

# Differential recruitment of nuclear receptor coactivators may determine alternative RNA splice site choice in target genes

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**The biological consequences of steroid hormone-mediated transcriptional activation of target genes might be difficult to predict because alternative splicing of a single neosynthesized precursor RNA can result in production of different protein isoforms with opposite biological activities. Therefore, an important question to address is the manner in which steroid hormones affect the splicing of their target gene transcripts. In this report, we demonstrate that individual steroid hormones had different and opposite effects on alternative splicing decisions, stimulating the production of different spliced variants produced from genes driven by steroid hormone-dependent promoters. Steroid hormone transcriptional effects are mediated by steroid hormone receptor coregulators that also modify alternative splicing decisions. Our data suggest that activated steroid hormone receptors recruit coregulators to the target promoter that participate in both the production and the splicing of the target gene transcripts. Because different coregulators activating transcription can have opposite effects on alternative splicing decisions, we conclude that the precise nature of the transcriptional coregulators recruited by activated steroid receptors, depending on the promoter and cellular contexts, may play a major role in regulating the nature of the spliced variants produced from certain target genes in response to steroid hormones.**

**G**ene expression regulation is a multistep process, including the synthesis of the pre-mRNA or transcription; the 5' and 3' end maturation of the transcript or capping and polyadenylation, respectively; the removal of the introns from the pre-mRNA; and the export to the cytosol of the mRNA and its translation (1). Steroid hormones play a major role in the control of cellular fate and cellular homeostasis by modulating the expression of genes, the products of which are involved in cellular programs such as apoptosis, proliferation, differentiation, and in cellular metabolism (2, 3). Most of the studies of steroid hormone action focus on transcriptional effects that are mediated by the binding of steroid hormone receptors to hormone response elements localized in target gene promoters (4). Nevertheless, the biological consequences resulting from the modulation of the transcriptional activity of genes cannot be precisely predicted because of alternative splicing. Approximately 60% of human pre-mRNAs undergo an alternative splicing process that results in the synthesis from one gene of different mRNAs encoding different proteins having different biological actions (5). For instance, the products of many genes involved in apoptosis are alternatively spliced and this splicing can result in the synthesis of isoforms that antagonize each other by having pro- vs. antiapoptosis effects (6). Therefore, the alternative splicing process can change considerably the biological consequences resulting from the transcriptional modulation of steroid hormone target genes and an important question to address is the mechanism by which steroid hormones control the splicing process at their target genes. Supporting the biological importance of this possibility, it was shown that steroid hormones can affect the nature of spliced variants (7–9).

Alternative splicing decisions are regulated by splicing factors that control the choices of the splicing sites used during the splicing process (10). Two important families of splicing factors have been identified: the heterogeneous nuclear ribonucleoprotein (hnRNP)-related proteins containing various RNA-binding domains, including the RNA-recognition motif (RRM) associated with a variable auxiliary domain (Aux.D) (11); and the serine/arginine-rich (SR)-related splicing factors containing various RNA binding domains and an arginine/serine-rich (RS) domain (12). Both families of splicing factors are known to antagonize each other in certain splicing decisions and it seems likely that the final splicing decision depends on the ratio of functional splicing factors (13, 14). Therefore, by modulating the expression level of certain specific splicing factors, steroid hormones could indirectly affect various splicing decisions. Supporting the existence of such a mechanism, recent experiments have indicated that steroid hormones can affect the expression level of various splicing factors (15–18). However, in each case, it was also shown that steroid hormone treatment affects differentially the expression level of spliced variants encoded by the splicing factor genes (15–18). These observations suggest the existence of other more complex mechanisms. Steroid hormones could affect splicing decisions through nongenomic hormonal actions that are now well characterized and that could result in posttranslational modifications of various splicing factors affecting either their activity or their localization, as recently postulated (18, 19). Nevertheless, such mechanisms do not explain how steroid hormones could specifically control alternative splicing of the transcripts of their transcriptional target genes. Moreover, we have recently shown that steroid hormones can affect splicing decisions in a promoter-dependent manner, which suggests the existence of co-transcriptional mechanisms for regulation of alternative splicing by steroid hormones (9).

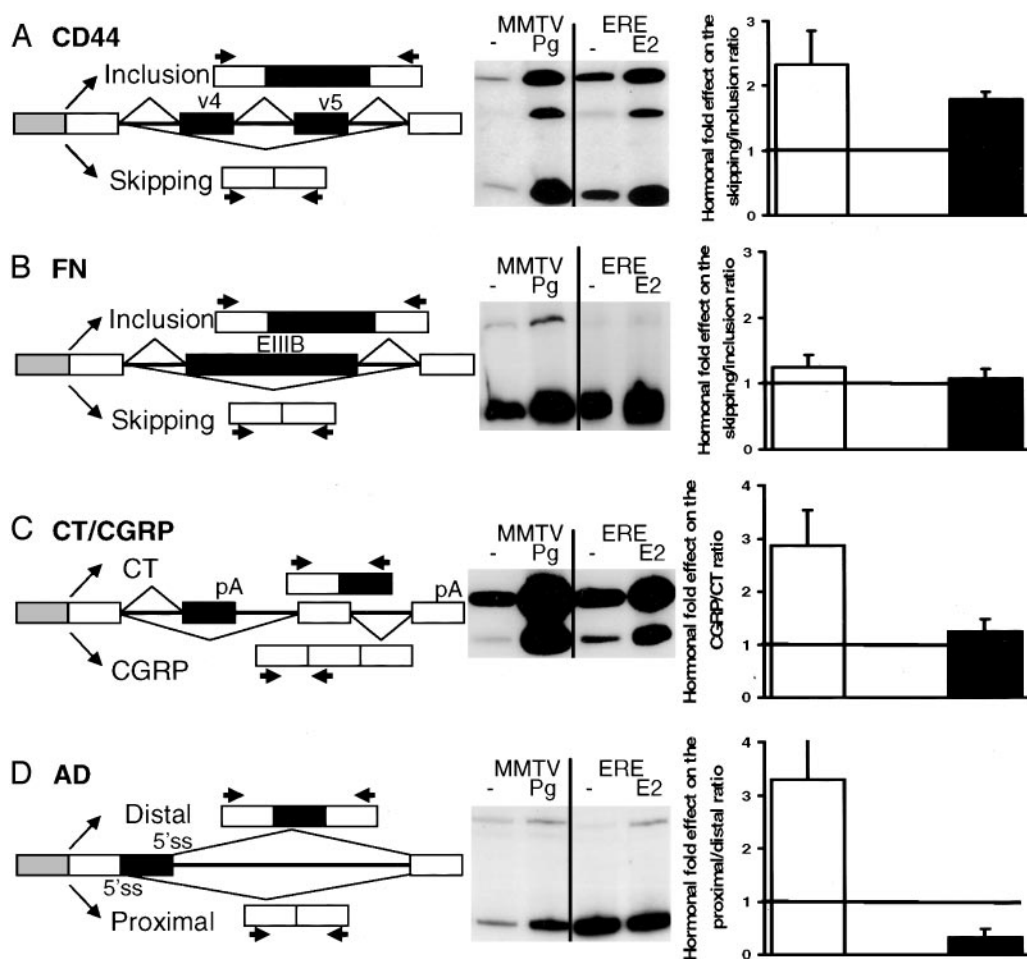
To investigate how steroid hormones could control the splicing process of the products of their target genes, we used reporter genes driven by hormone-responsive promoters, the products of which undergo alternative splicing. We observed that different hormones activating different steroid hormone receptors had distinct impacts on various splicing decisions. Moreover, we demonstrated that different transcriptional coregulators recruited to a promoter by activated steroid hormone receptors can mediate steroid hormone effects on promoter activity that result in opposing effects on exon

Abbreviations: Pg, progesterone; PR, Pg receptor; PRE, Pg-responsive element; MMTV, mouse mammary tumor virus; E<sub>2</sub>, estradiol; ERE, estrogen-responsive element; FN, fibronectin; CT/CGRP, calcitonin/CT gene-related peptide; ss, splice site; RRM, RNA-recognition motif; RS, arginine/serine-rich; SR, serine/arginine-rich; hnRNP, heterogeneous nuclear ribonucleoprotein; A1, hnRNP A1; p50, 50-kDa protein; Aux.D, auxiliary domain; ASC, activating signal cointegrator.

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**Fig. 1.** Different steroid hormones have different effects on alternative splicing decisions. (*Left*) Minigenes giving rise to different spliced variants were cloned downstream of the MMTV or ERE-TATA promoters and were transfected along with either PR or estrogen receptor  $\alpha$ , respectively, in HeLa cells, incubated 24 h in the absence or the presence of Pg or E<sub>2</sub>. After RNA preparation, the ratio of different minigene-spliced variants was determined by low-cycle RT/PCR by using a specific set of radiolabeled primers as described in *Methods*. (*Center*) Autoradiographic films of the radiolabeled PCR products obtained in representative experiments are shown. (*Right*) Average ( $\pm$ SD,  $n = 3$ ) of the Pg (white boxes)- or E<sub>2</sub> (black boxes)-mediated effects on the CD44 skipping/inclusion (A), FN skipping/inclusion (B), CGRP/CT (C), or proximal/distal (D) ratios are shown. In each experiment, the hormonal "fold effect" was obtained by dividing the spliced variant ratio obtained in the presence of hormone by the ratio obtained in the absence of hormone.

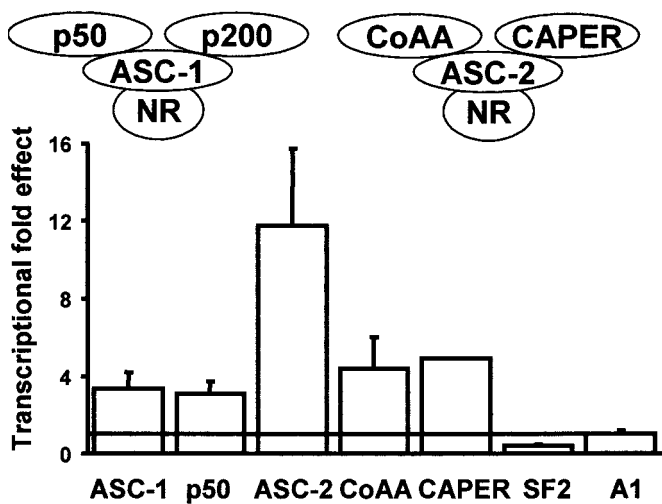
choice. In particular, we showed that the activating signal cointegrator (ASC)-1 and the ASC-2 transcriptional coregulators, as well as their associated proteins, enhance the production of transcripts synthesized from steroid hormone target genes and control the alternative splicing of these transcripts in an opposite manner. Our results suggest that nuclear receptors recruit transcriptional coactivators to target genes, and, depending on the ratio of specific coactivators recruited, can determine the nature of the alternatively spliced variants produced by the target gene.

## Methods

**Transfection.** Mouse mammary tumor virus (MMTV), progesterone (Pg)-responsive element (PRE)-, or estrogen-responsive element (ERE)<sub>2</sub>-TATA promoters were cloned upstream of the previously described CD44, fibronectin (FN), calcitonin/CT gene-related peptide (CT/CGRP), or adenovirus-based test genes (20–23). The precise cloning steps are available on request. Transfection experiments were done in triplicate by using 12-well plates. A transfection master mix was prepared for three wells. Five nanograms per well of steroid receptor expression vectors were cotransfected with 300 ng per well of reporter genes and either 300 ng per well of pBlueScript plasmid (Fig. 1) or 300 ng per well of different coregulator expression vectors (Figs.

2–4), by using Lipofectamine reagent (Invitrogen) and following manufacturer instructions. After six hours of incubation, the medium was replaced with medium containing 5% stripped FBS and either Pg ( $10^{-8}$  M) or estradiol (E<sub>2</sub>) ( $10^{-9}$  M). After 24 h of incubation at 37°C and 5% CO<sub>2</sub>, cells were harvested by using either RLT buffer (Promega) for luciferase assay or 1 ml of TRIzol (Invitrogen) for each set of triplicate wells for RNA isolation following manufacturer instructions.

**DNase Treatment and RT-PCR.** A DNase treatment master mix containing avian myeloblastosis virus Reverse transcriptase/Thermus flavus DNA Polymerase reaction buffer (Access RT-PCR system, Promega), MgSO<sub>4</sub> (2.5 mM final concentration), and RQ1 DNase (1 unit; Promega) was prepared and aliquoted to digest plasmid DNA contamination from RNA preparations for 1 h at 37°C, followed by 15 min of DNase heat inactivation at 65°C. An aliquot of this reaction was used for RT-PCR by using the Access RT-PCR system (Promega). Following the manufacturer's instructions, RT-PCR master mix was prepared containing radiolabeled primers at 1  $\mu$ M. The primers were radiolabeled by using [ $\gamma$ -<sup>32</sup>P]ATP (4,500 Ci/mmol; 1 Ci = 37 GBq) and T4 kinase (Invitrogen) following the manufacturer's instructions. Sense CD44, AGACACCATGCATGGTGCACC;



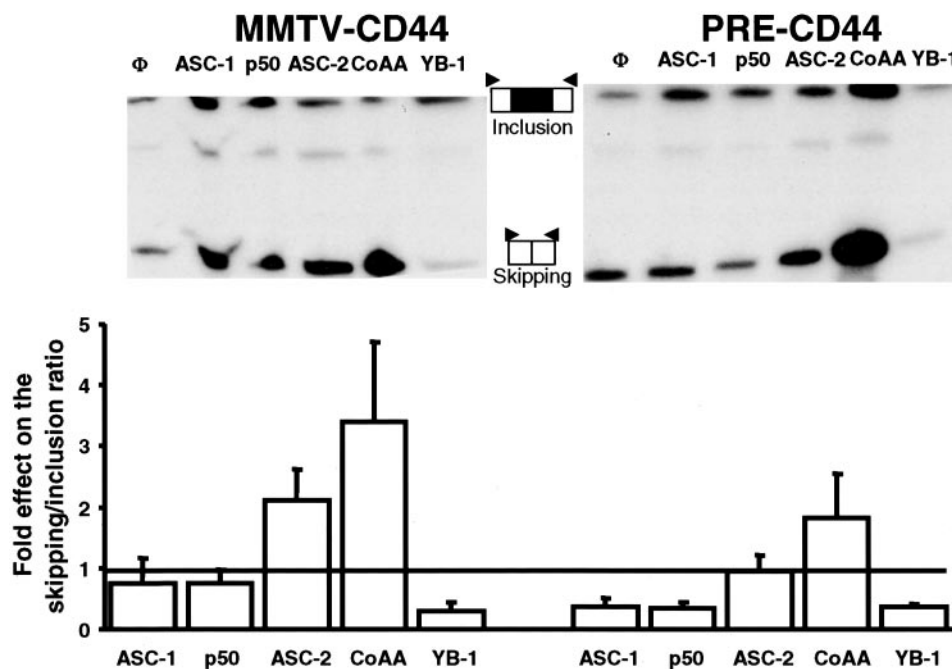
**Fig. 2.** The ASC-1 and ASC-2 complexes enhance Pg-mediated transcriptional effects. The nuclear receptor interacting proteins ASC-1 and ASC-2 interact with various proteins (either p50 and p200 or CoAA and CAPER, respectively) that are structurally related to splicing factors. These proteins, but not the splicing factors SF2 or A1, enhanced the transcriptional activity of a Pg-activated MMTV promoter driving the luciferase reporter gene, as illustrated by the graph. This graph represents the average of three separate but identical experiments in which the luciferase activity obtained in the presence of various expression vectors as indicated was divided by the control luciferase activity obtained in the presence of the empty expression vector.

Antisense CD44, CCATAACAGCATCAGGAGTG; sense CT/CGRP, CATCGCTGTCTGCGAGGGCC; antisense CT/CGRP (exon 4), GAGTTTAGTT GGCATTCTGG; Antisense

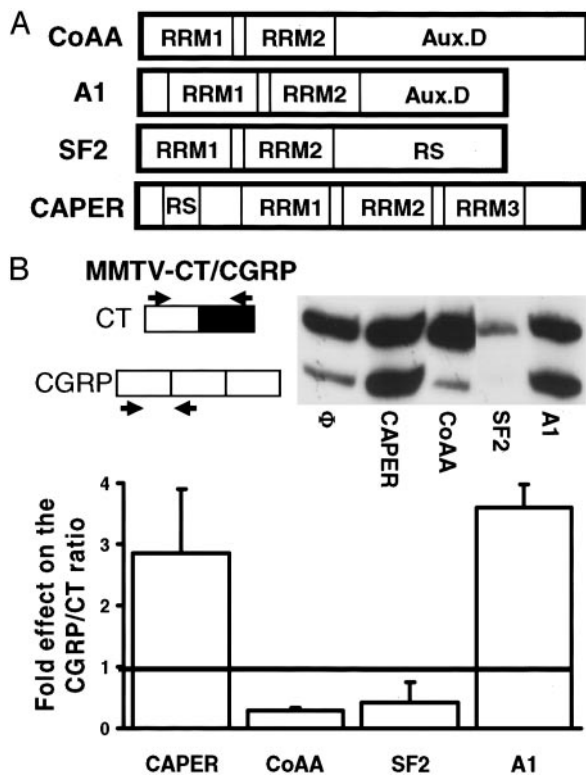
CT/CGRP (exon 5), CTGCTCAGGCTTGAAGGTCC; anti-sense AD, GAAAGACCGCGAAGAGTTTGTCC; sense AD, ATCCCACTGCTTACTGGCTTATC GA; antisense FN, CCCAGTGGCGGTGAATGAGTTGG; and sense FN, ATCA-CAGAGCCTC GCCTTTGCCGATCCG. The precise PCR amplification conditions for the different target minigene products are available on request. Radioactive RT-PCR products derived from either the FN, Adenovirus-based, or CT/CGRP minigenes were fractionated on nondenaturing 5% polyacrylamide gels. Radioactive RT-PCR products derived from CD44 minigenes were fractionated on denaturing 5% polyacrylamide gels. Dried gels were exposed to autoradiographic films or placed in PhosphorImager cassettes to allow quantification by using the PhosphorImager system (Molecular Dynamics).

## Results

**Different Steroid Hormones Have Different Effects on Alternative Splicing Decisions.** To carry out the present study, we cloned different reporter genes, the products of which undergo various alternative splicing decisions, downstream of either the MMTV promoter or an ERE-TATA promoter sensitive to either Pg or E<sub>2</sub>, respectively. The first minigene tested was derived from the human CD44 gene that contains 21 exons, 11 of which can be alternatively spliced (20). The reporter gene described in Fig. 1A contained only two of these variable exons, v4 and v5, that can be either included or excluded during the splicing process. When driven by the MMTV or an ERE promoter, Pg or E<sub>2</sub> treatment, respectively, resulted in an expected increase in the amount of the transcripts produced by the reporter gene and favored the production of the spliced variant that did not contain the two variable exons (skipping), compared with the spliced variant containing both variable exons (inclusion). Therefore, the skipping/inclusion ratio increased  $\approx 2.5$ - and  $\approx 2$ -fold under Pg or E<sub>2</sub>



**Fig. 3.** The ASC-1 and ASC-2 complexes mediate opposite effects on the alternative splicing of CD44 transcripts. MMTV- or PRE-CD44 reporter genes were transfected with PR and various expression vectors in HeLa cells incubated 24 h in the presence of Pg. (Upper) Autoradiographic films of the radiolabeled-RT/PCR products obtained in representative experiments are shown. (Lower) The average ( $\pm$ SD,  $n = 3$ ) of the effects of the different proteins on the CD44 skipping/inclusion ratio. In each experiment, the fold effect was obtained by dividing the spliced variant ratio obtained in the presence of the different expression vectors by the control ratio obtained in the presence of the empty expression vector ( $\Phi$ ). ASC-1 and its interacting protein, p50, stimulated CD44 exon inclusion preferentially on the PRE-CD44 reporter gene products, whereas ASC-2 and its interacting protein, CoAA, stimulated CD44 exon skipping preferentially on the MMTV-CD44 reporter gene products. The splicing factor YB-1 had the same effect on both reporter gene products.



**Fig. 4.** Two coactivators related either to hnRNP proteins or to SR splicing factors mediate opposite effects on alternative splicing of CT/CGRP transcripts. (A) The coactivator CoAA containing two N-terminal RNA-binding domain (RRMs) and a C-terminal Aux.D are structurally related to the splicing factor A1. The coactivator CAPER is structurally related to SF2 characterized by the presence of the RS domain. (B) The MMTV-CT/CGRP reporter gene was transfected with PR and various expression vectors in HeLa cells incubated 24 h in the presence of Pg. (Upper) The autoradiographic film of the radiolabeled RT/PCR products obtained in a representative experiment is shown. (Lower) The average ( $\pm$ SD,  $n = 3$ ) of the effects of the different proteins on the CGRP/CT ratio are shown. In each experiment, the fold effect was obtained by dividing the spliced variant ratio obtained in the presence of the different expression vectors by the control ratio obtained in the presence of the empty expression vector ( $\emptyset$ ).

treatment, respectively (Fig. 1A). In contrast, neither Pg nor E<sub>2</sub> affected the alternative splicing of an FN-derived reporter gene containing one variable exon (21), which demonstrates that steroid hormones do not affect the splicing of all variable exons (Fig. 1B). Interestingly, steroid hormones did not always induce the same splicing effect because by using a minigene derived from the human CT/CGRP gene (22), activation of the Pg receptor (PR) by Pg resulted in an increase in the production of the CGRP RNA compared with the production of the CT RNA ( $\approx$ 3-fold); in contrast, E<sub>2</sub> activation of the estrogen receptor  $\alpha$  had no effect on the CGRP/CT ratio (Fig. 1C). Moreover, by using a minigene (AD) containing two competitive 5' splice sites (5' ss) (23), Pg and E<sub>2</sub> mediated opposite effects on splicing. Pg treatment stimulated the use of the proximal 5' ss (the amount of RNA resulting from the proximal 5' ss use was increased  $\approx$ 3-fold compared with the amount of RNA resulting from the distal 5' ss use), whereas E<sub>2</sub> treatment stimulated the use of the distal 5' ss (the proximal/distal ratio was decreased  $\approx$ 3-fold; Fig. 1D). Altogether, these results demonstrate that steroid hormones and promoter context can affect various splicing decisions in a gene-specific manner and different hormones and receptors can have different impacts on RNA splicing of target genes.

**The ASC-1 and ASC-2 Complexes Enhance Pg-Mediated Transcriptional Effects.** Steroid hormone receptors activated by their hormones mediate their transcriptional effects by recruiting coregulators to their target gene promoters (21, 24). Therefore, we tested whether a subset of these coregulators could mediate the steroid hormone effects on splicing described in Fig. 1. Of particular interest were the ASC-1 and ASC-2 coregulators that interact with different steroid hormone receptors (25, 26) and with various proteins that are structurally related to proteins involved in the splicing process (Fig. 2). ASC-1 copurifies with a 50-kDa protein (p50) that contains a KH domain, a well characterized RNA-binding domain present in many splicing factors, and with a 200-kDa protein (p200) very similar to a protein in the U5 small nuclear ribonucleoprotein, a ribonucleoprotein complex involved in splicing process (25). ASC-2, also called TRBP, interacts with CoAA, an hnRNP-like protein; and CAPER, an SR-like protein (27, 28). As shown in Fig. 2, all of these proteins, and in particular the splicing factor-related proteins, p50, CoAA, and CAPER, enhanced Pg-activated MMTV promoter transcriptional activity, whereas two classical splicing factors, hnRNPA1 (A1) and SF2, did not do so. This result demonstrates that only a subset of proteins structurally related to splicing factors can enhance the steroid hormone-mediated effects on transcription, leading to an increase in the amount of total mRNA. Because not all splicing factors had an effect on steroid-mediated transcriptional effects, it suggested that the ASC-1- and ASC-2-interacting proteins have a specific transcriptional function.

**The ASC-1 and ASC-2 Complexes Mediate Opposite Effects on the Alternative Splicing of CD44 Transcripts.** After demonstrating that the ASC-1 and ASC-2 coregulators and their associated proteins stimulated the production of Pg-regulated gene transcripts, we then tested whether these proteins could change the nature (exon content) of these transcripts. Interestingly, by using the CD44 reporter gene driven by the MMTV promoter or a PRE-TATA promoter, we observed that the ASC-1 and ASC-2 complexes mediated opposite effects on splicing in a promoter-preferential manner (Fig. 3). ASC-1 and p50 favored the production of the spliced variants containing both CD44 variable exons (inclusion) compared with the spliced variants that did not contain these exons (skipping), when the CD44 minigene was driven by the PRE-TATA promoter but not by the MMTV promoter (Fig. 3 Right). In contrast, ASC-2 and CoAA favored the production of the spliced variants that did not contain the CD44-variable exons compared with the spliced variants containing both variable exons preferentially when the CD44 transcripts were produced from the MMTV promoter (Fig. 3 Left). As a control, the splicing factor YB-1 that we had shown previously to stimulate exon inclusion (20) had the same quantitative effect on the alternative splicing of the CD44 transcripts produced from either the MMTV or the PRE-TATA promoters (Fig. 3). This result suggests that in addition to their transcriptional effects, transcriptional coregulators can impact splicing decisions in a promoter-specific manner on this specific target gene. Because both ASC-1 and ASC-2 proteins stimulated transcription but mediated opposite splicing effects, these data demonstrate also that the splicing phenotype induced by these proteins is not simply a consequence of their transcriptional effects.

**Two Coactivators Related Either to hnRNP Proteins or to SR Splicing Factors Mediate Opposite Effects on Alternative Splicing of CT/CGRP Transcripts.** Alternative splicing decisions are regulated by the binding of multiple protein factors to regulatory sequences within or near to the pre-mRNA-regulated exon. Frequently, both positive- and negative-acting factors bind to the same region and the final splicing decision is dictated by the ratio of these antagonizing factors. One well studied example of this type of regulation occurs in the recognition of alternative exons containing multiple 5' or 3' ss; in this case, the antagonism is between an hnRNP protein and an SR protein (13, 14). Two of

the factors studied here resemble either hnRNP or SR proteins. CoAA has a domain structure similar to A1, containing two N-terminal RRM domains and a C-terminal Aux.D rich in tyrosine and glycine residues. CAPER, in contrast, resembles the SR protein SF2, with several RRMs and an RS domain (Fig. 4).

By using the adenovirus-based reporter gene that contains two competitive 5' splice sites (Fig. 1), we could not observe significant opposite action of CoAA vs. CAPER (data not shown). Nevertheless, by using the CT/CGRP reporter gene containing two competitive 3' terminal exons (Fig. 1), the choice of which can be differently regulated by SF2 vs. A1 (Fig. 4; ref. 29), we observed that CoAA and CAPER induced opposite splicing decisions. CoAA favored the production of the CT-spliced variant, whereas CAPER favored the production of the CGRP-spliced variants (Fig. 4). Very interestingly, CoAA acted like SF2, whereas CAPER acted like A1. Therefore, although structurally related to well defined splicing factors, our results suggest that the transcriptional coregulators affect splicing by different mechanisms than did classical splicing factors.

## Discussion

By altering the coding sequences of the mRNAs produced from a target gene, the alternative splicing process can considerably change the biological consequences, resulting from transcriptional stimuli such as steroid hormones (5, 7–9). To examine the regulation of alternative splicing choices by steroid hormones, we cloned reporter genes, the products of which undergo alternative splicing (20–23), downstream of promoters responsive to steroid hormones. We observed that different hormones activating different steroid hormone receptors had differential and even opposite impacts on splicing decisions (Fig. 1). These observations demonstrate that in the context of our four test genes, different transcriptional stimuli can stimulate the synthesis of different spliced variants produced by their target genes. Because steroid hormone actions are known to be mediated by receptor dependent recruitment of transcriptional coregulators at the promoter level (4, 24), we tested whether subsets of coregulators could also mediate the steroid hormone effects on splicing. We demonstrated that the ASC-1 and ASC-2 coregulators and their respective interacting partners (25–28) stimulate the production of transcripts from steroid hormone-regulated genes, but that ASC-1 and ASC-2 had opposite effects on the alternative splicing of the target gene products (Figs. 2 and 3). Our observations suggest that steroid hormone receptors induce formation of transcriptional complexes that both stimulate tran-

script production and control the nature of the spliced variants produced from these genes.

The specific set of coregulators that are recruited by activated steroid receptors to a target gene promoter and stimulate the production of a specific spliced variant from this target gene will depend on several parameters. First, as shown in Fig. 1, the nature of the spliced variant produced will depend on the nature of the steroid hormone activating its receptor and the structural organization of the gene itself. Second, the nature of the coregulators engaged in the transcriptional complex will depend on the promoter context. Indeed, the presence of other transcriptional factors present at a given promoter could participate in stabilizing different combinations of receptor transcriptional complexes (4, 24). This possibility was suggested by the promoter-specific splicing effects mediated by ASC-1 and ASC-2 on the MMVT vs. the PRE promoter (Fig. 3). Third, the ability of a given activated steroid receptor to recruit a specific set of coregulators will depend on their cellular availability. In other words, because different cells express different levels of various coregulators, the recruitment of a specific set of coregulators on a target gene promoter by activated steroid receptors and consequently the nature of the spliced variants produced from the target gene will be cell-specific. Finally, we also observed that two proteins known to interact with ASC-2 (27, 28) have differential effects on splicing (Fig. 4). ASC-2 could recruit either CoAA or CAPER, depending on the identity of the steroid hormone and cognate receptor interacting with ASC-2, on the promoter context in which ASC-2 is recruited, or on the relative expression level of CoAA and CAPER.

Finally, it is possible that the differential recruitment of CoAA and CAPER could depend on the posttranslational status of ASC-2. Indeed, like many other transcriptional coregulators, ASC-2 is known to be targeted by various signaling pathways (24, 30, 31). In other words, posttranslational modifications of ASC-2 by different signaling pathways could allow ASC-2 to preferentially interact with CoAA or CAPER, and therefore in response to steroid hormones, ASC-2 could recruit one or the other coactivator, depending on the signaling context within the cell. In this model, coregulators would act to integrate the various cellular signals and permit the synthesis of the correct amount of the specifically spliced variant in a given cellular context.

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