Molecular diversity through alternative splicing is important for cellular function and development. However, little is known about the factors that regulate alternative splicing. Here we demonstrate that one isoform of coactivator-associated arginine methyltransferase 1 (named CARM1-v3) associates with the U1 small nuclear RNP-specific protein U1C and affects 5′ splice site selection of the pre-mRNA splicing. CARM1-v3 was generated by the retention of introns 15 and 16 of the primary transcript of CARM1. Its deduced protein lacks the C-terminal domain of the major isoform of CARM1 and instead has v3-specific sequences at the C terminus. CARM1-v3, but not the other isoforms, strongly stimulates a shift to the distal 5′ splice site of the pre-mRNA when the adenoviral E1A minigene is used as a reporter and enhances the exon skips in the CD44 reporter. A CARM1-v3 mutant lacking the v3-specific sequences completely lost the ability to regulate the alternative splicing patterns. In addition, CARM1-v3 shows tissue-specific expression patterns distinct from those of the other isoforms. These results suggest that the transcriptional coactivator can affect the splice site decision in an isoform-specific manner.

It has been estimated that about 60% of human genes undergo alternative splicing (1). Commonly, alternative splicing determines the inclusion of a portion of coding sequence in the mRNA, giving rise to protein isoforms that differ in their peptide sequence and hence chemical and biological activity. The mechanism of alternative splicing permits diversity of translatable mRNAs, thereby increasing the proteome diversity encoded by a limited number of genes. Genetic switches based on alternative splicing are known to be important in many cellular and developmental processes, including sex determination, apoptosis, axon guidance, and tissue-specific differentiation (2–4).

Although a large variety of splicing decisions can be explained by the antagonistic effects of general splicing factors, such as serine/arginine-rich proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs), it is most likely that tissue-specific or developmentally regulated splicing factors have an important role in the regulation of alternative splicing. In Drosophila melanogaster, sex-lethal abnormal visual system is a gene-specific regulator of alternative splicing in sex determination (2), and the embryonic lethal abnormal visual system is a gene-specific regulator of alternative pre-mRNA processing in neurons (3). However, in mammals, only a limited number of splicing regulators have been identified to date, despite the large diversity of the mammalian gene transcripts, and little is known about the mechanisms by which alternative splicing is regulated.

Pre-mRNA splicing occurs in a large multicomponent ribonucleoprotein complex called the spliceosome, which is composed of U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein (snRNP) particles and many non-snRNP protein splicing factors (5). U1 snRNP recognizes the 5′ splice site and is among the first factors to interact with the pre-mRNA to form complexes (complex E in mammalian extracts) that commit the pre-mRNA to the splicing pathway (6).

U1 snRNP is composed of a U1 snRNA, seven different Sm proteins common to other snRNPs, and three U1-specific proteins: U1–70K, U1-A, and U1C (7). U1C does not interact directly with naked U1 snRNA, but a recent study indicated that U1C recognizes the sequence of the 5′ splice site and argued that this RNA-protein recognition precedes base pairing with U1 snRNA (8). In addition, the splicing regulator TIA-1 has been shown to interact with U1C to promote the recruitment of U1 snRNP to 5′ splice sites (9). Furthermore, we have demonstrated that the EWS/NOR1 fusion gene product identified in extraskeletal myxoid chondrosarcoma binds to U1C and affects pre-mRNA splicing (10). These observations suggest that some splicing regulators affect the 5′ splice site selection of the pre-mRNA splice site through their binding to U1C.

In the present study, to isolate splicing regulators that affect 5′ splice site selection, we searched for proteins associated with U1C using a yeast two-hybrid system. This strategy allowed the isolation of an isoform of coactivator-associated arginine methyltransferase 1 (CARM1), along with known splicing regulators Fox-1 (11), HRNBP2, and RBM4 (12). CARM1 has been shown to act as a transcriptional coactivator and cooperate synergistically with p300/CPB and p160 coactivators to enhance transcriptional activation by nuclear receptors (13). Here, we demonstrate that one isoform of CARM1 (named CARM1-v3), but not the other isoforms, strongly stimulates a shift to the distal 5′ splice site of the pre-mRNA splicing. Our results indicate that the transcriptional coactivator could allow...
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EXPERIMENTAL PROCEDURES

Plasmids—The U1C cDNA was isolated from human placental cDNA by PCR amplification and confirmed by DNA sequencing. The fetal forebrain cDNA library was generated from primary cultured rat forebrain cells (embryonic day 17) in pAD-GAL4–2.1, as described previously (14). The full-length CARM1 cDNA was cloned from the rat fetal forebrain cDNA library. Various deletion constructs of CARM1, UD1 (amino acids 120–573), UD2 (240–573), LD4 (1–480), and LD5 (1–539), were generated by PCR amplification, and methylationtransferase-defective mutants of CARM1 were generated using a QuickChange site-directed mutagenesis kit. These cDNA fragments were confirmed by DNA sequencing and restriction enzyme mapping. For yeast two-hybrid screening, the U1C cDNA was inserted into the pGBK7 vector (Clontech). For mammalian two-hybrid systems, the cDNA constructs were inserted into the pCMV-AD or pCMV-BD vectors (Stratagene). The plasmids pFR-Luc, pRL-Luc, and phBGW were purchased commercially (Promega). Expression plasmids for FLAG or myc-tagged proteins were constructed by inserting the cDNA into the pCMV-Tag2 or pCMV-Tag3 vectors (Stratagene). pCS3-NT-E1A, pMMTV-CDD4, pG5.1HA GRIP1, ERE-Luc, GST-U1P, and Dlxin-1 cDNA were kindly provided by Drs. F. Moreau-Gachelin, B. W. O’Mally, M. R. Stallcup, S. Ozawa, N. Tsuchiya, and K. Watanabe, respectively.

Cell Culture and Transfection—COS-7 and HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin/streptomycin. COS-7 and HEK293T cells were transiently transfected using FuGENE 6 (Roche Applied Science) and Polyfect (Qiagen), respectively, according to the manufacturer’s instructions.

Yeast Two-hybrid System—All experiments were performed in the yeast reporter strain YRG2 (Stratagene). pGBT-U1C was used as bait to screen a rat fetal forebrain cDNA library (in pAD-GAL4–2.1, Stratagene) by systematic transformation. Cells transformed with the two fusion plasmids were selected by growth on SD plates (-Trp, -Leu, and -His) and β-galactosidase expression. Plasmids were extracted from positive clones and introduced into Escherichia coli cells for the recovery and sequencing of plasmid DNA.

Mammalian Two-hybrid System—HEK293T cells were grown to 50% confluence in 24-well plates and then transfected with pCMV-BD, pCMV-AD, reporter plasmid pFR-Luc (Stratagene), and the Renilla luciferase expression plasmid phRGB (Promega). The total amount of plasmid DNA used for transfection was kept constant by adding an appropriate amount of vector plasmid. After incubation for 30 h, total RNA was extracted from one dish using the RNeasy column kit (Qiagen), and the cells from another dish were lysed with radioimmunoprecipitation assay buffer (Western blotting). For mammalian two-hybrid systems, the cDNA constructs were inserted into the pCMV-AD or pCMV-BD vectors (Stratagene) and sonicated briefly. Anti-myc antibody-agarose gel electrophoresis gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore). The proteins were detected with horse-radish peroxidase-conjugated goat anti-myc (Roche Applied Science) antibodies. AD fusion proteins were detected using rabbit anti-NF-kB p65 pAb antibody (Santa Cruz Biotechnology). Protein bands were visualized using the ECL Plus Western blot detection system (Amersham Biosciences).

UV Cross-link Analysis—RNA probes were synthesized by transcription of pEI or CD44-containing pCRII-TOPO plasmids (Invitrogen) linearized with BamHI using T7 RNA polymerase in the presence of α-[^32P]UTP. The RNA probes were mixed with a recombinant protein in binding buffer (10 mM Hepes-KOH, pH 7.9, 50 mM KCl, 1 mM EDTA, 100 μM yeast tRNA, and 0.1 mM phenylmethylsulfonyl fluoride) for 20 min at 20 °C. After UV cross-linking using UV-linker (FS-800, Funakoshi) for 4 min, the samples were treated with RNaseA (0.5 mg/ml) at 37 °C for 20 min by SDS-PAGE. The gel was then dried and subjected to autoradiography.

RNA-Protein Blot Analysis—Recombinant proteins were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes according to a standard method. The membranes were incubated with blocking buffer consisting of 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% bovine serum albumin, 1 mM EDTA, and 100 μM yeast tRNA at 4 °C for 12 h and further incubated in the same buffer containing 4 × 10^7 cpm/ml of a 32P-labeled probe at room temperature for 60 min. After washing in buffer consisting of 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5 μM EDTA for 14 min, they were subjected to autoradiography. Synthetic ribonucleotide homopolymers, poly(G), poly(A), poly(U), and poly(C), were used as probes, were end-labeled by T4 polynucleotide kinase.

In Vivo Splicing Assay—COS-7 cells grown in two sets of 60-mm dishes were transfected with 2.5 μg of FLAG-tagged CARM1 isoform expression plasmid and 0.5 μg of E1A or CD44 reporter plasmid. After incubation for 30 h, total RNA was extracted from one dish using the RNeasy column kit (Qiagen), and the cells from another dish were lysed with radioimmunoprecipitation assay buffer for Western blotting. Luciferase expression was performed using oligo(dT) primer extension for 5 min at 72 °C. Amplified products were separated by agarose gel electrophoresis and visualized with ethidium bromide. The gels were scanned by densitometry using an image analyzer (Quantity-One 4.4.1, Molecular Imager FX, Bio-Rad).

In Vitro Methylation Assays—In vitro methylation assays were performed as described (15). Briefly, HEK293T cells were transfected with FLAG-tagged CARM1 expression plasmid. After incubation for 35 h, cells were harvested in lysis buffer. Cell lysates were prepared and sonicated briefly. Antibodies against CARM1 were added to the cell lysates supernatant using dual-luciferase reporter reagent (Promega). Luciferase activity was normalized to Renilla luciferase activity.

Immunoprecipitation—HEK293T cells were transfected with FLAG-tagged CARM1-v3 expression plasmid and myc-tagged U1C expression plasmid. Cells were washed with phosphate-buffered saline and then lysed with lysis buffer (Promega). Luciferase assays were performed with the cell lysates supernatant using dual-luciferase reporter reagent (Promega). Luciferase activity was normalized to Renilla luciferase activity.

RESULTS

Isolation of U1C-binding Proteins as Candidates for Splicing Regulators—U1 snRNP recognizes the 5′ splice site and among the first factors to interact with the pre-mRNA to form complexes that commit the pre-mRNA to the splicing pathway. To identify the cellular factors that affect 5′ splice site selection, we screened the rat fetal brain cDNA library for proteins associated with U1C, one of the U1-specific proteins, using a yeast two-hybrid system. We isolated dozens of clones and identified seven factors that showed high levels of HHS3 and β-galactosidase reporter gene activation in the system (Fig. 1B). Three clones encoded known splicing regulators: RNA-binding protein Fox-1 (11), hexahexonucleotide-binding protein 2 (HRNP2), and RNA-binding motif protein 4 (RBMA) (12), while the rest encoded far upstream element binding protein 1 (FBP1) (16), 20 S proteasome α subunit C8 (C8) (17), a member of the necdin/melanoma-associated antigen family (Dlxin-1)
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The diagrams show the adenovirus E1A (upper panel) and the human CD44 (lower panel) minigenes and their major splicing products. The arrows denote primers for RT-PCR. In vivo splicing assays were performed in COS-7 cells transiently cotransfected with the E1A or CD44 reporter and expression vectors as indicated (right panels). Total RNA was isolated and subjected to RT-PCR, and the splicing products were analyzed by agarose gel electrophoresis. The list of isolated clones, the results of two-hybrid screen and splicing assays, the predicted amino acid motifs, and features. RRM, RNA recognition motif.

(18), and a variant of coactivator-associated arginine methyltransferase 1 (named here CARM1-v3), which were not known to be involved in regulating splicing.

We first examined the effect of these proteins on alternative splicing in vivo by using the adenoviral E1A minigene as a splicing model (Fig. 1A, upper). The E1A minigene (pCS-MT-E1A) was coexpressed with expression vectors of the isolated cDNA clones, and thereafter, alternatively spliced isoforms of E1A were detected by reverse transcription (RT)-PCR. Accord- ing to the presence of three alternative 5′ splice sites in the E1A pre-mRNA, three major isoforms (13 S, 12 S, and 9 S) and two minor isoforms (11 S and 10 S) can be transcribed from the E1A minigene (19). In COS-7 cells transfected with pCS3-MT-E1A and empty vector, we detected 13 S and 12 S isoforms as major RNA species (control), indicating that isoforms generated by proximal splice site selection are mainly detected in COS-7 cells. In contrast, transfection of hnRNP A1, used as a positive control, resulted in an increase of 9 S isoforms and a decrease in the relative proportions of 13 S and 12 S isoforms, indicating that hnRNP A1 modulates 5′ splicing site selection from proximal to distal sites, as reported previously (20). Similarly, a shift from proximal to distal sites in the 5′ splice site selection was also observed in COS-7 cells transfected with Fox-1, HRNP2B, FBP1, RBM4, and CARM1-v3. Notably, cells transfected with CARM1-v3 showed a great increase in 9 S isoforms and a concomitant decrease in the 13 S and 12 S isoforms. Cells transfected with C8 and Dlxin-1 showed little change in alternative splicing. We furthermore tested the effect of isolated factors on the pre-mRNA splicing of the CD44 minigene, since CD44 gene expression is extensively regulated by alternative splicing and since the resulting CD44 isoforms are known to be involved in tumor progression, immune responses, and embryonal development (21–23). The CD44 minigene contains the variable exons v4 and v5 of the human CD44 gene, along with their surrounding intron sequences (24). The transfection of COS-7 cells with the CD44 minigene and empty vector gave rise to two major spliced RNA products, containing either both variable exons (two-exon inclusion) or only one variable exon (one-exon inclusion) and a minor product with none of these exons (skipping) (Fig. 1A, lower, control). Cells cotransfected with Fox-1, HRNP2B, and CARM1-v3 showed an increase in the one-exon/two-exon ratio of spliced mRNAs as compared with the empty vector-transfected cells (control), and cells transfected with FBP1 and RBM4 showed a slight increase in the ratio. In contrast, the transfection of C8 and Dlxin-1 had little effect on the alternative splicing of the CD44 minigene. These results are summarized in Fig. 1B.

Our finding that Fox-1, HRNP2B, and RBM4 affected alternative splicing is consistent with reports that Fox-1 induced muscle-specific exon skipping of the human mitochondrial ATP synthase γ subunit gene (11), that HRNP2B is classified as a subtype of Fox-1, and that RBM4 acts antagonistically to serine/arginine-rich proteins in splice site and exon selection. Among newly isolated potential U1C-interacting proteins, we chose CARM1-v3, known as a positive regulator of transcription, for further analysis, since it showed the strongest effects on alternative splicing in the E1A reporter assay among the proteins examined and has not been demonstrated as a factor related to pre-mRNA splicing.

CARM1-v3 Interacts with U1C in Mammalian Cells—To confirm the association between CARM1-v3 and U1C in vivo, a test using a mammalian two-hybrid system was performed. Full-length U1C fused to the Gal4 DNA-binding domain in the mammalian-two hybrid vector pCMV-BD (BD-U1C) was cotransfected into HEK293T cells with CARM1-v3 fused to the NF-κB activation domain in the pCMV-AD vector (AD-CARM1-v3) and a reporter plasmid pFR-Luc. Cotransfection of AD-CARM1-v3 with BD-U1C resulted in the production of high levels of luciferase activity (Fig. 2A). Cells transfected with the combination of BD-U1C and AD-vector or that of BD-vector and AD-CARM1-v3 showed little or no luciferase activity. To confirm the association further, coimmunoprecipitation studies were performed with both proteins coexpressed as tagged fusion protein in HEK293T cells (Fig. 2B). FLAG-tagged CARM1-v3 and myc-tagged U1C were cotransfected into HEK293T cells, and lysates from the cotransfected cells were used for immunoprecipitation. An anti-myc antibody coimmunoprecipitated myc-U1C along with FLAG-CARM1-v3. A normal mouse IgG did not coimmunoprecipitate myc-U1C and FLAG-CARM1-v3. These results indicate that CARM1-v3 interacts with U1C, not only in yeast but also in mammalian cells.

To delineate the region of CARM1-v3 responsible for the interaction with U1C, deletion plasmids of AD-CARM1-v3 were constructed. Mammalian two-hybrid assays were performed on this series of CARM1-v3 deletions with BD or BD-U1C (Fig. 2A). High levels of luciferase activity were observed when BD-U1C was cotransfected with AD-UD1 (amino acids 120–573 in CARM1-v3) or AD-LD5 (1–539). Other constructs, AD-UD2 and -LD4, did not show stronger luciferase activity than the AD vector. Therefore, the region required for interaction between CARM1-v3 and U1C comprised the central region of CARM1-v3.

### Table

| Clones       | Two-hybrid | Motifs         | Splicing | Features                          |
|--------------|------------|----------------|----------|-----------------------------------|
| Fox-1        | +          | RRM            | +        | splicing regulator                |
| HRNP2B       | +          | RNA binding    | +        | subtype of Fox-1                  |
| FBP1         | +          | splicing      | +        | transcription factor              |
| RBM4         | +          | promoter      | -        | protein                            |
| Dlxin-1      | +          | MAGE family   | -        | cofactor                           |
| CARM1-v3     | +          | -              | +        |                                   |
CARM1-v3 Does Not Bind to the Pre-mRNA Directly—

Many splicing regulators have been shown to possess RNA recognition motifs and to bind RNA directly. In fact, isolated U1C-binding clones Fox-1, HRNBP2, and RBM4, all of which showed splicing activity, had RNA recognition motifs or activity to bind RNA. However, CARM1-v3, which showed a strong effect on alternative splicing, does not have any RNA recognition motifs. Therefore, we examined by UV cross-link analysis whether CARM1-v3 was able to bind to the pre-mRNA directly.

Fig. 3. CARM1-v3 does not bind to the pre-mRNA directly. A, GST-fused Fox-1, used as a positive control, or CARM1-v3 protein was incubated with the ^32P-labeled E1A or CD44 pre-mRNA. After UV cross-linking, samples were treated with RNase A and subjected to SDS-PAGE and autoradiography. B, RNA binding activity of CARM1-v3 was analyzed using synthetic ribonucleotide homopolymers, poly(A), poly(C), poly(U), and poly(G), as probes. GST-fused UP1, used as a positive control, and CARM1-v3 were separated, transferred to PVDF membranes, and incubated with ^32P-labeled ribonucleotide probes. The names of probes used are listed at the top of the panels. The asterisk indicates signals from impurities.

Four Isoforms Were Transcribed from the CARM1 Gene—

CARM1-v3 was not identical to the previously reported CARM1 (referred to here as CARM1-v1) (Fig. 4). A comparison of the two sequences indicates the insertion of nucleotides 1,709–1,986 and 2,056–2,184 in CARM1-v3 relative to CARM1-v1 cDNA. To determine other variants of CARM1 further, we searched for them in various tissues by using RT-PCR and library screening. We cloned an additional two types of CARM1 along with v1 and v3 and named them CARM1-v2 and -v4 (Fig. 4, A and B). The deduced protein sequences of CARM1-v1, -v2, -v3, and -v4 contain 608, 651, 573, and 585 amino acids, respectively. They commonly contain the arginine methyltransferase domain and GRIP1-binding domain (15). The rat genome sequences from the GenBank™ data base indicate that they are alternatively spliced variants of the primary transcript of CARM1.
the CARM1 gene, which is located on chromosome 8 and contains 16 exons (Fig. 4C). CARM1-v3 was generated by retention of two intron sequences of the primary transcript, resulting in a lack of the C-terminal amino acids of CARM1-v1 and instead the insertion of v3-specific amino acid sequences (amino acids 540–573). CARM1-v2 was generated by the retention of intron 15, and CARM1-v4 by skips of exon 15. Next, we examined the association of the four isoforms with U1C using a mammalian two-hybrid system. High levels of luciferase activity were observed when BD-U1C was cotransfected with AD-CARM1-v1, -v2, -v3, and -v4. No significant luciferase activity was observed when the BD vector was cotransfected with AD-CARM1 isoforms. This result is consistent with that of the mammalian two-hybrid experiment with deletion plasmids of CARM1-v3.

CARM1-v3, but Not -v1, -v2, or -v4, Affects Alternative Splicing—We isolated four CARM1 isoforms, v1, v2, v3, and v4, all of which associated with U1C in the mammalian two-hybrid system. To examine whether the splicing activity shown by CARM1-v3 is also exhibited by other isoforms, we examined the effects of the four isoforms on pre-mRNA splicing of the E1A and CD44 minigenes. Transient transfection experiments with both CARM1 isoforms and E1A expression vectors were performed in COS-7 cells that express relatively low levels of endogenous CARM1 mRNA (data not shown). All isoforms were tagged with an FLAG epitope to monitor their expression levels in transfected cells by Western blotting with the anti-FLAG antibody. As shown in Fig. 5A, expression of CARM1-v3 caused increased usage of the distal 5' splice site, resulting in greatly increased levels of the 9S isoform and decreased levels of the relative amounts of the 13 S and 12 S isoforms. CARM1-v1, -v2, and -v4 had some effect on the E1A splicing profile, but it was much less extensive than that of CARM1-v3. Despite the extremely low expression level of CARM1-v3, as revealed by Western blotting, there was a dramatic change in the ratio of the E1A isoforms, indicating that CARM1-v3, but not -v1, -v2, or -v4, strongly promotes the choice of the distal 5' splice site in COS-7 cells. We furthermore examined the effect of CARM1 isoforms on the CD44 minigene (Fig. 5B). Cells transfected with CARM1-v3 showed about a 2-fold increase in the one-exon/two-exon ratio of spliced mRNAs as compared with those transfected with control vector. Cells transfected with CARM1-v2 showed a slight increase in the ratio. CARM1-v1- or -v4-transfected cells did not show any significant change in alternative splicing. These results indicate that the effects on alternative splicing by CARM1 are specific to isoform v3 despite the common property of U1C binding among the isoforms.

Functional Differences between CARM1-v3 and the Other Isoforms—The isoform-specific regulation of alternative splicing by CARM1 prompted us to check whether CARM1-v3 could have properties different from those of other isoforms. We first tested the methyltransferase activity of CARM1 isoforms. FLAG-tagged CARM1 isoforms were expressed in HEK293T cells by transient transfection, isolated by immunoprecipitation, and incubated with histone H3 in the presence of [methyl-3H]-adenosyl-L-methionine, and methylated histone H3 was detected by SDS-PAGE and fluorography. To normalize the protein level of each CARM1 isoform, a large amount of transfected cells (~6 × 10^6 cells) were immunoprecipitated with a small amount of anti-FLAG antibody-agarose beads (15 μl). Equality of the protein levels used in the assay were monitored by Western blotting with the anti-FLAG antibody. CARM1-v1 strongly methylated histone H3, as reported previously (15) (Fig. 6A). Similarly, the other isoforms v2, v3, and v4 methylated histone H3 efficiently at comparable levels with CARM1-v1.

Next, we investigated the coactivator activity of CARM1 isoforms in nuclear receptor-mediated transcriptional regulation, since CARM1 interacts with GRIP1, a member of the p160 family of nuclear receptor coactivators, and acts as a coactivator for nuclear receptors. The estrogen-responsive element-luciferase reporter construct (ERE-Luc) was tested in COS-7...
products were subjected to agarose gel electrophoresis (RT-PCR, and the splicing reporter and expression vector of FLAG-tagged protein as indicated.

In vivo

FIG. 5. Effect of CARM1 isoforms on in vivo alternative splicing. In vivo splicing assays were performed with the E1A reporter (A) or CD44 reporter (B). COS-7 cells were transiently cotransfected with the reporter and expression vector of FLAG-tagged protein as indicated. Total RNA was isolated and subjected to RT-PCR, and the splicing products were subjected to agarose gel electrophoresis (upper panels in both A and B) and quantitative analysis using an image analyzer (Molecular Imager FX, Bio-Rad). The experiments were repeated at least three times, and the relative abundance of the major splicing products is shown as a percentage (mean ± S.D., middle panels). hnRNP A1 and EWS/NOR1 were used as positive controls. The expression of each protein was confirmed by Western blotting (WB) with anti-FLAG antibody (lower panels). Long exposures are shown for CARM1 isoforms to detect low levels of expression in the CD44 reporter assays, while short exposures are shown for hnRNP A1 and EWS/NOR1.

cells with the ERα and GRIP1 expression plasmids in the presence or absence of CARM1 isoforms. Consistent with the previous report (15), ERα and E2 transactivated the ERE-Luc reporter expression in the presence of GRIP1 greater than in the absence of GRIP1 (Fig. 6B). CARM1-v1 further enhanced the reporter gene expression induced by E2, ERα, and GRIP1. The extent of CARM1-v1 activity was similar to that of CARM1-v2 or -v4 activity. CARM1-v3 showed significantly greater activity as a coactivator than did CARM1-v1, -v2, and -v4 in both the presence and absence of GRIP1.

Arginine Methyltransferase Activity Is Not Required for the Splicing Activity of CARM1-v3—CARM1-v3 retained arginine methyltransferase activity and showed greater coactivator activity than the other isoforms. Thus, we examined the requirement of arginine methyltransferase activity of CARM1-v3 for the regulation of alternative splicing and for the coactivator function. As shown in Fig. 7A, the CARM1-v1 mutant (CARM1-v1AAA), in which Val-Leu-Asp at position 189–191 was replaced with Ala-Ala-Ala, lost the methyltransferase activity, as reported by Chen et al. (26). Similarly, the CARM1-v3 mutant (CARM1-v3AAA), which had the same amino acid replacements at the same position, also lost the activity. As shown in Fig. 7B, CARM1-v1 and -v1AAA did not have any effect on pre-mRNA splicing, but both CARM1-v3 and -v3AAA had a strong effect on alternative splicing. This result indicates that arginine methyltransferase activity is not required for the splicing activity of CARM1-v3.

To examine the requirement of arginine methyltransferase activity for the coactivator function, the coactivator activity of CARM1-v3AAA was also examined by ERE-Luc reporter assay, in which the CARM1 expression vector was cotransfected with ERE-Luc, ERα, and GRIP1, into COS-7 cells, in the presence of E2. As shown in Fig. 7C, the major isoform CARM1-v1 showed reduced coactivator activity when arginine methyltransferase activity was lost. However, the coactivator activity of CARM1-v3AAA was almost the same as that of CARM1-v3 in the ERE-Luc reporter assay. This result suggests that the coactivator function of CARM1-v3 is independent of the arginine methyltransferase activity.
arginine methyltransferase activity and that CARM1-v3 have properties different from those of CARM1-v1.

The C-terminal End of CARM1-v3 Is Necessary for Splicing—To determine the respective contributions of the CARM1-v3 domains to the selection of the 5' splice site, we generated CARM1-v3 deletion mutants in which the N-terminal domain and C-terminal v3-specific domain were selectively deleted. The splicing activity of CARM1-v3 was compared with that of the deletion constructs in a transient E1A splicing assay, and levels of expression in transfected cells were monitored by Western blotting with the anti-FLAG antibody.

In Fig. 7, arginine methyltransferase activity of CARM1-v3 is not required for the splicing activity. A, arginine methyltransferase activity of CARM1-v1 and -v3 mutants (CARM1-v1AAA and -v3AAA), which contain three amino acid replacements (Val-Leu-Asp → Ala-Ala-Ala) at position 189–191 in both mutants, were examined using histone H3 as a substrate. The reaction conditions were the same as described for Fig. 6.

B, in vivo analysis of splicing activity of CARM1-v3 lacking methyltransferase activity. COS-7 cells were cotransfected with the E1A reporter and CARM1 expression vectors as indicated. The assay conditions were the same as described for Fig. 5. The histogram represents the mean ± S.D. calculated from at least three independent experiments (lower panel). C, coactivator activity of CARM1-v1 or -v3 lacking methyltransferase activity. COS-7 cells were transiently transfected with the ERE-Luc reporter plasmid and expression vectors encoding ERα, GRIP1, and CARM1 mutants as indicated. The assay conditions were the same as described for Fig. 6. The expression of CARM1 mutants were confirmed by Western blotting (WB) with anti-FLAG antibody (lower panel).

In Fig. 8, in vivo analysis of splicing activity of CARM1-v3 deletion mutants. A, schematic representation of the CARM1-v3 deletion mutants. The top diagram represents CARM1-v3. The gray boxes correspond to the v3-specific region. B, COS-7 cells were transiently transfected with the E1A reporter plasmid and expression vectors encoding CARM1-v3 or deletion mutants as indicated. The E1A isoforms were amplified by RT-PCR from RNAs extracted from transfected cells and subjected to agarose gel electrophoresis (upper panel). The histogram represents the mean ± S.D. calculated from at least three independent experiments (middle panel). C, coactivator activity of CARM1-v3 deletion mutants. COS-7 cells were transiently transfected with the ERE-Luc reporter plasmid and expression vectors encoding ERα, GRIP1, and CARM1-v3 mutants as indicated. The assay conditions were the same as described for Fig. 6.
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**Fig. 9. CARM1 isoforms are natural transcripts present in different tissues.** Normal rat tissues were screened by RT-PCR for expression of CARM1-v1, -v2, -v4 (upper panel), -v3 (middle panel), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (lower panel). Arrowsheads, white boxes, and gray boxes indicate primer sets for PCR, CARM1 exons, and the v3-specific region, respectively.

The 13 S and 12 S E1A species counterbalanced by an increase in 9 S mRNA levels. These results suggest that the effect of CARM1-v3 on the selection of the 5' alternative splice site requires its C-terminal v3-specific region in addition to the region conserved among the isoforms (amino acids 1–539).

To analyze the correlation between the effect on alternative splicing and coactivator activity, we also examined coactivator activity of CARM1-v3 deletion mutants by ERE-Luc reporter assay (Fig. 8C). The coactivator activity of the UD1 mutant was almost the same as that of CARM1-v3 in the ERE-Luc reporter assay, but UD2, LD4, and LD5, all of which showed little effect on alternative splicing, showed reduced coactivator activity.

**Tissue Distribution of CARM1 Isoforms—**The effects on alternative splicing by CARM1 were specific to isoform v3. Therefore, we examined the expression pattern of the endogenous CARM1 isoforms in various tissues using RT-PCR. To avoid contamination of PCR fragments derived from the genomic DNA, PCR primers were designed to amplify multiple exons of CARM1 isoforms. As shown in Fig. 9, CARM1-v1 was predominantly detected, being present in fetal brain, adult brain, liver, skeletal muscle, and testis. CARM1-v3 was expressed in fetal brain and adult spleen, liver, and kidney at high levels. CARM1-v4 was present at comparable levels with CARM1-v1 in spleen, lung and kidney. CARM1-v2 was detectable in brain, liver, and testis. Differences in the tissue distribution suggest that the CARM1 primary transcript is processed differently in various tissues.

**DISCUSSION**

Alternative splicing is controlled by the cooperative regulation of factors within the spliceosome and associated proteins. In this report, we identified proteins that associate with a U1 snRNP-specific protein, U1C, using yeast two-hybrid screening. We found that one isoform of the transcriptional coactivator CARM1 interacts with U1C and favors the selection of the distal 5' splice site during the maturation of E1A pre-mRNA used as an alternative splicing model. Given that U1C is responsible for the initial recognition of the 5' splice site sequence (8), our observations suggest that CARM1-v3 can affect selection of the 5' splice site through its association with U1C. This possibility is consistent with reports that several other proteins interacting with U1C are implicated in splicing regulation. The apoptosis-promoting factor TIA-1 has been shown to interact with U1C and modulate alternative splicing of fibroblast growth factor receptor 2 (9). In addition, we previously showed that the oncogenic fusion gene product EWS/NOR1 interacted with U1C and had the ability to alter the 5' splice site selection (10). This fusion protein could complement loss-of-function mutants of snu23p, a component of yeast spliceosomes. The EWS/FLI1 fusion gene product has also been shown to interact with U1C and inhibit RNA splicing mediated by YB-1 (27). Our observation that CARM1-v3 associates with U1C suggests a close linkage with the common transcriptional apparatus and role in pre-mRNA splicing. Hence, CARM1 isoforms may play an important role not only in transcription but also in RNA metabolism.

Using yeast two-hybrid screening, we have also identified Fox-1, HRNBP2, and RBM4 as U1C-associated proteins. These proteins have been shown to be involved in splicing; Fox-1 induced muscle-specific exon skipping of the human mitochondrial ATP synthase γ subunit gene (11), and RBM4 suppressed the use of the proximal splice sites in the E1A reporter (12); however, spliceosomal targets for these factors have not yet been identified.

Our observation that Fox-1, HRNBP2, and RBM4 possibly associated with the U1C protein suggests that these factors regulate the splicing site decision through binding to the U1 snRNP. This is consistent with previous findings (11, 12) and our results showing that Fox-1 and RBM4 affected 5' splice site selection, since U1 snRNP is a factor recognizing the 5' splice site when pre-mRNA splicing occurs (6). Many splicing regulators have been demonstrated to interact with spliceosomal factors, such as SF1, U2AF65, and UsnRNPs, and categorized by spliceosomal targets (28). It may be possible that U1C-binding factors fall into a new category of splicing regulators.

Several lines of evidence support the conclusion that CARM1-v3 is a naturally occurring splice variant and not an artifact. First, we isolated full-length CARM1-v3 cDNA containing a poly(A)+ tail from the rat fetal forebrain cDNA library. Second, various primer sets located on different exons yielded CARM1-v3 products that correctly corresponded to the v3 nucleotide sequences (data not shown), suggesting that the results of RT-PCR for CARM1-v3 were not accidental. Third, the same CARM1-v3 sequence was obtained from different fetal and adult tissues, where a distinct tissue-specific expression was observed. Fourth, CARM1-v3 mRNA is estimated to escape “nonsense-mediated mRNA decay,” which is a mechanism for degrading mRNAs containing a premature termination codon located more than 50–55 bp upstream from the last exon-exon junction (29), because the termination codon of CARM1-v3 was located on the last exon generated by the retention of intron 14 and 15. Although the existence of the endogenous CARM1-v3 protein must be confirmed experimentally, these findings demonstrate that CARM1-v3 is a naturally existing transcript.

To explain the effects of CARM1-v3 on alternative splicing, several possibilities can be proposed. One possibility is that the association of CARM1-v3 with U1C affects the binding of U1 snRNP to the 5' splice site. Previous studies have revealed that the usage of alternative splice sites is often influenced by the competitive binding of splicing factors. For instance, hnRNPA1, which has been shown to affect 5' splice site selection, can disturb 5' splice site occupancy by U1 snRNP (30). The association of CARM1-v3 with U1C may influence the assembly of splicing factors. Another possibility is that CARM1-v3 and associated proteins participate in the alternative splicing cooperatively.
Many splicing regulators possess RNA-binding motifs in their sequences and play a role in splicing through direct binding to the target RNA. However, it is unlikely that the role of CARM1-v3 in splicing proceeds via recognition of a specific RNA sequence, since GST-fused CARM1-v3 did not bind to RNA in vitro (Fig. 3). Recently, two RNA-binding proteins, PABP1 and HuR, were identified as substrates of CARM1 (31, 32). Also, the main pool of proteins that are arginine-methylated possess RNA binding properties (33). Thus, there is a possibility that factors interacting with both RNAs and CARM1 may be involved in the splicing activity of CARM1-v3. The third possibility is that a structural change in CARM1-v3 may cause an alteration of components of the transcription machinery. Since transcription and splicing are coordinated processes at the functional and structural levels (34), the transcription machinery can influence splicing decisions. We observed that CARM1-v3 showed greater coactivator activity than the other isoforms, and the coactivator function of CARM1-v3, but not CARM1-v1, is independent of the arginine methyltransferase activity. Moreover, CARM1-v3 deletion mutants UD2, LD4, and LD5, all of which showed reduced coactivator activity, have little effect on alternative splicing (Fig. 8). It is possible that CARM1-v3 affects alternative splicing through alteration of transcriptional components. The fourth possibility is that functional/structural change in CARM1-v3 affects the RNA polymerase II elongation rate, since previous reports demonstrated that RNA polymerase II elongation rate affects pre-mRNA splicing (35). CARM1 interacts with a p160 coactivator, which is associated with RNA polymerase II, and they synergistically transactivate nuclear receptor-dependent transcription (13, 15). CARM1-v3 may affect the RNA polymerase II elongation rate through interaction with a p160 coactivator. Further investigation will be required to determine the mechanisms by which CARM1-v3 affects alternative splicing.

Although CARM1-v1, -v2, and -v4 were associated with U1C in the mammalian two-hybrid assay, they did not have any effect on alternative splicing. Therefore, the association of CARM1 with U1C is not sufficient and may require additional factors or mechanisms for efficient activity. Given our finding that the CARM1-v3 mutant devoid of the C-terminal v3-specific region did not affect the selection of 5′ splice sites in the E1A pre-mRNA, additional partners interacting with the v3-specific region might be involved in the splicing activity of CARM1-v3.

We observed that the selection of alternative 5′ splice site was affected by CARM1 in an isoform-specific manner. Analysis of the expression pattern of CARM1 isoforms in different tissue types demonstrated that whereas CARM1-v1 was mainly detected in brain, liver, skeletal muscles, and testis, CARM1-v3 was detected in other tissues, such as spleen and kidney. Since developmentally or tissue-specific regulated splicing factors have been expected to have important biological roles, CARM1-v3 might be involved in selecting splicing sites in tissues where CARM1-v3 is expressed at higher levels. Further study will be required to determine the biological roles of the CARM1 isoforms.

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