

Switched alternative splicing of oncogene CoAA during embryonal carcinoma stem cell differentiation

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ABSTRACT

Alternative splicing produces functionally distinct proteins participating in cellular processes including differentiation and development. CoAA is a coactivator that regulates transcription-coupled splicing and its own pre-mRNA transcript is alternatively spliced. We show here that the CoAA gene is embryonically expressed and alternatively spliced in multiple tissues to three splice variants, CoAA, CoAM and CoAR. During retinoic-acid-induced P19 stem cell differentiation, the expression of CoAA undergoes a rapid switch to its dominant negative splice variant CoAM in the cavity of the embryoid body. CoAM functionally inhibits CoAA, and their switched expression up-regulates differentiation marker Sox6. Using a CoAA minigene cassette, we find that the switched alternative splicing of CoAA and CoAM is regulated by the *cis*-regulating sequence upstream of the CoAA basal promoter. Consistent to this, we show that p54^{nrb} and PSF induce CoAM splice variant through the *cis*-regulating sequence. We have previously shown that the CoAA gene is amplified in human cancers with a recurrent loss of this *cis*-regulating sequence. These results together suggest that the upstream regulatory sequence contributes to alternative splicing of the CoAA gene during stem cell differentiation, and its selective loss in human cancers potentially deregulates CoAA alternative splicing and alters stem cell differentiation.

INTRODUCTION

Most eukaryotic genes contain interspersed introns that are removed from pre-mRNA by RNA splicing to yield functional mRNA (1,2). In addition to constitutive splicing, the majority of human gene transcripts is

alternatively spliced, producing distinct mRNA products which contribute greatly to the complexity of the human proteome (3,4). Alternative splicing is cell-type-specific and developmentally regulated (5–7). Splicing variants yield functionally different proteins which are often critical for specific cellular functions. In contrast, altered alternative splicing patterns are often associated with different types of diseases (8), and aberrant alternative splicing is one of the characteristics of cancer cells (9).

Alternative splicing has been extensively shown to be coupled with transcriptional regulation. A number of regulatory proteins may be involved in alternative splicing decisions. These proteins are in the spliceosome particles, the SR protein family (10), the heterogeneous nuclear ribonucleoproteins (hnRNPs) and a number of cofactor proteins involved in transcriptional regulation (11). Although intronic and exonic sequence elements within pre-mRNA are required for splicing, substantial evidence also suggests that protein complexes recruited by promoter sequences determine, in a transcription-coupled manner, the choices and patterns of alternative splicing (12,13). For example, steroid hormones have been shown to regulate alternative splicing through nuclear hormone receptors and their coactivators in promoter complexes (12). One mechanism by which promoter sequences may influence splicing is by directing the assembly of splicing factors on the RNA polymerase II (RNAP) C-terminal domain during RNA synthesis (14,15).

Coactivator CoAA (coactivator activator) was originally cloned as a nuclear receptor coactivator TRBP/NcoA6-interacting protein (16,17). CoAA stimulates transcriptional activation mediated by nuclear receptors and transcription factors, and also regulates alternative splicing through a number of steroid hormone receptor-stimulated promoters (12,18). CoAA (also known as paraspeckle protein 2, PSP2) is a major component of nuclear paraspeckles and is colocalized with splicing factor p54^{nrb} (19,20). CoAA is a member of the hnRNP family and contains two N-terminal RNA recognition motifs (RRMs) and a C-terminal transcriptional

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activation domain with 27 repeating tyrosine- and glutamine-rich motifs (16,21). Both RRM1 and the C-terminal activation domain are essential for CoAA activities. The C-terminal repeats are homologous to sequences in a family of oncoproteins containing EWS (Ewing's sarcoma) and TLS/FUS (translocation/fusion in liposarcoma) (21), which regulate alternative splicing. The human CoAA gene itself is also alternatively spliced. One of the splice variants, termed CoAM (coactivator modulator), contains the N-terminal RRM1 but lacks the C-terminal activation domain (16). CoAM serves as a natural dominant negative form antagonizing the functions of CoAA in transcription and alternative splicing (16,18).

We have recently shown that the CoAA gene at chromosome 11q13 is amplified in human cancers including lung and skin cancers (21). Molecular analysis reveals the CoAA-coding sequence and its basal promoter are retained within the amplicon, whereas an upstream sequence is not (21). The recurring gene aberration with loss of the CoAA regulatory sequence implies a potential functional importance of the CoAA gene regulation, including its alternative splicing. In this study, using an embryonal carcinoma (EC) P19 stem cell neuronal differentiation system, we demonstrate that the expression of CoAA dramatically switches to its alternative splicing variant CoAM in cells destined to form the cavity of embryoid bodies (EBs). Overexpression of CoAM induces differentiation marker Sox6 in the absence of retinoic acid (RA), suggesting the switch from CoAA to CoAM expression may play an important role in promoting differentiation. Furthermore, we show that deletion of a CoAA upstream regulatory element, which is lost in a number of cancers, impairs the switch in alternative splicing from CoAA to CoAM in a CoAA minigene cassette. In particular, CoAA down-regulation is absent. Data thus support a model in which loss of the regulatory element disturbs the balance of alternative splicing of the CoAA gene and results in constant CoAA expression and defective CoAM expression. This defect may promote maintenance of a stem cell phenotype and prevent differentiation. In summary, our study indicates that switched alternative splicing of the CoAA gene is regulated by its upstream sequence during stem cell differentiation.

MATERIALS AND METHODS

Immunohistochemistry

Polyclonal anti-CoAA antibody (CoAA specific, against 307–545 aa) and anti-RRM1 antibody (against 1–156 aa of CoAM) were prepared in rabbits by immunization with GST fusion proteins (Covance). His-tagged CoAA protein as antigen was crosslinked to the Affi-gel 10 resin according to the manufacturer's protocol (Bio-Rad), and was used for affinity purification of anti-CoAA (21). Sagittal sections of mouse embryonic tissue at E12.5 and E15.5 were stained with affinity purified anti-CoAA at a dilution of 1:250. The P19 EBs were paraffin-embedded and the sections were stained with anti-CoAA, anti-RRM1 antibodies at dilution of 1:500 and with anti-active caspase-3 peptide antibody at 1:200 (USBiological,

C2087-16A). Antibody binding was detected using biotinylated anti-rabbit or anti-mouse IgG F(ab)₂ secondary antibody followed by detecting reagents (DAKO). Sections were counterstained with hematoxylin.

Immunoblotting

Endogenous CoAA and CoAM in P19 cells were detected using whole-cell extracts at each differentiating stage and probed with anti-RRM1 antibody. For antibody evaluation, CoAA and CoAM were overexpressed in CV1 cells under the control of a CMV promoter in pcDNA3 vector (Invitrogen). Immunoblots were probed with anti-CoAA and anti-RRM1 primary antibodies at a dilution of 1:200 and detected with the ECL system (Amersham Pharmacia).

P19 cell and ES cell culture and differentiation

Mouse EC P19 cells were maintained in α -modified minimum essential medium supplemented with 7.5% bovine calf serum and 2.5% fetal bovine serum, 100 U/ml penicillin and 0.1 μ g/ μ l streptomycin. Cells were incubated in 5% CO₂ at 37°C. Undifferentiated murine EC P19 cells were induced by 500 nM all-*trans* RA (Sigma) up to 4 days in suspension culture to form EBs (EB2–EB4). The EBs were trypsinized and plated in tissue culture dish. The cells were further differentiated (D3–D12) for an additional 12 days in the absence of RA. P19 cells were transfected with the plasmid or siRNA of CoAA (25 nM) (21), when applicable, using Lipofectamine 2000 reagent (Invitrogen) for 24 h before harvest. Total amounts of DNA for each well were balanced by adding vector DNA. Mouse embryonic stem (ES) cells (D3, 129S2/SvPas blastocysts, ATCC) were maintained on gamma-irradiated (30 Gy) mouse embryonic fibroblast feeder layers. The culture and differentiating conditions were as previously described (22). Briefly, neuronal differentiation of ES-cell-derived EBs was induced by 1000 nM RA for 6 days to form EBs (EB2–EB6). Undisrupted EBs were differentiated in culture in the absence of RA for an additional 15 days (D3–D15) before harvest.

Luciferase assay

CV-1 cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 0.1 μ g/ μ l streptomycin, and were incubated in 5% CO₂ at 37°C. CV-1 or P19 cells were transfected in triplicate in 24-well plates using Lipofectamine 2000 (Invitrogen). Cells were incubated with ligand dexamethasone (100 nM) to induce MMTV-luciferase reporter, when applicable, for 16 h before harvest. Total amounts of DNA for each well were balanced by adding vector DNA. Relative luciferase activities were measured by a Dynex luminometer. Data are shown as means of triplicate transfections \pm standard errors.

Construction of CoAA minigene

To facilitate promoter analysis, a shortened CoAA minigene was constructed as shown in Figure 5A. The CoAA minigene was designed to prevent the expression of functional CoAA and CoAM proteins that otherwise might interfere with splicing of its own minigene. Excluding the promoter region, the human CoAA gene spans ~11 kb containing three exons, nt 1–432, 7589–9053 and 9836–10718 (Figure 5A). Deletions were introduced to the minigene within the first intron (645–7419), within the second exon (7876–8801) that encodes the activation domain, and within the third exon (10044–10718) containing the 3' untranslated region. A 1-nt (G) insertion in the first exon disrupted the open reading frame and prevented the production of RRM domains. Expression of CoAA and CoAM transcripts was detected using vector-specific primers that distinguish the minigene and the endogenous gene transcripts. A cassette consisting of the CoAA minigene linked naturally to various fragments of its own promoter was inserted into a promoter-less pcDNA3 vector (BglII to XhoI). As a control, a separate construct was prepared with the CoAA minigene expressed under the control of a CMV promoter in pcDNA3.

RT-PCR and real-time quantitative PCR

Normalized first-strand cDNAs from multiple normal human tissues and cancer cell lines (MTCTM panels, Clontech) were analyzed by PCR using primer pairs common to all CoAA splicing forms. For P19 cells, total RNA was isolated at each differentiation stage using Trizol reagent (Invitrogen), treated with DNase I, and normalized for their concentrations before use. RT-PCR was performed using the one-step RT-PCR kit (Qiagen). In transfection experiments, P19 cells were cotransfected with CoAA minigenes and p54^{nrB} or PSF expression plasmids. Primer pairs used in RT-PCR are as follows: (from 5' to 3') primers common to endogenous CoAA, CoAM and CoAR splicing forms, atgaagatattctgtgggcaa, ctaaaccgctcggaacc; CoAM-specific, tctccaccaagggtatggt, ctacatcgccgctggta; Nanog, agggctctgactgagatgctctg, caacctggttttctgccaccg; Oct4, ctgaggccaggcaggagcag, ctgtagggaggcttcgggcaactt; Sox6, cagcggatggagagg aagcaatg, cttttctgttcatcatgggctgc; MAP2, ggacatcagcct cactcacaga, gcagcatgtcaaagtctcacc; GFAP, gaatgactcct cactccctgc, cgctgtgaggtctggcttggc; and GAPDH, accaca gtcatgccatcac, tcaccaccctgttctgtga. Primer pairs for detecting transcripts from the CoAA minigene are as follows: F, aatgtgtcggctgcatgc; B, ctaaaccgctcggaacc; C, tatgaagagatacggcctggttc; V, atggctgcaactagaaggcag; J, tctccaccaagggtatggtt; and A, gctgtgctgcccggtag. Real-time quantitative RT-PCR primers: CoAA, ctctgcac taccagcaggctttt, cgctcagaggcggccacataag; and CoAM, caaa gaagtgaagggaagc, aatccagcaggacttggc. Quantitative RT-PCR was performed on iCycler (BioRad) using SYBR Green I (Invitrogen).

Chromatin immunoprecipitation (ChIP)

P19 cells at each stage of differentiation were treated with 1% formaldehyde for 10 min, and the crosslinking was

stopped by 125 mM glycine. Cells were lysed and sonicated in the buffer containing 20 mM Tris pH 8.0, 75 mM NaCl, 75 mM KCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM DTT and protease inhibitors. Immunoprecipitation was carried out using salmon sperm DNA-blocked protein A/G resin (Upstate), and anti-RNAP II (8WG16, Covance), or anti-SRP20 (Santa Cruz), or anti-NF-YA (Santa Cruz), or anti-p54^{nrB} (BD biosciences) in the above buffer except with 0.1% Triton at 4°C overnight. The resin in the absence of antibody was a control. The crosslinking was reversed by eluting with 0.1 M NaHCO₃, 1% SDS, 0.3 M NaCl at 65°C for 4 h. Purified DNA (Qiagen kit) was subjected to PCR analysis. Input was 1% extract before immunoprecipitation. Primer pairs used are the following: –80 bp, ggccggaggtagctcttctgac, ctccacaggaatg ctggcgac; –3000 bp, attagaatgccttcaagggg, ctccggctgata gtggacatac; and –8500 bp, caggcggactcgggtctttag, caaacctcatatacggagtgc.

Sequence analyses and CoAR accession

Alu repeats within the regulatory sequences of the CoAA gene were identified using Censor Server at Genetic Information Research Institute (www.girinst.org) and the BLAST search at the National Center for Biotechnology Information (NCBI). Transcription-factor-binding sites within the CoAA basal promoter were predicted by the TFSEARCH program at the Computational Biology Research Center of Japan (www.cbrc.jp). Human CoAR sequences reported in this article have been deposited at GenBank/EBI Data Bank with accession number DQ294957.

RESULTS

Alternative splicing of CoAA gene transcripts

The human CoAA gene (gene symbol *RBM14*) contains three exons spanning ~11 kb. We have previously shown that the CoAA gene is alternatively spliced and produces CoAA and CoAM transcripts through competitive 5' alternative splicing events between the second and third exons (Figure 1A) (16,21). The resulting CoAA and CoAM proteins share two N-terminal RRM domains, but only CoAA possesses the C-terminal activation domain containing repeated YxxQ motifs (16, 21). Consequently, CoAA is a potent transcriptional coactivator, whereas CoAM competes with CoAA via shared RRM domains and represses CoAA activities in both transcription and splicing (16,18). In this study, using RT-PCR analysis we identified a third splice variant of CoAA, designated here as CoAR (coactivator regulator), in multiple human adult and embryonic tissues, and in cancer cell lines (Figure 1B). Sequencing analysis indicated that the CoAR transcript was derived by joining the first and third exons, skipping the entire second exon and altering the reading frame for the third exon. CoAR contains only one RRM domain encoded by the first exon. The transcriptional activity of CoAR is moderately repressive when expressed in a transient transfection assay (Figure 1C). This splice variant was previously undetected due to its low

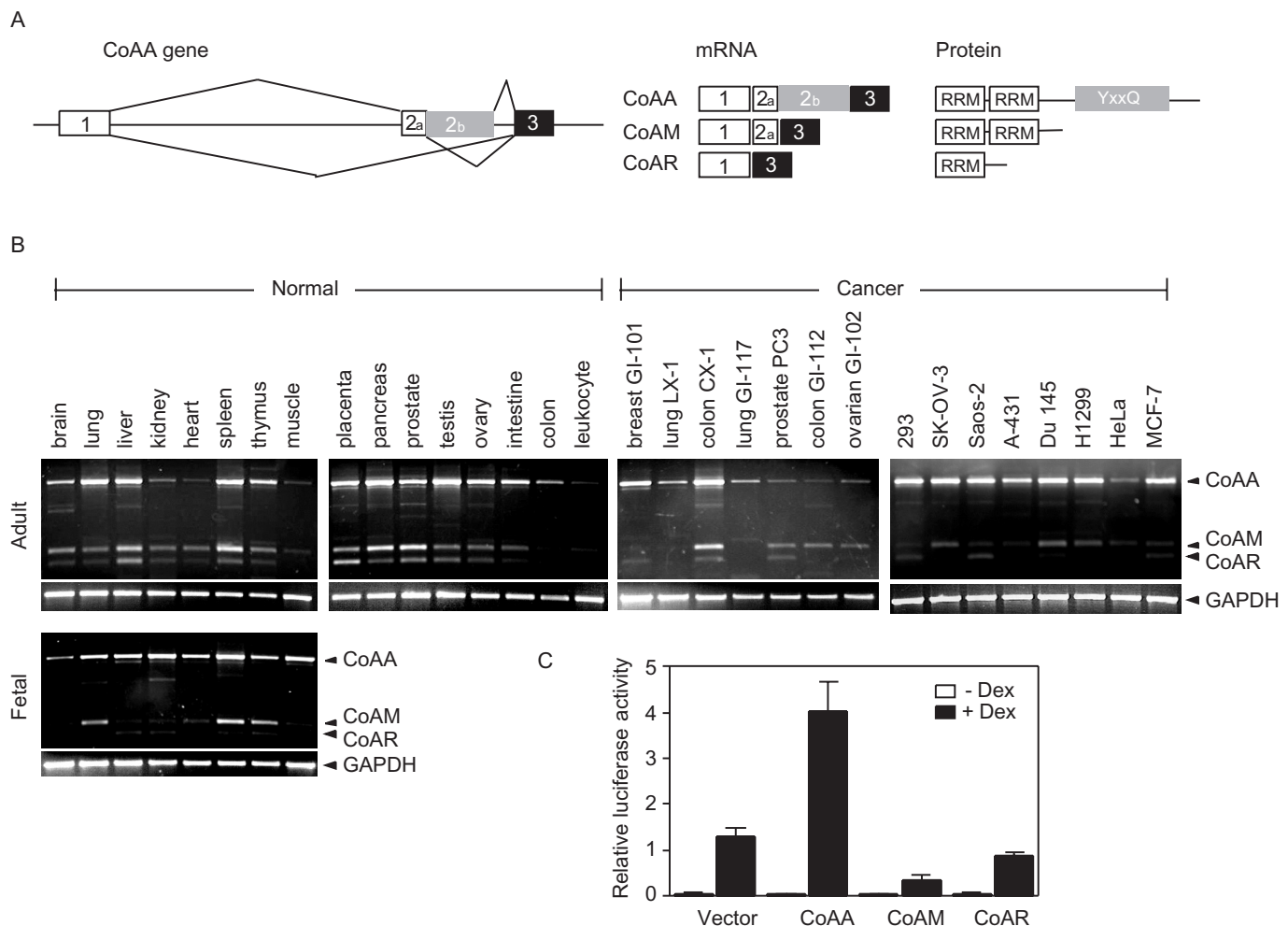


Figure 1. Alternative splicing of the CoAA gene. (A) Schematic representation of the CoAA gene structure. Introns are shown as lines and the three exons as numbered boxes with alternative splicing events depicted. The spliced mRNAs and their respective protein structures are shown at right. (B) RT-PCR analysis of endogenous CoAA, CoAM and CoAR in human adult and fetal tissues as well as cancer cell lines using GAPDH as a control. (C) Transcriptional activities of CoAA splicing variants were analyzed in CV-1 cells using MMTV-luciferase reporter system, in the presence or absence of dexamethasone (Dex). CoAA activates, and CoAM and CoAR repress the transcriptional activity.

abundance in HeLa cells (Figure 1B), in which CoAA and CoAM were originally cloned. The nucleotide and amino-acid sequences of CoAR have been deposited at the NCBI with accession number DQ294957.

The CoAA mRNA transcript is expressed in all tissues and cells examined as shown by RT-PCR (Figure 1B), a result consistent with previous northern blot analysis (16). Sequence analyses confirmed that the three PCR products observed are the three CoAA splice variants. The CoAA transcript is relatively abundant in both normal tissues and cancer cell lines (Figure 1B). However, the relative levels of CoAA, CoAM and CoAR mRNAs vary between samples. In some human fetal tissues and cancer cell lines, CoAM or CoAR expression is below the limits of detection. The variation in the expression ratios of CoAA isoforms, which could partially be due to the alternative splicing regulations, represents a possible cell-specific control of CoAA activity through its inhibitory splice variants.

Switched alternative splicing of CoAA and CoAM during P19 stem cell differentiation

To investigate CoAA alternative splicing in a physiological context, we analyzed a murine EC stem cell line, P19, during neuronal differentiation. P19 is a teratocarcinoma-derived pluripotent stem cell line that can give rise to all three germ layers in mice (23). Undifferentiated P19 EC cells in culture can be induced by RA to differentiate to neuronal and glial cells (24,25) or by DMSO to cardiac and skeletal muscles (26). Treatment of P19 EC cells with RA for up to 4 days induces non-adhering aggregates called EBs which resemble the inner cell mass of embryos (27,28). Prolonged culture in the absence of RA for an additional 12 days induces neuronal differentiation into a mixture of cell types including differentiated neurons and glial cells, as well as a variety of partially differentiated or undifferentiated cells (23).

We analyzed the expression of endogenous CoAA and its splicing variant transcripts using RNA isolated at

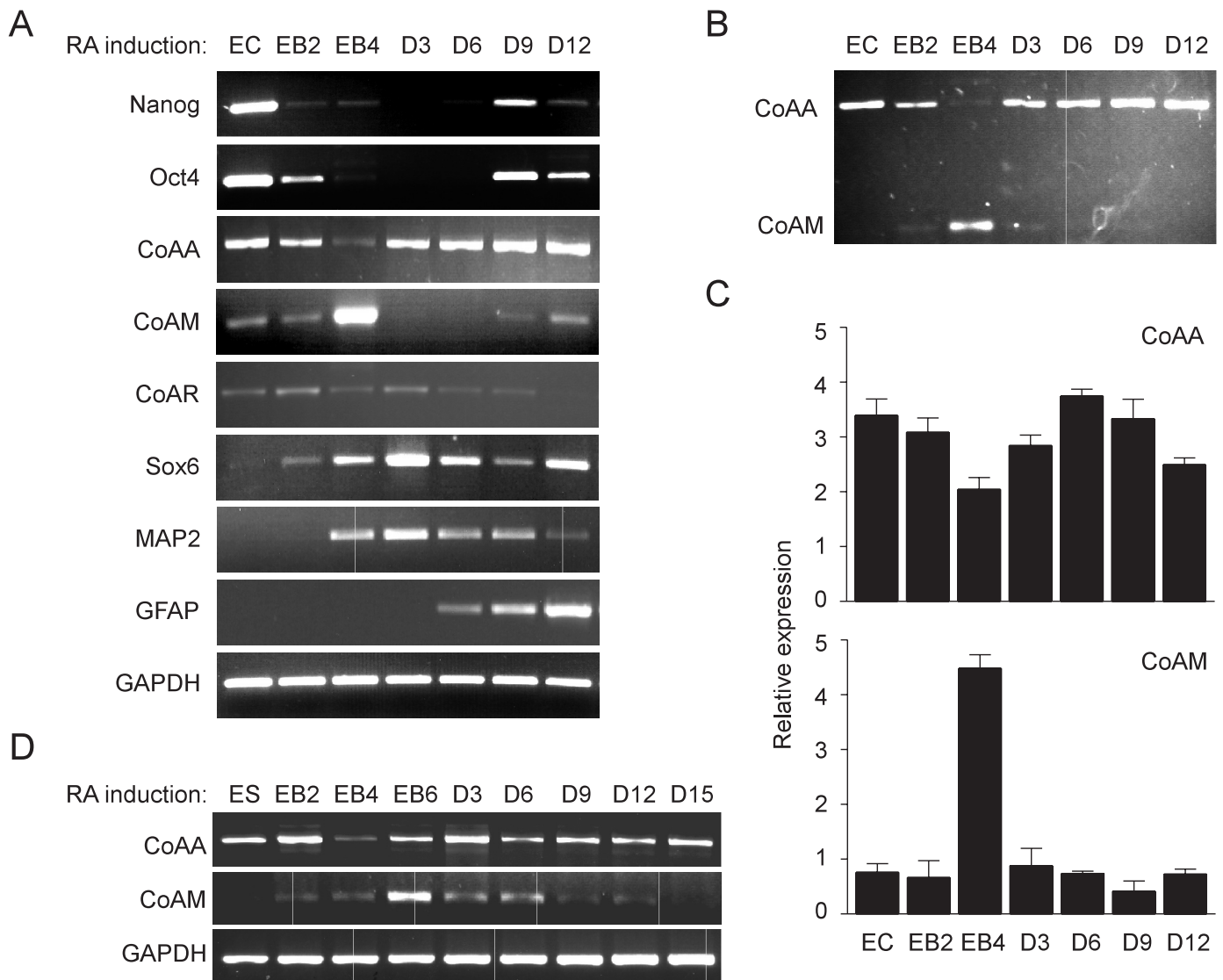


Figure 2. Alternative splicing switch from CoAA to CoAM during P19 embryonal carcinoma cell differentiation. (A) Undifferentiated P19 cells (EC) were induced with 500 nM retinoic acid (RA) in suspension culture up to 4 days to form embryoid bodies (EB2–EB4) which were trypsinized and further differentiated in the tissue culture dish for an additional 12 days in the absence of RA (D3–D12). Total RNA was isolated and normalized at each stage and analyzed using gene-specific primers as indicated by RT-PCR. GAPDH was the control. (B) CoAA and CoAM were analyzed by RT-PCR using shared primers. (C) The relative quantity of CoAA and CoAM was analyzed by quantitative real-time PCR. (D) Mouse ES cells were induced with 1 μ M RA for 6 days. The EB was undisturbed in continuous culture for 15 days in the absence of RA. RT-PCR was carried out using RNA isolated at each stage.

different time points during P19 cell differentiation. Expression of CoAA shows a rapid but transient decrease, reaching the minimum at Day 4 corresponding to the EB formation (Figure 2A). In contrast, the expression of CoAM is drastically increased during EB formation, reaching the maximum level at Day 4 and declining rapidly during further differentiation. These data indicate that an alternative splicing switch between CoAA and CoAM occurs during EB formation. The expression of CoAR is very low and without significant change at the EB stage (Figure 2A). We focused our subsequent analysis on CoAA and CoAM. The switched expression was confirmed using primer pairs common to both CoAA and CoAM (Figure 2B), and was quantitatively measured

by real-time PCR analysis (Figure 2C). A number of differentiation markers were analyzed simultaneously as controls. The expression of Nanog (29) followed by Oct4 (30,31), and then by CoAA declines upon RA treatment (Figure 2A). Meanwhile, the expression levels of Sox6, a member of the Sox (SRY box) gene family (32–34), microtubule-associated protein-2 (MAP2), a neuronal marker, and glial fibrillary acidic protein (GFAP), a glial cell marker, increase sequentially during neuronal differentiation. These data together suggest that a switched expression of CoAA transcripts occurs during the EB stages of P19 cell differentiation. We also examined mouse ES cells and switch from CoAA to CoAM expression was also found in ES cells during EB formation (Figure 2D).

Since the EB of ES cells was undisrupted in continued culture condition, the down-regulation of CoAM was delayed, correlating to the presence of EB. In addition to RA-induced neuronal differentiation, the switched expression of CoAA and CoAM was also detected during DMSO-induced muscle differentiation of P19 cells (not shown). These results indicate that the increase of CoAM, which inhibits CoAA transcriptional activity, might be common in different type of stem cell differentiation at the EB stage.

To detect the expression of CoAA and CoAM at the protein level, we evaluated the two CoAA polyclonal antibodies. One was raised against the two N-terminal RRM domains (anti-RRM) for detecting both CoAA and CoAM, and the other was raised against the CoAA-specific C-terminal activation domain (anti-CoAA). A CoAM-specific antibody is not feasible due to its overlapping primary sequence with CoAA. The two antibodies detected both endogenous and overexpressed CoAA in 293 cells (Figure 3A). The endogenous CoAM protein level is very low in this cell line, which is consistent with its mRNA level (Figure 1B).

Since CoAM mRNA is significantly elevated in EBs of P19 cells, we compared the staining pattern of the two antibodies to infer the expression pattern of CoAM. EBs are large multi-cell aggregates that form cavities at Day 4 of RA induction (Figure 3B) (25,27). CoAA expression, assessed by anti-CoAA, was detected in almost all cells of early-stage EBs, but only in the outer layer of mature EBs. CoAA was not detected in the EB cavity (Figure 3C). In contrast, the high level of expression of CoAM, detected by anti-RRM with the comparison of anti-CoAA, was found predominantly within the EB cavity (Figure 3C). The majority of signals within the cavity is unlikely contributed by CoAR when the mRNA level is considered, especially, when further confirmed by western blot analysis (Figure 3D). Most of the CoAM protein appears to be in cytoplasm although a low level of nuclear CoAM may also be present (Figure 3C). This pattern was also confirmed by overexpression of a Flag-tagged CoAM in P19 cells (Supplementary Figure S3). A mechanism in regulating the nuclear-cytoplasm shuttle of CoAM at this stage is unclear, however, other splicing regulators have been reported to be shuttled between the nucleus and the cytoplasm (35). The rapid decline of CoAM expression in later stages of differentiation might be due to the loss of CoAM-containing cells through apoptosis in the cavity, where a high level of cleaved active caspase-3 was present (Figure 3C). CoAM mRNA levels appear to decline more rapidly than CoAM protein levels (Figures 2A and 3D). At the D3 stage, CoAM protein still remains but CoAM mRNA is almost absent. This is possibly due to the longer protein half-life and the shorter mRNA half-life during degradation processes when cells become apoptotic. Our data together suggest that cells expressing CoAM are located in the EB cavity. Since CoAM potently represses transcription (16), it may regulate target gene expression and splicing during essential steps of cavitation at the EB stage.

To confirm the involvement of CoAA in neuronal differentiation, we compared the endogenous CoAA

protein expression patterns in mouse embryonic tissues at gestational stages of E12.5 and E15.5 by immunohistochemistry. In the developing brain, CoAA is widely expressed in the neocortex at E12.5. The expression is reduced in the ventricular zone at E15.5, but is enriched in neurons migrated to the cortical plate that raises cerebral cortex (Figure 3E). Due to the high expression level of CoAA and low expression levels of CoAM and CoAR, the anti-RRM antibody that detects all three CoAA splicing forms was unable to distinguish from anti-CoAA in immunohistochemical staining (data not shown). However, the data did not exclude the possibility that CoAM and CoAR are present in certain tissues, in which their mRNAs were detected by PCR (Figure 1B). In addition, high levels of CoAA expression were detected in almost all fetal tissues at E12.5 (Supplementary Figure S1). By contrast, at E15.5, CoAA expression appeared to be restricted to certain cell types during differentiation (Supplementary Figure S2). These results nonetheless suggest that the CoAA is a predominant form and is expressed in a cell-specific manner during differentiation in multiple tissues including brain.

Decreased CoAA and increased CoAM induce Sox6 expression in P19 cells

The ability of CoAM to antagonize CoAA function, together with the dramatic decrease of CoAA and the increase of CoAM in the EB cavity, raise the possibility that the switch of CoAA and CoAM might be involved in regulating differentiation. To test this hypothesis, we altered CoAA and CoAM levels by overexpressing or RNAi, and monitored the expression level of differentiation marker genes, including Sox6 and MAP2. Sox6 is a member of a protein family defined by a high mobility group protein domain, called SRY box. Sox family proteins are widely involved in differentiation including sex determination, and in brain, bone and muscle development (36). Sox6 is known to be up-regulated during neuronal differentiation in P19 cells (32) and has a function in pre-mRNA splicing (37). Since the up-regulation of CoAM is immediately followed by that of Sox6 (Figure 2A), we chose Sox6 as one of the marker genes for analysis. We overexpressed CoAM or applied CoAA RNAi in undifferentiated P19 cells in the absence of RA. The siRNA for CoAA was evaluated in P19 cells (Supplementary Figure S4) and was previously described (21). While earlier differentiation markers such as Nanog or Oct4 did not change levels in the absence of RA induction, the expression of Sox6 and the neuronal marker MAP2 was induced by overexpressing CoAM or by the treatment with CoAA siRNA in the absence of RA (Figure 4). The endogenous CoAA level was decreased during both treatments. The data indicate that CoAA and CoAM may be specific in regulating a subset of genes including Sox6, especially in their downstream differentiation pathways. However, the increased Sox6 level was transient in contrast to the effect of RA induced differentiation, which produced a sustained elevation of Sox6. The relapse effect might be due to the transient

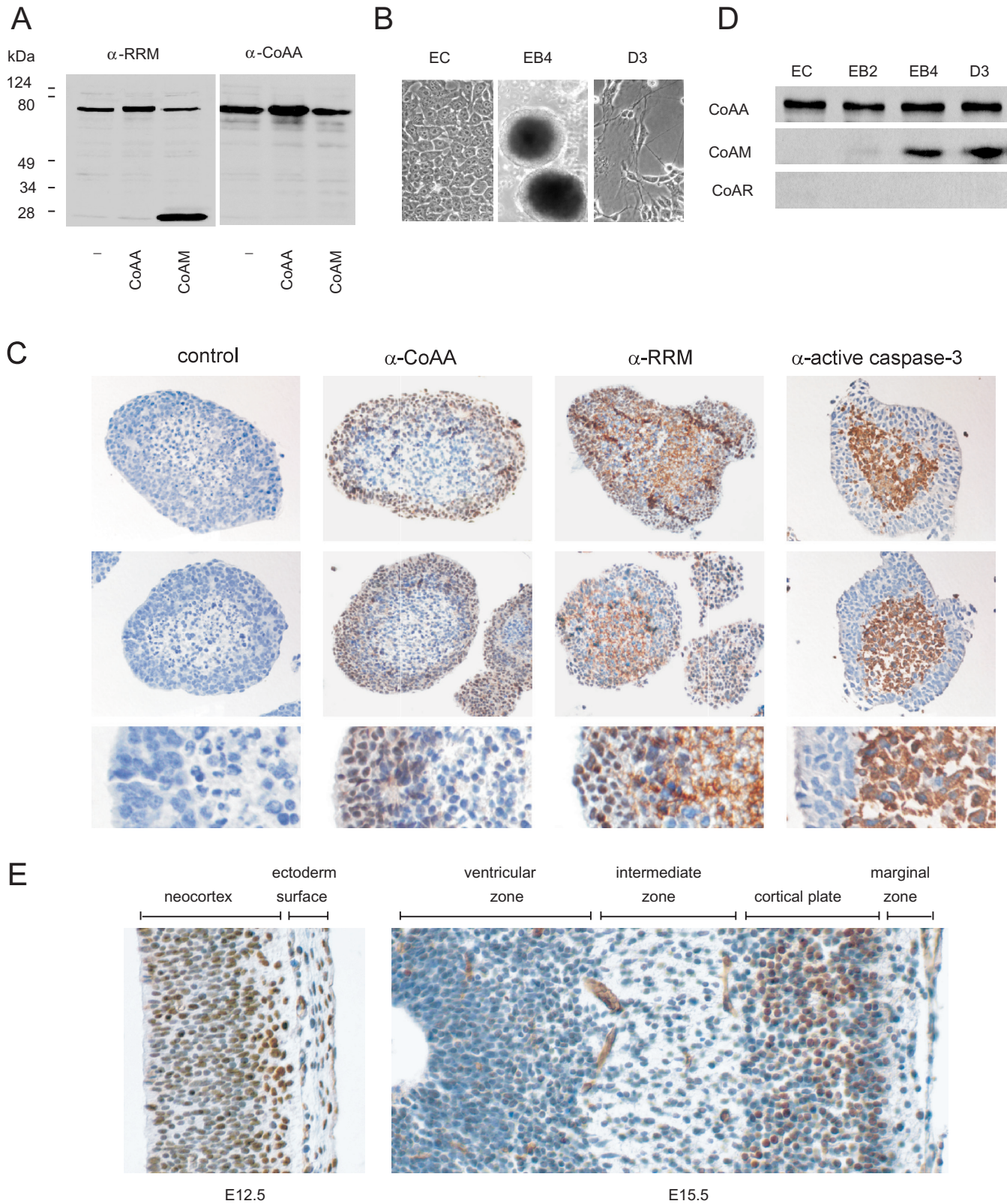


Figure 3. Expression of CoAA in embryoid body of P19 cells and in brain of mouse embryo. (A) Evaluation of anti-RRM and anti-CoAA antibodies using western blot analysis by overexpression of CoAA and CoAM in 293 cells. (B) Light microscopy of P19 cells at indicated differentiation stages ($\times 400$). (C) Embryoid body at Day 4 was paraffin-embedded, sectioned and stained with anti-RRM (against both CoAA and CoAM), anti-CoAA (against CoAA only) and anti-active caspase-3 (against cleaved caspase-3) antibodies. Two representative views are shown ($\times 400$). Enlarged views are shown below. The results show CoAM and active caspase-3 staining in the EB cavity. (D) Western blot analysis of CoAA, CoAM and CoAR at indicated differentiation stages of P19 cells. (E) Immunohistochemistry analyses of mouse embryonic brain at gestation stages of E12.5 and E15.5. The sagittal sections were stained with affinity-purified anti-CoAA antibody (1:200) and counterstained with hematoxylin ($\times 400$).

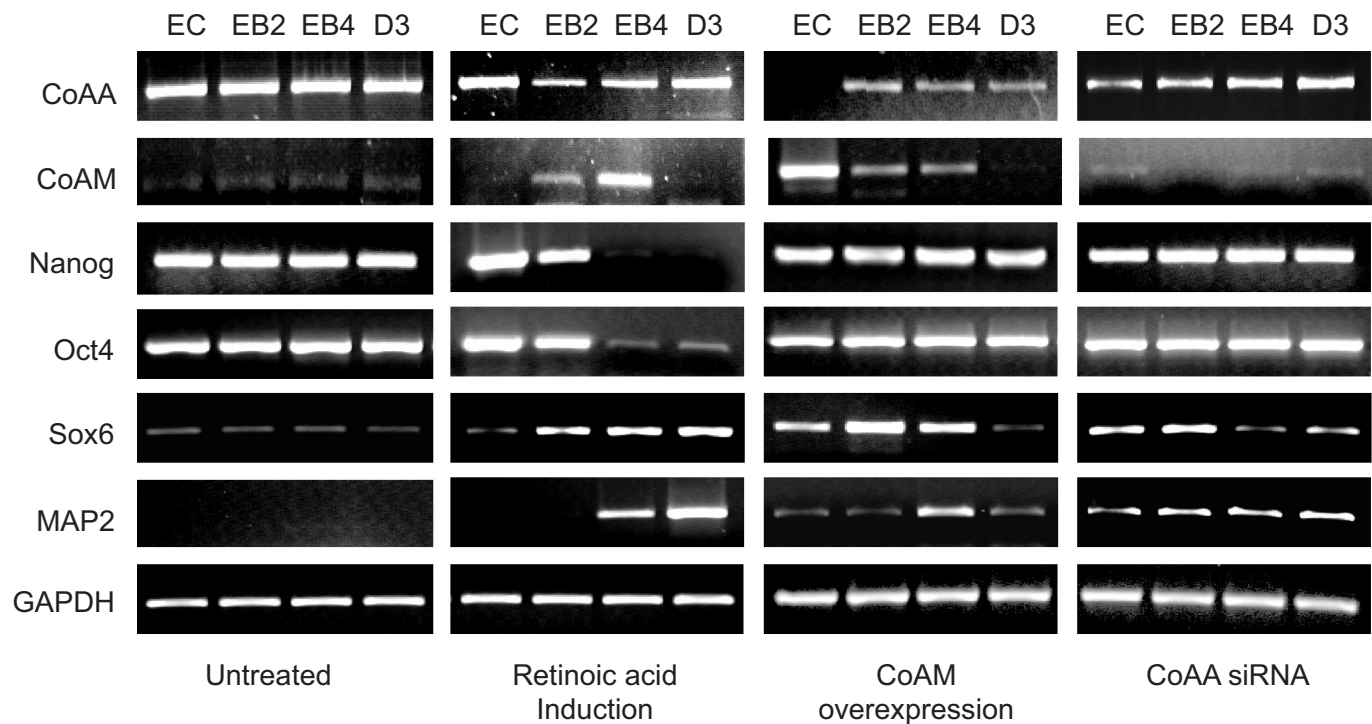


Figure 4. Overexpression of CoAM and treatment with CoAA siRNA induce Sox6 expression in the absence of retinoic acid. P19 cells were transfected with CoAM or with CoAA-specific siRNA (25 nM) at the EC stage, and then were cultured in suspension for 4 days in the absence of RA induction. The cells were trypsinized, plated and further cultured for 3 days. The untreated and RA-induced (for 4 days) P19 cells were similarly cultured as controls. RT-PCR analyses were carried out with indicated marker genes at each indicated stage.

increase of CoAM or the transient decrease of CoAA (Figure 4). These data nevertheless suggest that CoAA and CoAM are able to directly or indirectly regulate Sox6. It is still yet to be known whether CoAA and CoAM are necessary or sufficient to maintain the differentiation program in the absence of RA induction. In summary, the ratio of CoAA and its dominant negative CoAM may be critical during P19 stem cell differentiation, particularly at the EB stage.

Construction of CoAA minigene

Alternative splicing is regulated at multiple levels, particularly when coupled with transcriptional activation (11,14). Evidence has suggested that alternative splicing can be regulated through intronic or exonic enhancers and silencers as well as differential usage of promoter and regulatory sequences (10,13). We have previously identified the loss of *cis*-regulatory sequences of amplified CoAA gene in human cancers (21), suggesting that this sequence may play an important regulatory role. To investigate if this sequence regulates the alternative splicing of the CoAA gene in P19 cells, we constructed a shortened CoAA minigene with intact splicing junctions. The minigene is under the control of CoAA promoter with or without its native *cis*-regulating sequences. Thus, the CoAA minigene splicing can be analyzed in P19 cells using transient transfection approach.

The design of the minigene ensures that no functional CoAA or CoAM protein is expressed but alternative splicing capability is preserved. The rationale for this

design is to eliminate the potential effect of the CoAA protein in regulating its own splicing. The CoAA minigene was constructed with a shortened first large intron, a reading frame-shift mutation in the first exon and a deletion within the second exon encoding the activation domain (Figure 5A) (see Materials and methods section). A minimum of 160 nucleotides surrounding each splicing site was left intact. The minigene was first evaluated under a CMV promoter by RT-PCR using vector-specific primers to avoid endogenous interference (Figure 5A and B). The results demonstrate that the CoAA minigene was capable of producing both CoAA and CoAM equivalent transcripts, when CoAA and CoAM cDNAs were used as positive controls (Figure 5B). We also verified that the CoAA minigene has no coactivator effect on activation of an MMTV-luciferase reporter, which is consistent with the predicted absence of CoAA or CoAM functions (Figure 5C). Although potential intronic or exonic regulatory elements in the minigene could be affected, our data indicate that the CoAA minigene preserves its splicing capability (Figure 5B), as well as its switching capacity (see below).

The minigene was then constructed under control of the CoAA basal promoter with various lengths of its upstream *cis*-regulatory sequence to evaluate the regulation of the alternative splicing switch in P19 cells. The human CoAA gene contains a highly GC-rich basal promoter with predicted transcription-factor-binding sites for Sp1 and NF-Y (Figure 5D and Supplementary Figure S5). These transcription-factor-binding sites and

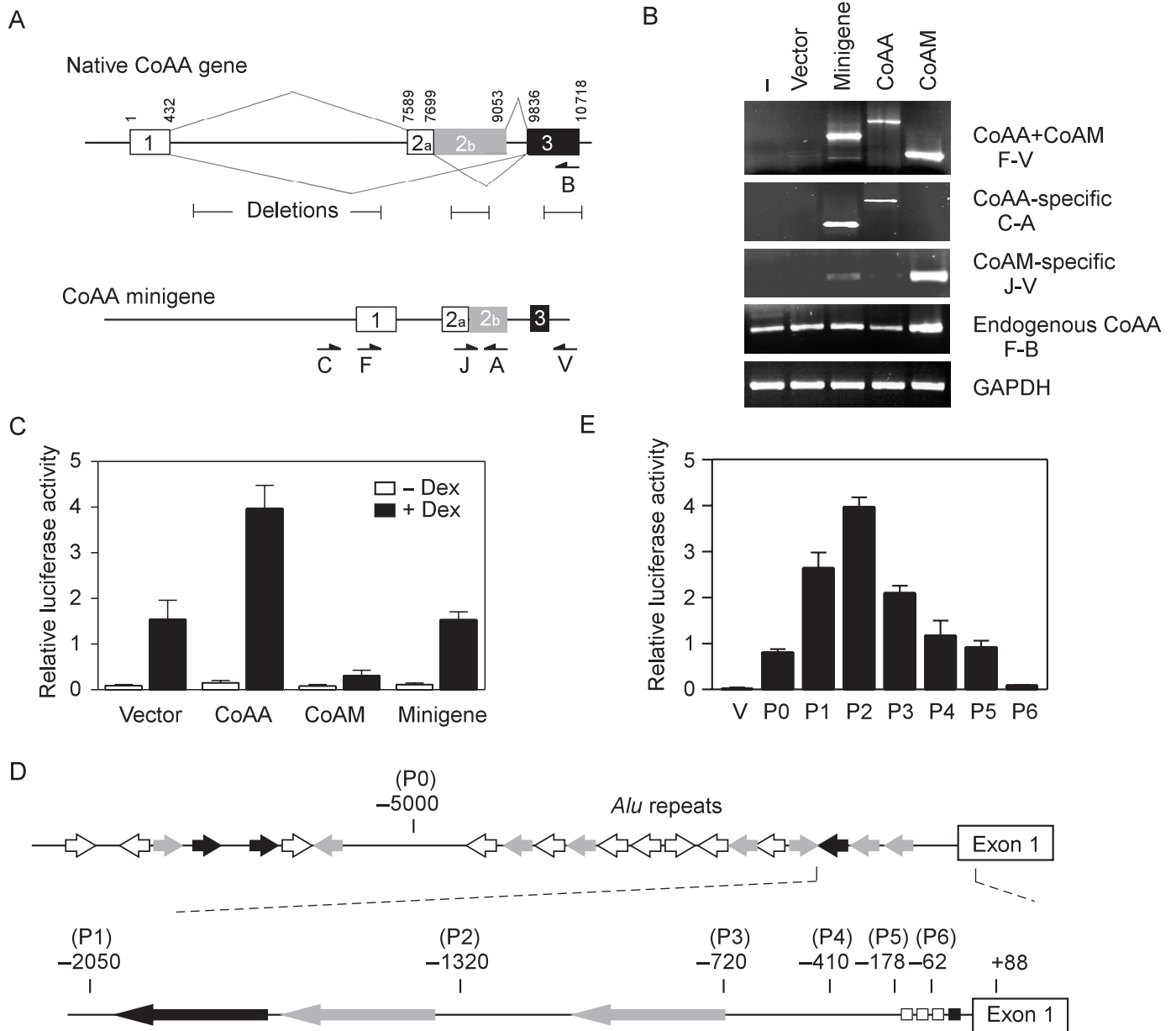


Figure 5. Construction of CoAA minigene. (A) Diagrams show structure of the native CoAA gene and minigene constructions (not to scale). Three CoAA exons are shown as numbered boxes with their alternative splicing events indicated. In the CoAA minigene, the first intron, the second exon (2b) coding the activation domain and 3' untranslated region within the third exon were shortened. A frame-shift mutation was introduced into the first exon to prevent production of RRM domains. The minigene was linked to a CMV promoter or to its natural 5' sequences (not shown). (B) CMV-minigene was transfected and its CoAA and CoAM equivalent transcripts were analyzed by RT-PCR using overexpression of their cDNAs as controls. Vector-specific and isoform-specific primers are indicated in (A). (C) The CoAA minigene shows the absence of transcriptional activity in a luciferase reporter assay using CoAA and CoAM as controls. (D) Diagram illustrates the CoAA first exon and its upstream regulatory sequences. Orientated arrows depict *Alu* repeats, *Alu-Y* in black; *Alu-S* in gray; and *Alu-J* in white. Enlarged basal promoter region shows predicted Sp1 and NF-Y binding sites as black or white squares, respectively (Supplementary Figure S5). Numbers indicate the length of promoter fragments analyzed. (E) The CoAA promoter fragments, indicated in (D), were linked to luciferase reporter and analyzed by transient transfection assays in P19 cells.

their spacing are highly conserved among human, mouse and rat species. Within a 10 kb sequence upstream of the human CoAA gene, there are 21 *Alu* repeats, which belong to the short interspersed repetitive sequences (SINES) (Figure 5D). A similar pattern of SINES is also present in the mouse CoAA gene. We have previously cloned 5 kb of the human CoAA upstream sequence and generated a series of deletion fragment constructs with a luciferase

reporter (Figure 5D and E) (21). Serial deletions of this *Alu*-rich sequence significantly increased the reporter expression in P19 cells, suggesting that this region contains a *cis*-acting silencing element in transcription (Figure 5E). Further deletion of the basal promoter region successively reduced the transcription activity, indicating that an intact basal promoter is essential for gene activation. Together, these data suggest that an *Alu*-containing

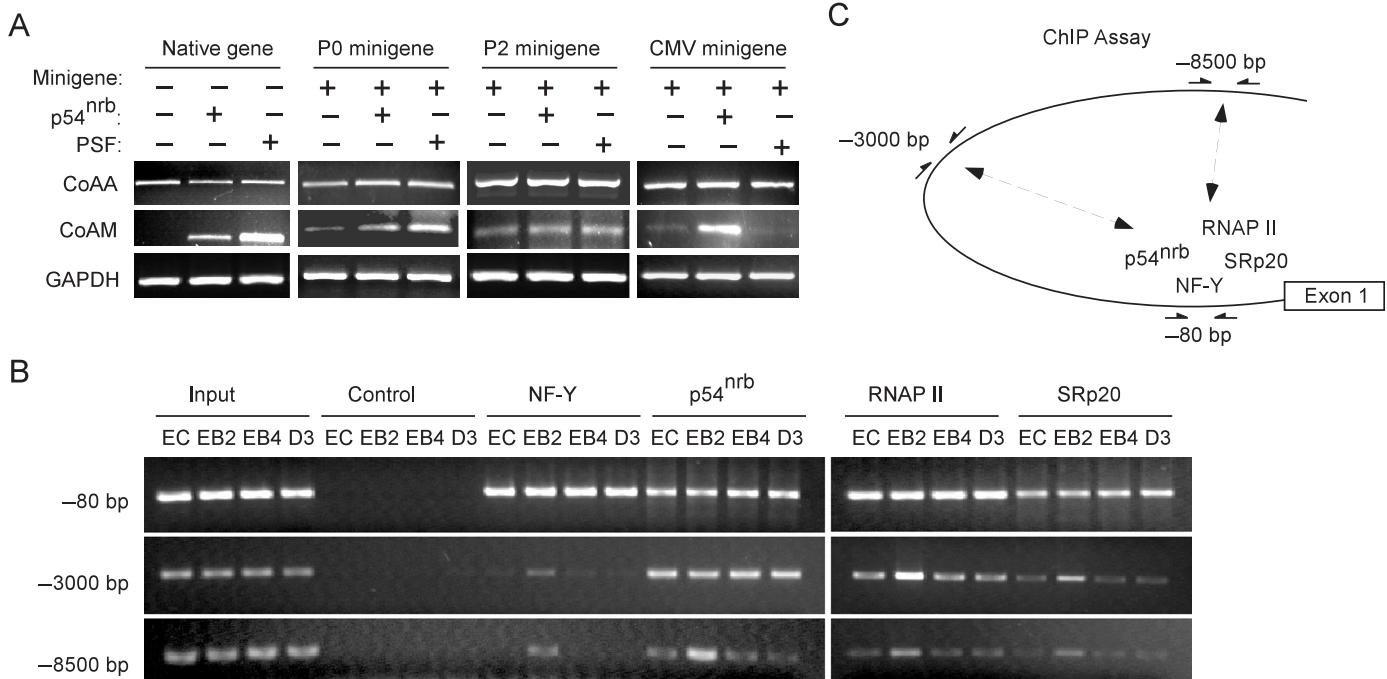


Figure 6. PSF and p54^{nrb} regulate alternative splicing of the CoAA gene through the *cis*-regulating sequences. (A) Undifferentiated P19 cells were transfected with CoAA P0- or P2-minigene, or CMV minigene together with splicing factors p54^{nrb} or PSF. The expression of CoAA and CoAM equivalent transcripts was analyzed by RT-PCR. GAPDH was a control. (B) Chromatin immunoprecipitation (ChIP) analysis of CoAA basal promoter region (-80 bp) and upstream *cis*-regulating sequences (-3000 and -8500 bp) using anti-NF-Y, anti-p54^{nrb}, anti-RNAP II and anti-SRp20 during P19 cell differentiation. Input was 1%. The resin in the absence of antibody was a control. (C) The figure depicts the interactions (dashed arrows) between *cis*-regulating sequences and potential splicing and promoter complexes containing RNAP II, p54^{nrb}, SRp20 and NF-Y. Paired arrows indicate the primer locations.

cis-regulating element and the CoAA basal promoter are functionally required in P19 cells. The various lengths of the *cis*-regulating sequences in the CoAA minigene were then compared in subsequent splicing analysis.

p54^{nrb} and PSF are involved in regulating of the alternative splicing of CoAM

We first tested the alternative splicing of the CoAA minigene in P19 cells using two well-characterized splicing regulators, polypyrimidine tract binding protein-associated splicing factor (PSF) and p54 nuclear RNA-binding protein (p54^{nrb}). PSF and p54^{nrb} are RRM-containing proteins that have been suggested to regulate pre-mRNA splicing and to associate with transcription and splicing complexes (38–43). Both endogenous PSF and p54^{nrb} are present in undifferentiated P19 cells (not shown). In the absence of RA induction, overexpression of either PSF or p54^{nrb} in undifferentiated P19 EC cells induced a very significant elevation of endogenous CoAM, but not of CoAA (Figure 6A). The stimulation of CoAM expression by PSF and p54^{nrb} was also evident in cells transfected with the P0 minigene, that carries the *cis*-regulating *Alu*-containing sequence (-5000 to +88) (Figure 6A). However, there was much less induction of CoAM transcript when the CoAA P2 minigene was driven only by the CoAA basal promoter (-1320 to +88) under the same condition. In addition,

p54^{nrb}, but not PSF, significantly induced CoAM expression from the CoAA minigene driven by a CMV promoter. These data suggest that inclusion of the *cis*-regulating sequence in the minigene promotes CoAM splicing similar to that in the endogenous gene. In contrast, CMV has a different promoter protein complex assembly, which may permit regulation by p54^{nrb} but not by PSF. The p54^{nrb} mRNA is relatively constant while PSF mRNA increased in RA-induced P19 cells as detected by RT-PCR analysis (not shown). There is no apparent correlation between the expression levels of p54^{nrb} and PSF and the switch from CoAA to CoAM during RA-induced differentiation. Thus, the involvement of p54^{nrb} and PSF in regulating alternative splicing of the CoAA gene might be due to their protein modifications at the particular differentiation stage. RA may induce certain signaling pathways that lead to the p54^{nrb}/PSF activation in a splicing complex responsible for CoAM expression. Our data minimally suggest that the alternative splicing of CoAM can be regulated through PSF and p54^{nrb}, although other splicing factors might also be involved. The data also indicate that the promoter and upstream *cis*-regulating sequences are involved in alternative splicing decisions.

To seek potential mechanism for the involvement of CoAA promoter and regulatory sequence in alternative splicing, we performed chromatin immunoprecipitation (ChIP) analysis using antibodies against p54^{nrb}, RNAP II, one of the SR proteins named SRp20, and transcription

factor NF-Y, whose binding sites are present in the CoAA promoter (Supplementary Figure S5). ChIP analysis suggests that there are physical interactions of protein complexes containing p54^{nrb}, RNAP II, SRp20 and NF-Y with the CoAA basal promoter region as well as the *Alu*-containing *cis*-regulating sequences (Figures 6B and C). NF-Y, RNAP II, p54^{nrb} and SRp20 all strongly interact with the CoAA basal promoter region (−80 bp) at all time points of RA induction. However, they have varied interaction strength with the *cis*-regulating sequences (−3000 and −8500 bp) during differentiation. In particular, their interactions are generally increased at the EB2 stage. p54^{nrb}, however, has a different interacting pattern, in which the interaction with the −3000-bp region is more constant than the interaction with the −8500-bp region (Figure 6B). The alteration of interaction level during RA-induced differentiation is correlated with the early stage of EB formation. Although the details of the interacting pattern during differentiation may be complex, our data suggest that there are interactions among promoter/enhancer complexes and splicing factors including p54^{nrb}. These data further support that alternative splicing regulation may require the *cis*-regulating sequence and the basal promoter of the CoAA gene.

The *cis*-regulating sequence is responsible for alternative splicing switch from CoAA to CoAM during P19 cell differentiation

To examine the upstream *cis*-regulating sequence in regulating alternative splicing of CoAA and CoAM during P19 cell differentiation, we compared the CoAA minigene with different lengths of the *cis*-regulating sequence. During RA-induced EB formation, the endogenous CoAA gene undergoes a dramatic switch in alternative splicing from CoAA to CoAM (Figure 7). The P0 (−5000 to +88) minigene, though at a reduced expression level, undergoes a similar switch, which has similar timing to that of the endogenous CoAA gene at stage EB4. In contrast, the P2 (−1320 to +88) and the P4 (−410 to +88) minigenes containing the basal promoter region have relatively high levels of expression of both CoAA and CoAM but are unable to induce a complete switch after RA induction (Figure 7). There was up-regulation of CoAM, but the down-regulation of CoAA was totally absent. Thus, when compared to the endogenous gene or P0 minigene, the expression of CoAM transcript from the P2 and P4 minigene fails to remain dominant at the EB4 stage. In addition, the CMV-driven minigene, in which the CoAA basal promoter was substituted with a CMV promoter, shows an incomplete switch in expression between CoAA and CoAM upon RA induction. Quantification of the PCR products in the gel is shown in the lower panels of Figure 7. The transcriptional level appears to be linked with alternative splicing on the CoAA minigene. The higher transcriptional rates in the P2 or P4 minigene are correlated with inadequate CoAM splicing (Figures 5E and 7), whereas a lower transcriptional rate in the P0 minigene shows an almost complete switch from CoAA transcript to CoAM transcript. These data imply a linked transcription rate

and alternative splicing regulation, and again suggests that regulatory sequence and promoter context are involved in regulating alternative splicing decisions. As switched expression of CoAA and CoAM is correlated with EB cavitation during stem cell differentiation, the CoAA gene at this stage might be regulated at the alternative splicing level through its promoter and *cis*-regulating sequence.

DISCUSSION

Increasing evidence indicates that alternative splicing plays an important role in differentiation and development. We show here, during neuronal stem cell differentiation, the alternative splicing of CoAA switches to its dominant negative CoAM during the cavitation of the EB. To our knowledge, this study is the first to describe the switched expression of alternative splicing variants with antagonizing functions during EB cavitation. The switch subsequently induces the expression of differentiation marker Sox6. In addition, we show that the *cis*-regulating element of the CoAA gene is required for the switch, which leads to suppressed CoAA activity through both reduced CoAA expression and functional inhibition by CoAM. As shown in the proposed model (Figure 8), while the basal promoter region activates transcription and stimulates the production of CoAA, the *cis*-regulating sequence represses transcription and stimulates alternative splicing of CoAM. This balance is shifted, presumably by regulating factors, binding the *cis*-regulating sequence during EB cavitation. In human cancers, in which the *cis*-regulating sequence was lost from the amplified CoAA gene, this balance might be irreversibly altered so that CoAA is overexpressed, consistent with our previous observation (21); and CoAM may be underexpressed in stem cells with disturbed normal differentiation. Sustained coactivator CoAA expression would be predicted to impact target genes with respect to both transcription and alternative splicing, perhaps contributing to maintaining an undifferentiated cell phenotype.

The mRNA expression pattern of the endogenous CoAA gene is complex. All three isoforms are identified by RT-PCR in the majority of human tissues and are confirmed by subsequent nucleotide sequencing. Their relative ratio and abundance in each tissue partially reflect the alternative splicing regulation of the CoAA gene. Because of the absence of additional upstream open reading frame in the CoAA gene, the ratio change is less likely due to the nonsense-mediated decay of mRNA (44). Our analysis of protein levels is limited by the overlapping nature of the primary sequences, and the generally low levels of CoAM and CoAR. The endogenous CoAM protein is difficult to detect except in EB. CoAA, however, is found in all cell lines analyzed, including P19 cells, and is abundant in many tissues analyzed with immunohistochemistry. Although CoAR is a functional repressor for CoAA, CoAR may not contribute significantly to the suppressed CoAA activity in the EB cavity, largely because the low levels of expression and the absence of up-regulation at EB stage (Figure 2A). This is probably because both CoAA and CoAR share splicing junctions

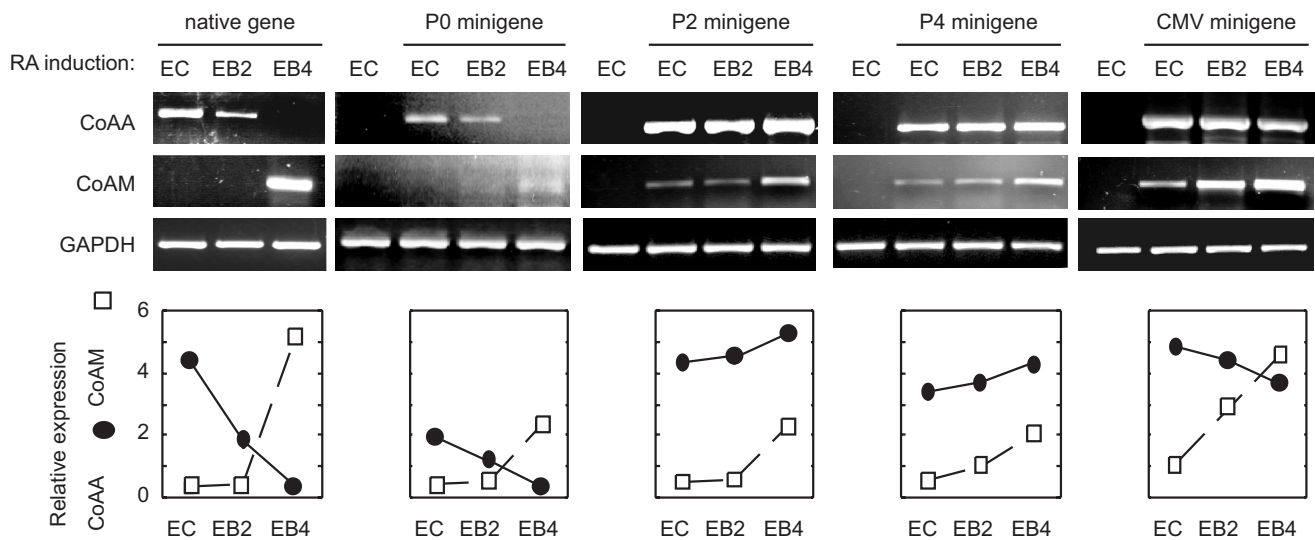


Figure 7. Switched alternative splicing of CoAA and CoAM is regulated by the upstream *cis*-regulating sequence. P19 cells were induced by 500 nM retinoic acid for 4 days. The cells were transfected with P0-, P2-, P4-, or CMV-minigene at each stage and the minigene transcripts were analyzed by RT-PCR using isoform-specific primers. GAPDH served as a control. Endogenous CoAA and CoAM transcripts from native CoAA gene were controls. Quantification of PCR products in gel are shown below.

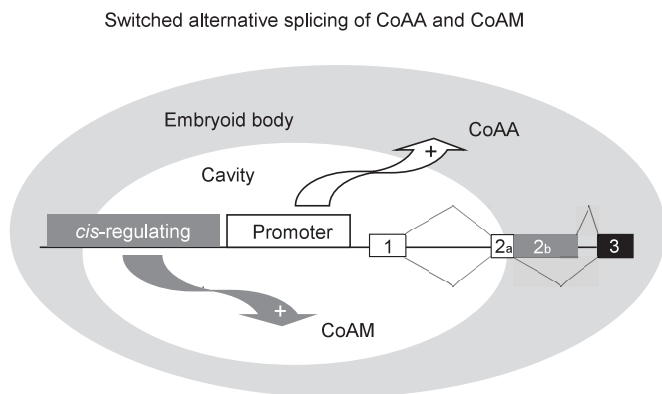


Figure 8. Tentative model of CoAA alternative splicing regulation in the embryoid body of stem cells. CoAA is produced by alternative splicing with inclusion of the second exon. A competitive 5' alternative splicing event excluding a portion of the second exon (2b) produces a dominant negative variant, CoAM, which lacks the activation domain. The basal promoter of the CoAA gene, shown as an open box, promotes splicing of the CoAA form, which is expressed in the outer layer of the embryoid body (gray). The upstream *cis*-regulating sequence, shown as a filled box, promotes alternative splicing of CoAM, which is expressed in the cavity of EB (white). The loss of upstream *cis*-regulating element in cancer may prevent the alternative splicing switch and disrupt stem cell differentiation.

at exon 1 and exon 3. In contrast, CoAM utilizes a unique splicing junction at exon 2, whose regulation may be separated from that of CoAA. Thus, the observed protein increase in EB cavity is contributed by CoAM. In addition, CoAA protein is expressed in most cells at the mouse E12.5 stage (Supplementary Figure S1). At later stages of development, the expression appears to become gradually restricted to cells undergoing further differentiation (21). In adult mouse, CoAA expression is also quite abundant in some tissues examined, including in germ cells of testis (Supplementary Figure S2). The CoAA

expression pattern suggests a functional role of CoAA in multiple tissues. Its dominant negatives, whose levels in specific cells remain to be determined, may regulate CoAA by changing their relative ratios through alternative splicing.

The basal promoter of the CoAA gene appears to be highly conserved among human, mouse and rat species (Supplementary Figure S5). The *cis*-regulating sequence is also quite conserved with a similar high density of *Alu* repeats in human and B1 repeats in mouse. These sequence features suggest that the CoAA gene may share a similar regulation cross species. Although protein factors associated with the CoAA regulatory sequences are largely unknown, there are three conserved NF-Y sites and one conserved Sp1 site located within the CoAA basal promoter. In contrast, the CMV promoter contains four cyclic AMP-responsive elements (CREs), three NF- κ B sites and one Sp1 site. The CMV promoter does not show apparent homology with the CoAA promoter. However, their distinct but overlapping protein-binding profiles might provide the explanation that both p54^{nrb} and PSF induce CoAM expression through the protein interactions on the CoAA promoter, while only p54^{nrb} but not PSF induces CoAM expression through the CMV promoter (Figure 6A). Presumably, the protein complexes on each promoter have differential interactions with p54^{nrb} and PSF. Our ChIP analysis indeed suggests that there are dynamic protein interactions between transcription/splicing complexes and CoAA promoter sequence, or *Alu*-rich *cis*-regulating sequence during differentiation (Figure 6). Especially, the protein complexes increase the interaction with the *cis*-regulating sequence at early stage of EB formation (EB2). Thus, it is evident that the CoAA gene may be regulated through its *cis*-regulating sequences during EB formation. The functions of *Alu* repeats have been shown to be associated with gene regulation including CpG methylation-mediated gene silencing

(45–47), and hormone-induced gene activation (48). Evidence has also suggested that *Alu* repeats contain alternative splicing enhancers and silencers, and participate in alternative splicing regulation (49). In addition, the repetitive nature of *Alu* repeats may facilitate DNA rearrangement, which explains the aberration of the CoAA gene in human cancers (50,21). Although the precise size of the CoAA *cis*-regulating element has not been defined, our results suggest that the *Alu*-rich region critically regulates CoAA expression and alternative splicing, at least during stem cell differentiation.

Since both the RRM domains and the C-terminal activation domain are required for CoAA activity (16), the alternatively spliced CoAM may compete with CoAA through their shared RRM domains. The RRM domains have been shown to be the most abundant protein domains in nuclear proteins (51,52), however, several available crystal structure analyses indicate the structures of RRMs are highly specific. For example, the RRMs of hnRNP A1, U1A and sex lethal have their unique peptide architectures with specific interacting surfaces toward nucleic acids or proteins (53). In this regard, the RRM-containing CoAM may compete only with CoAA for the same binding partners and thus serve as a CoAA dominant negative. The abundance of CoAM will then become critical for controlling endogenous CoAA function. And therefore, the switched alternative splicing between CoAA and CoAM may be an important approach to fine-tune CoAA activity, especially during differentiation at the EB stage.

Cell fate determination in stem cell differentiation involves a complex pattern of gene regulation including alternative splicing (54,55). Although RA is a nuclear receptor ligand, its target genes during differentiation are largely undetermined. CoAA as a nuclear receptor coactivator may also regulate an array of genes, including their alternative splicing. A cascade of alternative splicing regulation during differentiation and development has been documented, which might facilitate the understanding of the CoAA function. One of the best examples is the *sex-lethal (Sxl)* gene in sex determination in *Drosophila*. *Sxl* regulates *transformer*, which in turn regulates *doublesex*, all through alternative splicing (6). The *Sxl* transcript is alternatively spliced producing an RRM-containing protein that stimulates its own splicing via a positive feedback mechanism. This establishes and maintains the status of *Sxl* alternative splicing and determines phenotype of male or female in cells (56). Similarly, the CoAA gene is also alternatively spliced producing splicing regulators with antagonized functions. In addition, CoAA regulates its own gene through positive feedback mechanism (21). In the absence of RA induction, overexpression of CoAM alone, which shares identical RRMs with CoAA, can significantly stimulate the production of the differentiation marker Sox6 (Figure 4). Interestingly, Sox6 and some Sox family members have also been shown to regulate alternative splicing (37). Thus, a potential cascade of splicing regulation involving CoAA might exist during stem cell differentiation. Further studies are needed to confirm if the on/off switch of CoAA controls specific differentiation programs in the mammalian system and

which factors, including PSF and p54^{nrb}, are responsible for the switch.

In summary, we find that the alternative splicing of CoAA switches to its dominant negative form CoAM during EB cavitation of EC cell differentiation. In addition, we show that CoAA promoter and *cis*-regulating sequences play an important role in regulating alternative splicing. The finding not only provides a potential explanation for oncogenic CoAA in regulating stem cell differentiation, but also supports the concept that the promoter/enhancer participates in alternative splicing decisions.

SUPPLEMENTARY DATA

Supplementary Data is available at NAR Online.

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