF143–3×Flag fusion protein in each clone was investigated by Western blotting with anti-Flag M2 antibody (Sigma).

Cell synchronization and cell cycle analysis. HeLa cells were synchronized in G1/S phase by a double thymidine block [19], and then harvested for electrophoretic mobility shift assay (EMSA) and luciferase reporter assay. In addition, after the release from a double thymidine block, the cells were allowed to grow in fresh serum-supplemented medium, and samples were harvested every 3 h for 24 h for cell cycle analysis and real-time reverse transcriptase (RT)-PCR assay. The cells were also arrested in G2/M phase using a thymidine–nocodazole block. In brief, the cells were first treated with 2 mM thymidine (Wako), released into fresh medium for 3 h, and then blocked with medium containing 100 ng/ml nocodazole (Sigma) for 18 h.

For cell cycle analyses, cells were stained with 50 μg/ml propidium iodide (Dojindo) containing 1 mg/ml RNase A (Wako). Cell cycle distribution was determined by analyzing the DNA content using Cellquest (Becton Dickinson) and ModFit LT (Verity Software House) software and a FACScan cytometer (Becton Dickinson).

RNA isolation and real-time PCR. Total RNA was prepared from HeLa cells using the RNeasy mini kit (Qiagen). Contaminating genomic DNA was removed using the RNase-free DNase set (Qiagen), and cDNA was synthesized from 0.5 μg of total RNA using the PrimeScript 1st strand cDNA synthesis kit (Takara) with random hexamers. Transcript levels were quantified by real-time PCR with QuantiTect SYBR green PCR mix (Qiagen) using a DNA Engine PTC-200 with a Chromo4 Real-Time module (MJ Research). The following primers were used in real-time PCR: *ESCO2*, forward primer 5′-TAATGAATTGGGCTTCCAGC-3′ and reverse primer 5′-AGGGGTGTTGCAGTACTTGG-3′; and *GAPDH* (internal control), forward primer 5′-TGCACCACCAACTGCTTAGC-3′ and reverse primer 5′-GGCATGGACTGTGGTCATGAG-3′. Cycling parameters consisted of 30–40 cycles at 95 °C for 20 s, 58–59 °C for 30 s, and 72 °C for 30 s (depending on the primer pair).

Electrophoretic mobility shift assays. Recombinant ZNF143 and ZNF76 were synthesized using TnT quick-coupled transcription/translation systems (Promega) according to the manufacturer's instructions. Proteins synthesized *in vitro* were detected using Transcend nonradioactive translation detection systems (Promega).

Nuclear extracts from HeLa cells were prepared using the EpiQuik nuclear extraction kit I (Epigentek).

To prepare whole cell extracts, HeLa cells transfected with constructs expressing recombinant ZNF143 or ZNF76 were lysed at 2×10^5 cells/μl in a buffer containing 20 mM Hepes (pH 7.4), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mM NaF, 1 mM Na-orthovanadate, protease inhibitors (nacalai tesque), and 1% NP-40. The insoluble fraction was then pelleted and removed.

EMSA analyses were performed as follows: SBS probe (5′-GACCGAGAACTACAATTCCCAGAATGCACCTAAACAAGG-3′; 50,000 cpm) end-labeled using [α-^{32}P]dCTP (PerkinElmer) and the Klenow fragment of *Escherichia coli* DNA polymerase I (Takara) were incubated with 2 μl *in vitro* translation reaction products, 15 μg nuclear extracts, or 5 μg whole cell extracts from HeLa cells expressing ZNF143 or ZNF76 protein, respectively. For supershift assays, antibodies were added to the binding assay mixtures after adding a radiolabeled probe. Rabbit antiserum against ZNF143 was kindly provided by Dr. Kubota, and the IgG fraction was purified from this rabbit antiserum by protein G-Sepharose (Amersham Biosciences) column chromatography. Control normal rabbit IgG was purchased from Jackson ImmunoResearch. DNA–protein complexes were separated on 4–6% polyacrylamide gels. Images were captured using a FLA-9000 (FujiFilm).

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed as described [20]. In brief, soluble chromatin was prepared from 1% formaldehyde-fixed HeLa cells, and immunoprecipitated with 10 μg control normal mouse IgG (Jackson ImmunoResearch) and anti-Flag M2 (Sigma) antibodies. Immunoprecipitates were brought down with Dynabeads M-280 (Invitrogen). DNA was purified from the bound and input fractions, and quantified by real-time PCR as described above. The following primers were used to detect SBS in the *ESCO2* promoter and *CCDC25* (GenBank GeneID 55246) intron (control region), located approximately 2 kb upstream of the transcription start site of *ESCO2*: SBS, forward primer 5′-AATTTCTTACCCCGTGACCC-3′ and reverse primer 5′-GCTGTTTCCAAGATTCTCGC-3′; and CCDC25 intron, forward primer 5′-TTGTTTTCTGTTGCATCCCA-3′ and reverse primer 5′-TTCTTCCCACCTTCCATCTG-3′. Cycling parameters consisted of 40 cycles at 95 °C for 20 s, 54 °C for 30 s, and 72 °C for 30 s.

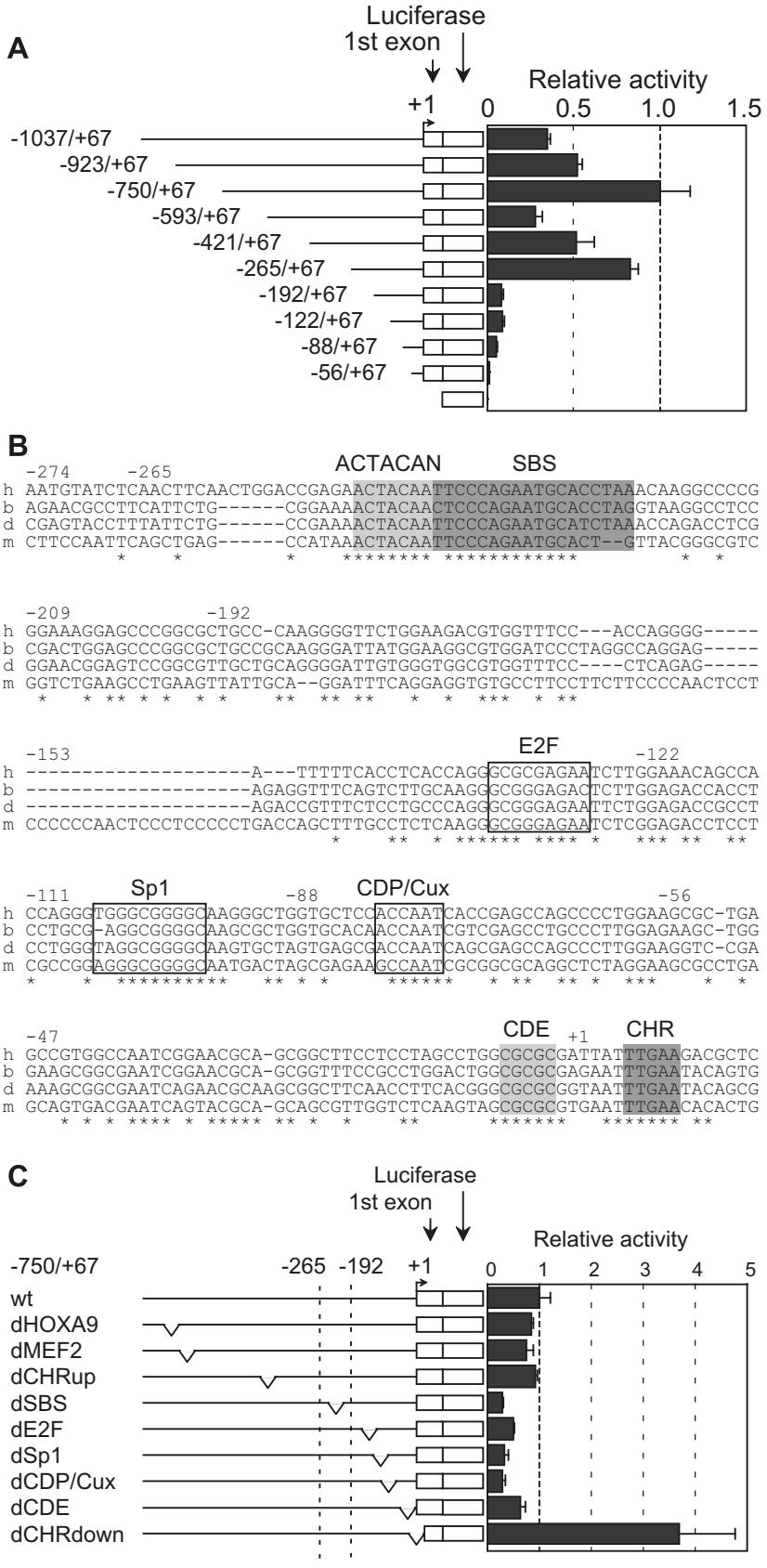

Fig. 1. Deletion analyses and cross-species conservation of the *ESCO2* promoter region. (A) Schematic representation of 5′-deleted *ESCO2* promoter–luciferase constructs. Values are expressed as relative reporter activity normalized to the construct −750/+67. Data are means ± SD in triplicate. A representative result of three independent experiments is shown. (B) Nucleotide sequence comparison of human (h), bovine (b), dog (d), and mouse (m) *ESCO2* promoters. Multiple sequence alignments were performed with ClustalW. Identical nucleotides are indicated by asterisks. (C) Schematic representation of site-directed deletion *ESCO2* promoter–luciferase constructs. Relative reporter activity was normalized to the wild-type construct −750/+67. Data are presented as in (A).

Results and discussion

Conserved cis-element responsible for transcriptional activity of ESCO2

Deletion analysis was conducted in the −1037/+67 region of the *ESCO2* promoter (Fig. 1A). Among the various reporter constructs based on the pGL4.10 vector, the −750/+67 region showed the highest luciferase reporter activity, followed by the −265/+67 region, whose activity revealed 83% of the construct −750/+67. The −192/+67 construct diminished the reporter activity by 90% as compared to the construct −265/+67. There was also a slight downward trend in reporter activity from −192 to −88. On the contrary, the construct −56/+67 almost completely abolished reporter activity.

Computational analysis of the *ESCO2* promoter sequence using DiAlign TF software (Genomatix; www.genomatix.de/index.html) was performed to search for possible cis-acting elements that were highly conserved across species (human, bovine, dog, and mouse). Eight predicted elements were found in the 2 kbp upstream of the transcription start site of *ESCO2*, located adjacent to the 3′-flanking site of the *CCDC25* gene. The locations of the six predicted elements and the corresponding transcription factors were as follows: region −690/−674, HOXA9; −648/−626, MEF2; −240/−218, ZNF143/76; −140/−124, E2F; −108/−94, Sp1; and −84/−66, CDP/Cux (or NF-Y). Immediately upstream of the hStaf/ZNF143 binding site (SBS), the interspecies conserved 7-bp ACTACAN submotif was also found (Fig. 1B). However, this submotif has not been reported to bind with known transcription factors. The remaining two sites were both CHR elements that were located at regions −421/−409 and +2/+14. There was a conserved sequence, which was similar to the consensus motif of CDE (−6 to −2), 6 bp upstream of the CHR element located near the transcription start site.

To ascertain whether these nine putative cis-acting elements could regulate *ESCO2*, further deletion analyses was performed to the −750/+67 region using pGL4.10 reporter constructs (Fig. 1C). Each deletion construct for the binding sites of ZNF143/76, E2F, Sp1, and CDP/Cux (or NF-Y) led to reduction in reporter activity to less than half of the wild-type activity. The reporter activity of the deletion construct for SBS was the minimum (29% of the wild-type level). Moreover, it was striking that the deletion construct of the CHR element near the transcription start site revealed more than 3.5-fold reporter activity compared to the wild type (Fig. 1C).

These findings suggest that the −265/−193 region in the *ESCO2* promoter contained an essential transcription activator and the −265/+67 region was the core promoter. Considering that SBS was located within region −265/−193, SBS was thought to be a major contributing element to *ESCO2* transcription.

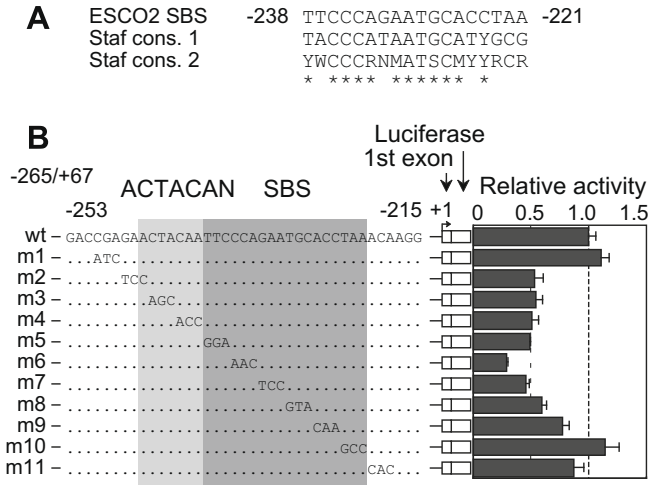

Fig. 2. Mutation analyses of the ACTACAN submotif and SBS in the *ESCO2* promoter. (A) Comparison of the SBS sequence of the *ESCO2* promoter and consensus sequences determined by binding site selection under highly (Staf cons. 1) or moderately (Staf cons. 2) stringent conditions (Y, W, R, N, M, and S stand for T/C, A/T, A/G, any nucleotide, A/C, and G/C, respectively). Identical nucleotides are indicated by asterisks. (B) Triple point mutations were introduced into the *ESCO2* promoter–luciferase construct −265/+67 as indicated. Residues that are the same as in the wild type are indicated by dots. Values are expressed as relative reporter activity normalized to the wild-type construct −265/+67. Data are means ± SD in triplicate. A representative result of three independent experiments is shown.

Effects of the ACTACAN submotif and SBS on ESCO2 promoter activity

In Fig. 2A, *ESCO2* SBS was aligned with the reported consensus SBS sequences determined by binding selection [10,21], and 12 out of 18 bp were identical to the consensus sequence. Next, to examine the sequence specificity of the conserved ACTACAN submotif and SBS, triple point mutations were serially introduced into the segment extending from −250 to −218 of the *ESCO2* promoter, and the transcriptional activities of these mutations were analyzed by luciferase reporter assay (Fig. 2B). Mutation constructs of m2, 3, 4, and 5 corresponding to the −247/−236 region showed approximately half the reporter activity of the wild type. Mutation in the CCA sequence (m6: region −235/−223) revealed the strongest reduction in *ESCO2* transcription (29% of the wild-type level). Interestingly, reporter activity gradually recovered as the mutation site moved from 5′ to 3′ direction along the CCAGAATGCACCTAA sequence (m6, 7, 8, 9, and 10: region −235/−221) and finally reached the wild-type level.

These findings indicated that both SBS and the ACTACAN submotif were essential for *ESCO2* transcription. To our knowledge, this is the first report demonstrating that the SBS-adjacent ACTACAN submotif in a native endogenous promoter has a transcriptional function.

ZNF143 binds to the ESCO2 promoter in vitro and in vivo

The ^{32}P-labeled DNA probe (fragment −253/−215) encompassing the ACTACAN submotif and SBS of the *ESCO2* promoter was incubated with *in vitro* translated recombinant ZNF143, and the complex between the hot probe and ZNF143 was observed in EMSA (Fig. 3A). The competition assay with the wild-type and mutant cold probes showed that the CCA sequence at SBS (m6: region −233/−235) was most important for this binding capacity and the ACC sequence (m9: region −226/−224) partially contributed to the binding, however the AGC sequence at the ACTACN submotif (m3: region −242/−240) and the TAA sequence at SBS (m10: region −223/−221) did not engage in the binding. Furthermore, native endogenous and intracellularly expressed ZNF143 from HeLa nuclear extracts also bound to the above ^{32}P-labeled DNA probe. The specificity of binding was confirmed using the competition assay. The presence of endogenous ZNF143 in the complex was assessed by supershift analysis using anti-ZNF143 antibody.

In addition, the binding of ZNF143 to the *ESCO2* promoter was confirmed by a ChIP assay (Fig. 3B). As expected, we observed that the *ESCO2* promoter displayed strong binding of ZNF143–3×Flag fusion protein, whereas the control region, *CCDC25* intron, showed weaker binding. Only background signals were shown in negative controls.

Moreover, binding of the ^{32}P-labeled probe with recombinant full-length ZNF143/76 and DBD of ZNF143, which were produced from whole cell extracts of transfected HeLa cells, was demonstrated and competed in the same manner as *in vitro* translated and endogenous ZNF143 (Fig. 3C).

These results indicated that SBS was recognized by both recombinant ZNF143/76 and endogenous ZNF143 in a sequence-specific manner through its DBD. Moreover, the binding affinity of ZNF143

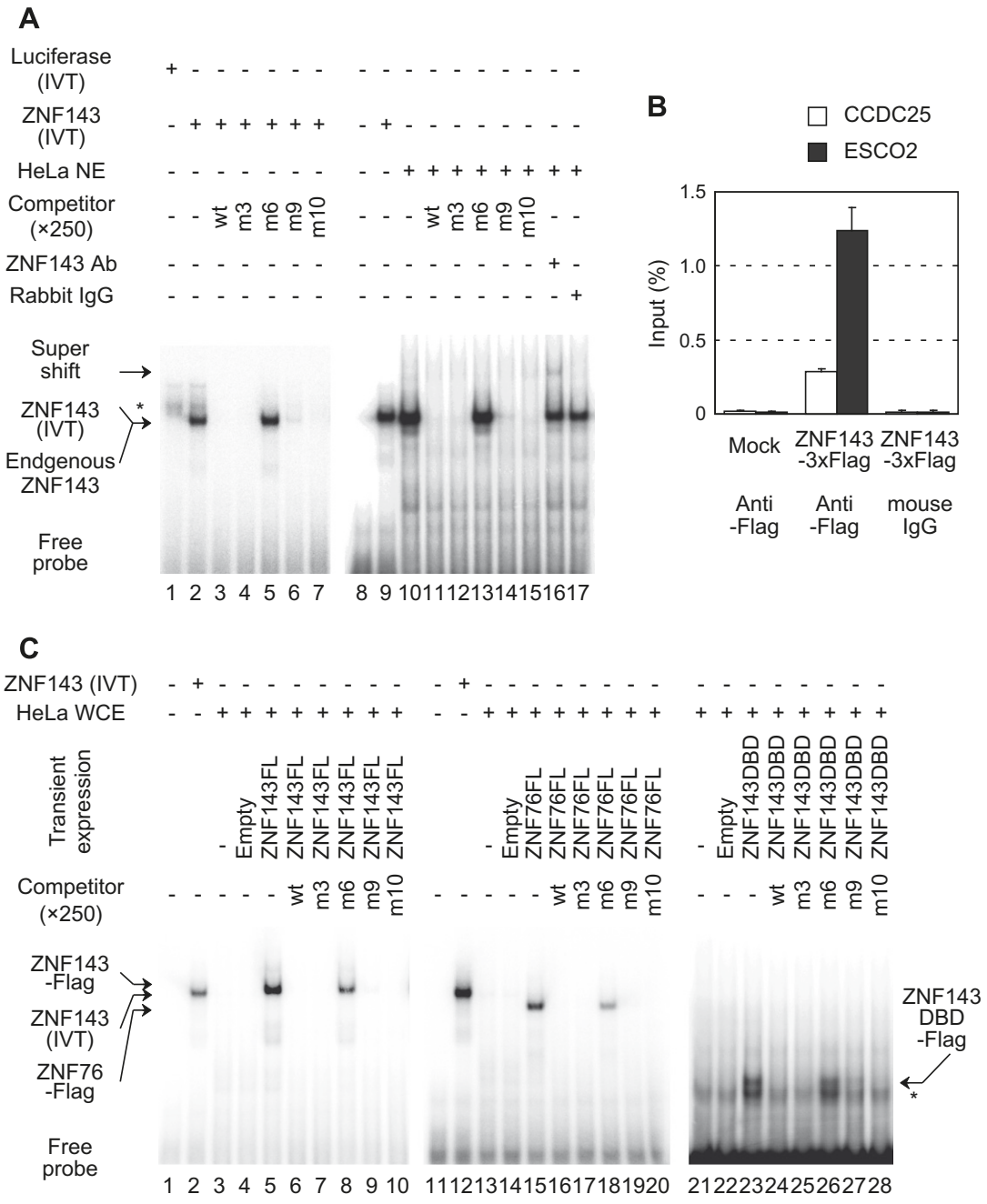

Fig. 3. ZNF143 binds to the *ESCO2* promoter *in vitro* and *in vivo*. (A) The ^{32}P-labeled DNA probe encompassing region −253/−215 of the *ESCO2* promoter was incubated in the absence of protein (lane 8) or presence of *in vitro* translated luciferase (lane 1) or ZNF143 (lanes 2–7, 9) and nuclear extracts (NE) from HeLa cells (lanes 10–17). Competition assays were performed in the absence (lanes 2, 10) or presence of a 250-fold molar excess of unlabeled wild-type (lanes 3, 11) and mutant (lanes 4–7, 12–15) probes. The nucleotide sequences of wild-type and mutant probes are shown in Fig. 2B. Reactions were performed with rabbit anti-ZNF143 antibody (lane 16; 10 μg) or control rabbit IgG (lane 17; 10 μg). EMSA analyses in lanes 1–7 and 8–17 were performed in separate experiments. The positions of the shifted and supershifted complexes are shown by arrows. The asterisk indicates nonspecific bands. (B) ChIP assay was performed with stable transfected HeLa cells expressing ZNF143–3×Flag fusion protein or mock-transfected HeLa cells using anti-Flag M2 antibody or control mouse IgG. The bound and input fractions were quantified by real-time PCR, and ratios of bound to input are indicated as the percentage of input. Data are means ± SD in triplicate PCRs. A representative result of two independent experiments is shown. (C) Probes were incubated in the absence of protein (lanes 1, 11) or presence of *in vitro* translated ZNF143 (lanes 2, 12) and whole cell extracts (WCE) from HeLa cells untransfected (lanes 3, 13, and 21) or transiently transfected with pCAGGS/Flag–Empty (lanes 4, 14, and 22), pCAGGS/Flag–ZNF143–FL (lanes 5–10), pCAGGS/Flag–ZNF76–FL (lanes 15–20), and pCAGGS/Flag–ZNF143–DBD (lanes 23–28). Competition assays were performed as in (A) (lanes 6–10, 16–20, and 24–28). EMSA analyses in lanes 1–10, 11–20, and 21–28 were performed in separate experiments. The positions of the shifted and supershifted complexes are shown by arrows.

to the *ESCO2* promoter demonstrated by the competition assay (Fig. 3) was correlated with variations of luciferase reporter activities indicated by sequential mutation of SBS (Fig. 2B), and was in agreement with the previous studies on the minimal region of Staf for high-affinity binding to SBS [10–12]. However, the ACTACAN submotif is not necessary for this binding, although it was contribute to transcriptional activation of *ESCO2*.

Transcription of ESCO2 is cell cycle-regulated

Based on findings obtained by cDNA microarrays, transcription of *ESCO2* is regulated in a cell cycle-dependent manner during S phase [8]. We also confirmed S phase-specific expression of *ESCO2* by real-time RT-PCR (Fig. 4A). HeLa cells were synchronized in G1/S phase by a double thymidine block. The transcription level

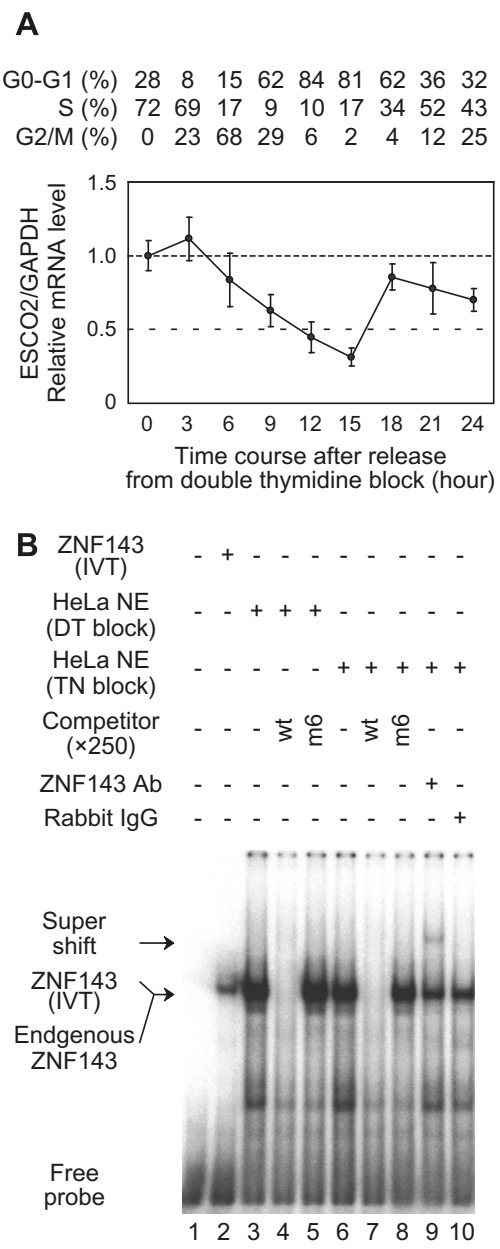

Fig. 4. Expression of *ESCO2* mRNA was cell cycle-regulated. (A) HeLa cells were synchronized by a double thymidine block and released. The distribution of cells over the cell cycle stages was determined. *ESCO2* mRNA levels were normalized to *GAPDH* mRNA levels. Data are means ± SD in triplicate PCRs. (B) EMSA with a ^{32}P-labeled DNA probe encompassing positions −253/−215 of the *ESCO2* promoter in the absence of protein (lane 1) or presence of *in vitro* translated (IVT) ZNF143 (lane 2) and HeLa nuclear extracts (NE) from cells synchronized by a double thymidine (DT) block (lanes 3–5) or thymidine–nocodazole (TN) block (lanes 6–10). Competition assays were performed in the absence (lanes 3, 6) or presence of a 250-fold molar excess of unlabeled wild-type (lanes 4, 7) and mutant (lanes 5, 8) probes. The nucleotide sequences of wild-type and mutant probes are shown in Fig. 2B. Reactions were performed using rabbit anti-ZNF143 antibody (lane 9; 10 μg) or control rabbit IgG (lane 10; 10 μg). Positions of the shifted and supershifted complex are shown by arrows.

peaked 3 h after release from the block (in S phase), declined gradually from 3 to 15 h through the G2/M–G1 phases, and increased again 18 h later (in the second S phase).

To investigate whether the binding of endogenous ZNF143 to SBS in the *ESCO2* promoter is cell cycle-dependent, we performed EMSA using the ^{32}P-labeled probe containing SBS (fragment −253/−215) and nuclear extracts from HeLa cells. The labeled probe and endogenous ZNF143 complexes were detected in cells synchronized both in G1/S phase by double thymidine and in G2/M phase by thymidine–nocodazole blocks (Fig. 4B). These results suggest that ZNF143 is not sufficient to account for the cell cycle-dependent transcription of *ESCO2*.

The tandem CDE/CHR element located near the transcription start site is a frequently observed element in cell cycle-regulated promoters and had been initially identified in S/G2 phase-specific transcription promoters such as *CDC2*, *CCNA2*, and *CDC25C* [22,23]. The *ESCO2* promoter also contains the CHR element conserved between species (Fig. 1B), which functioned as a transcriptional repressor (Fig. 1C). We thus speculate that the CHR element participates in cell cycle transcriptional regulation of *ESCO2*.

In conclusion, this study extended the role of SBS to that of an essential activator of the S phase-specific *ESCO2* promoter, but SBS was not involved in cell cycle-dependent expression. The future challenge is to investigate how the CDE/CHR element engages in cell cycle-dependent transcriptional regulation of *ESCO2*.

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