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Research Article

Splicing Factor Tra2-β1 Is Specifically Induced in Breast Cancer and Regulates Alternative Splicing of the *CD44* Gene

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Abstract

The human *CD44* gene undergoes extensive alternative splicing of multiple variable exons positioned in a cassette in the middle of the gene. Expression of alternative exons is often restricted to certain tissues and could be associated with tumor progression and metastasis of several human malignancies, including breast cancer. Exon v4 contains multiple copies of a C/A-rich exon enhancer sequence required for optimal inclusion of the exon and binding to the nucleic acid–binding proteins YB-1 and human Tra2-β1. Here, we show that hTra2-β1, a member of the extended family of serine/arginine-rich (SR) splicing factors, enhances the *in vivo* inclusion of CD44 exons v4 and v5. It increased inclusion of exons v4 and v5 and acted synergistically with YB-1. Activation required the C/A-rich enhancer within exon v4. Several other SR proteins had none or only a slight effect on CD44 exon inclusion. In contrast, SC35 inhibited exon usage and antagonized the effects of Tra2 or YB-1. In a matched pair analysis of human breast cancers and their corresponding nonpathologic tissue controls, we found a significant induction of Tra2-β1 in invasive breast cancer, both on the RNA and protein levels. Together with our functional data, these results suggest an important role for Tra2-β1 in breast cancer. Induction of this splicing factor might be responsible for splicing of CD44 isoforms associated with tumor progression and metastasis. (Cancer Res 2006; 66(9): 4774-80)

Introduction

Alternative pre-mRNA splicing is emerging as an important mechanism of genetic diversity (1–3). Recent microarray data show that 74% of human genes undergo alternative splicing, which generates different protein isoforms (4). This importance of splicing is underlined by the increasing number of diseases associated with missplicing. These events can be caused by either mutations in regulatory sequences, such as splice sites, branch point, and enhancer/silencer sequences, or alterations in *trans*-acting factors (5, 6). Changes in splice site selection have been frequently observed in cancer (e.g., *MDM2, Bin1*, and *FGFR-2* genes in breast, skin, and prostate cancers; refs. 7–10).

A striking example for changes in alternative splicing due to tumorigenesis is CD44. Human CD44 is able to produce several hundred functional mRNAs through the combinatorial inclusion of one or multiple in-frame alternative exons (11–13).

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Considerable attention has focused on CD44 variable exons v4 through v6 because the inclusion of these exons has been shown to correlate with both tumorigenesis and metastasis (14, 15) of several malignancies, including breast cancer (16). Molecularly, we know the most about the sequences and factors involved in the recognition of variable exons v4 and v5. Exon v5 contains both splicing enhancer and silencing elements (17–19).

Exon v4 (Fig. 1*A*) has several copies of a C/A-rich sequence (Fig. 1*B*) known to be a strong exon enhancer (20–23). We have shown previously that this sequence binds the single-stranded RNA and DNA-binding protein YB-1 (24). Increased YB-1 concentration induces exon v4 as well as exon v5 inclusion. The effect depends on the presence of the C/A-rich exon enhancer within exon v4 (24).

The best characterized C/A-rich exon enhancers are from the *Drosophila* doublesex gene. Multiple repeats of consensus sequence UCUUCAAUCAACA (dsxRE) are located within the female-specific exon and bind dTra, dTra2, and serine/arginine-rich (SR) proteins (25–31). Exon v4 also contains another enhancer element of the sequence AAAGGACAAAGGACAAAA (dsxPRE) that binds dTra2 and is required for maximal exon recognition (30, 31). In humans, two orthologues of dTra2 exist, hTra2-β1 and hTra2-α (32, 33). *In vitro*, Tra2-α and Tra2-β were shown to influence splice site selection by interacting with GAAGAA-rich enhancers (34–36). Recently, the binding sequence of Tra2-β1 was determined *in vivo* and found to contain considerably more pyrimidines than determined by *in vitro* analyses (37). This binding site GHVVGANR is present in exon v4 (Fig. 1*B*).

CD44 exons v4 and v5 contain both C/A- and purine-rich enhancer. The characterized important C/A-rich enhancer within exon v4 is similar to both the dsxRE and the dsxPRE (Fig. 1*B*). Exon v5 contains a 12-nucleotide purine-rich enhancer with a GAAGAA sequence similar to the binding site of human Tra2-β1. Furthermore, it contains one copy of the Tra2-β1-binding motif determined *in vivo* (37). The structure of the CD44 exons v4 and v5 suggested that hTra2-β1 might influence splicing of these exons.

Deregulation of hTra2-α and hTra2-β has been observed in several pathologic conditions [e.g., stroke (38, 39), atherosclerosis (40), nerve injury (41), and silicosis (42)]. We therefore wondered whether Tra2-α and Tra2-β expression changes in cancer. Recently, we showed in a model of breast cancer development a stage-dependent induction of alternative CD44 mRNA splicing, which was accompanied by an increase of distinct SR proteins in tumors and their metastasis (16). To test whether the molecular properties of Tra2-β1 are relevant in an oncologic context, we analyzed Tra2-β expression in invasive human breast cancer together with matched pairs of nonpathologic tissue specimen. We found that Tra2-β1 is significantly up-regulated in breast cancer, which could explain some of the observed changes in alternative splicing patterns.

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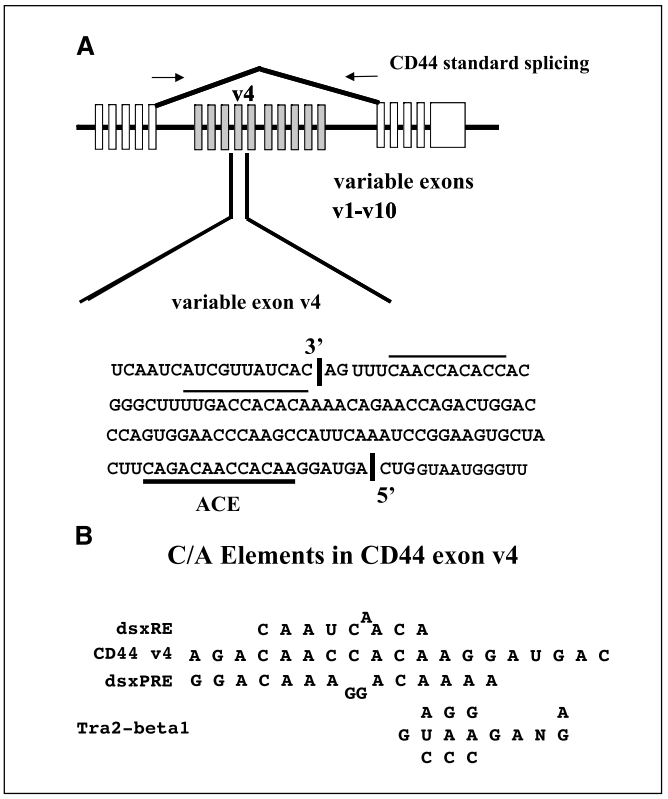

Figure 1. CD44 alternative splicing. *A,* genomic structure of human CD44. *Gray,* alternative exons; *white,* constitutive exons; *underlined,* major C/A-rich element (*ACE*); *overlined,* other C/A-rich sequences. *B,* comparison of the major C/A-rich enhancer sequence in CD44 exon v4 to the enhancer repeat element (dsxRE) and the purine-rich enhancer (dsxPRE) in the female-specific alternative exon from the *Drosophila melanogaster* doublesex gene and hTra2-β1-binding site.

Materials and Methods

Plasmids and transfections. The wild-type and mutant *CD44* minigenes used to study exons v4 and v5 inclusion have been described previously (24). *In vivo* splicing phenotypes were determined following transfection of this minigene into HeLa cells after 48 hours of transfection. Low cycle (15-20 cycles) of reverse transcription-PCR (RT-PCR) amplification of total cell RNA using radiolabeled primers specific for β-globin exons flanking the CD44 variable exons was used to determine exon inclusion levels. The RT-PCR protocol used has been shown to faithfully represent the relative amounts of RNA containing or missing exons v4 and v5 (24). When cDNAs representing the two products of the alternative splicing reactions were transfected in varying ratios, the RT-PCR amplification assay used detected relative amounts of produced RNA directly reflective of input DNA ratios, indicating both that the assay is linear and that there is no pronounced RNA stability difference between the two spliced products.

Tissues. Archived fresh-frozen samples of patients with primary invasive adenocarcinomas of the breast and matched pairs of surrounding nonpathologic breast tissue were analyzed for their Tra2-β expression profile. Samples were initially taken during breast conserving surgery or mastectomy in women diagnosed with breast cancer without prior primary systemic therapy. The local pathologist did in all cases the diagnosis of invasive tumor and corresponding nonpathologic tissue with the fresh-frozen section technique. Patients gave informed consent before the operation, and tissue collection was approved by the local ethic committee of the University of Freiburg (Freiburg, Germany). All tissue specimen were frozen in liquid nitrogen and stored at −80°C until further analysis. In addition, for immunohistochemical analysis, the corresponding paraffin-embedded tissue was analyzed by the same pathologist.

RNA isolation and RT-PCR. Tissues were minced on dry ice before they were treated with a tissue homogenizer (Polytron, Littau, Switzerland) in Trizol solution (Life Technologies, Inc., Gaithersburg, MD) with 1 mL/100 mg tissue thrice for 10 seconds. After 5 minutes of incubation at 26°C, 0.2 mL chloroform/1 mL Trizol was added, and the solution was shaken vigorously followed by incubation at 26°C for 5 minutes. After centrifugation for 15 minutes at 4°C and 12,000 × *g*, the aqueous phase containing the total RNA was precipitated with 0.5 mL isopropyl alcohol/1 mL Trizol for 10 minutes at 26°C and centrifuged for 10 minutes at 12,000 × *g* at 4°C. The pellets were rinsed with 75% ethanol (1 mL ethanol/1 mL Trizol reagent used for the initial homogenization), vortexed and centrifuged at 4°C for 5 minutes at 7,500 × *g*, and finally dissolved in RNase-free water and stored at −80°C for further analysis.

RNA (5 μg) was used for cDNA synthesis using Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer, Branchburg, NJ) and oligo(dT) primers followed by PCR (30 cycles) using primers specific for Tra2-β1: sense 5′-CATAGACGATCACGTAGCAGGT-′3 and antisense 5′-GAGAGCTGCCATAGGTAGGTC-′3 and 18S RNA as internal control sense 5′-CATAGACGATCACGTAGCAGGT-′3 and antisense 5′-GAGAGCTGCCATAGGTAGGTC-′3.

The expected amplicon size was 305 bp for Tra2-β and 418 bp for 18S RNA.

Protein isolation and Western blot. Total cellular protein was isolated from the interphase and phenol phase from the initial homogenate after precipitation of the DNA with 0.3 mL 100% ethanol/1 mL Trizol used for the initial homogenization. The samples were stored at 26°C for 5 minutes and afterwards centrifuged at 2,000 × *g* for 5 minutes at 4°C. The phenol-ethanol supernatant was than precipitated with 1.5 mL isopropyl alcohol/1 mL Trizol used for initial homogenization. After storage for 10 minutes at 26°C, the protein precipitates were centrifuged at 12,000 × *g* at 4°C for 10 minutes. Protein pellets were washed thrice in 0.3 mol/L guanidine hydrochloride in 95% ethanol for 20 minutes at 26°C and centrifuged at 7,500 × *g* for 5 minutes at 4°C. The protein pellets were vortexed after the final wash in 2 mL 100% ethanol, stored for 20 minutes at 26°C, and finally centrifuged at 7,500 × *g* for 5 minutes at 4°C. The pellets were resuspended in 1% SDS solution and incubated at 50°C for complete dissolution. Insoluble material was removed by centrifugation at 10,000 × *g* for 10 minutes at 4°C. Supernatants were stored for further analysis at −80°C. Protein (30 μg) was subjected to SDS/PAGE (12%), transferred onto enhanced chemiluminescence membranes (Amersham, Piscataway, NJ), incubated with rabbit hTra2 antibody, diluted 1:2,000 in 1× NET/gelatin [2.5 g/1,000; 10× NET buffer: 1.5 mol/L NaCl, 0.05 mol/L EDTA, 0.5 mol/L Tris (pH 7.5), 0.5% Triton X-100], and detected with an anti-rabbit antibody coupled to horseradish peroxidase (1:3,000; Amersham).

Immunohistochemistry. Routinely formalin-fixed and paraffin-embedded specimens were studied for the expression of Tra2-β by using a polyclonal anti-Tra2-β1 (43). For visualization of Tra2-β1 expression, antigen retrieval and indirect immunoperoxidase technique were applied as described recently (23).

Antigen retrieval was done by cooking the slides for 30 minutes in a citrate buffer (pH 6.0). The slides were incubated with the Tra2-β1 antibody (1:2,000) for 60 minutes at room temperature.

Statistical analysis. Analysis of the different splicing factor effects on CD44v4 and CD44v5 inclusion was done after 6 individual cotransfections for each factor.

Expression difference of Tra2-β1 RNA between tumor and normal breast tissue was calculated. The percentages of Tra2-β RNA compared with the amount of 18S RNA was used. The Kolmogorov-Smirnov test showed a nonnormal distribution of the results, which was not improved by logarithmic transformation. Therefore, the Wilcoxon test for paired samples was used. The Statistical Package for the Social Sciences software version 13.0.1 was used for statistical analysis.

Results

Increased expression of Tra2-β1 induces CD44 exons v4 and v5 inclusion. To test the effect of Tra2 on CD44 variable exon

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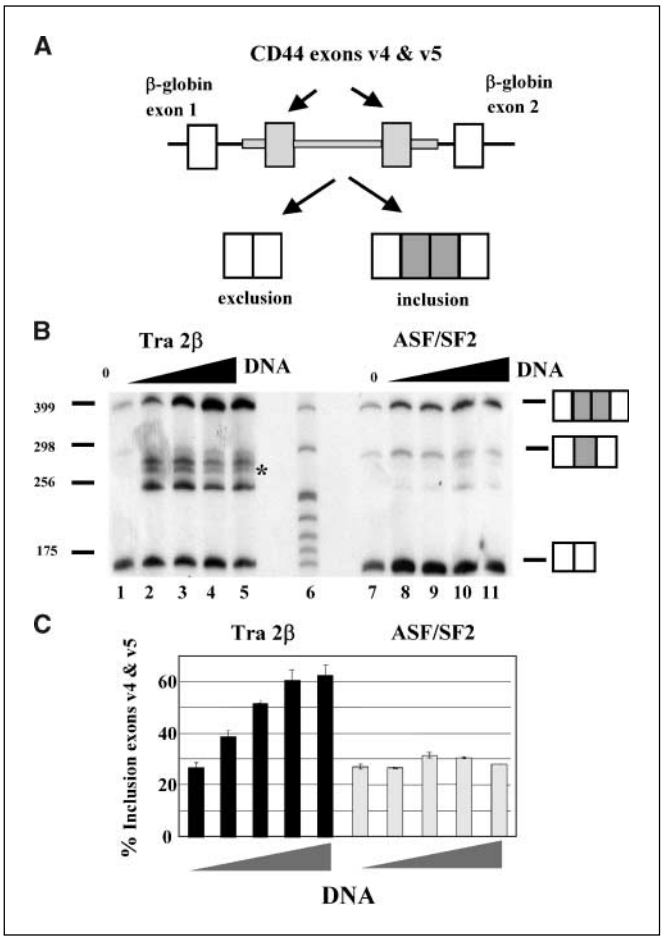

Figure 2. Human Tra2-β1 increases inclusion of CD44 exons v4 and v5. *A,* minigene used as a splicing reporter. Exons v4 and v5, along with the intron sequences between and surrounding them, were inserted into the first intron of the human *β-globin* gene. Product RNAs from this minigene either include or exclude exons v4 and v5. *B,* RNA splicing patterns by low-cycle RT-PCR. Cotransfection of the reporter in (*A*) and expression vectors (0, 1, 2, 3, or 4 μg) coding for either human Tra2-β1 or ASF/SF2. Major products resulting from normal splicing patterns. *Asterisk,* cotransfection with Tra2-β1 also induced the production of aberrant RNA. *C,* quantification of the effects of Tra2-β1 or ASF/SF2 from multiple experiments. Gels of amplification products were analyzed in the phosphorimager, and the percentage of RNA, including both exon v4 and exon v5, was plotted with respect to the amount of input Tra2-β1 DNA.

splicing, we used a transient expression vector, in which exons v4 and v5 and their surrounding intron sequences were inserted into an intron of the human *β-globin* gene driven by the cytomegalovirus promoter (Fig. 2*A*). This splicing reporter produces multiple-spliced RNA products on transfection of HeLa cells (24). The majority product (75%) of the RNA lacks both CD44 variable exons (Fig. 2*B*), whereas the minority product includes both exons. An even less frequent RNA results from the inclusion of one variable exon. We have shown previously that this splicing reporter responds to increasing concentrations of YB-1 and the RNA helicase p72 to induce the inclusion of both exons to >80% (24, 44).

Cotransfection of this reporter construct with human Tra2-β1 (Fig. 2*B*) altered splicing of the construct and increased the inclusion of both exons from 19.5 ± 5.78% to 65 ± 4.74% (mean ± SD; Fig. 2*C*). In contrast, cotransfection with the related SR protein ASF/SF2 had little to no effect on CD44 alternative splicing with an induction of exons v4 and v5 inclusion up to 31.6 ± 3.48% (Fig. 2*B* and *C*).

In *Drosophila*, dTra2 works in concert with dTra to activate the C/A-containing doublesex exon. Both hTra2-α and hTra2-β can complement for dTra2 loss in *Drosophila* (32). Although a human orthologue to dTra does not exist, it was of interest to see if dTra could influence the ability of hTra2 to activate CD44 splicing. As shown in Fig. 3, cotransfection of the reporter minigene with dTra had only a minimal effect on inclusion. hTra2 was slightly inhibited in its ability to activate both exons in the presence of dTra. This result suggests that a human Tra-like protein probably does not participate in CD44 alternative splicing.

The effect of Tra2-β1 is exon specific. The action of hTra2-β1 protein could be due to recruitment of splicing factors to generic *cis*-elements. We therefore tested whether Tra2-β1 affects the inclusion of the constitutive middle exon of the reporter Dup 33.

This exon is flanked with weak signals derived from the human *β-globin* gene and weakened through internal deletions (45). Because Tra2-β1 did not significantly affect this minigene, we conclude that its effect is not the result of an induced binding to generic splicing signals (Fig. 4*A*). To determine which sequences within the CD44 exons were required to enhance exon inclusion by Tra2-β1, we assayed mutants of CD44 exon v4, in which the various C/A-rich sequences had been mutated. In earlier studies, we have reported that the C/A-rich sequence closest to the 3′-end of the exon was the most important sequence for recognition (24). Mutation of this sequence causes production of a RNA containing exon v5 but lacking exon v4 (24). Cotransfection with Tra2-β1 did not reverse the effect of the mutation or increased of exon v5 inclusion (Fig. 4*B, lanes 5* and *6*). This result suggests that the induction of exons v4 and v5 inclusion required the presence of the C/A-rich sequences within exon v4. Although the result with exon v4 is expected, the result with exon v5 is somewhat surprising given the presence of the sequence GAAGAA within exon v5. The mutant assayed in Fig. 4 altered the exon v4 enhancer sequence from CAGACAACCACAAGGA to CAGAuAAggACuAGGA. This not only changed the C/A richness of the enhancer and its similarity to the C/A-rich doublesex repeat elements but also converted the sequence into a sequence quite similar to the doublesex purine-rich element (sequence GGACAAAGGACAA). Despite this similarity, raising the concentration of human Tra2-β1 was not sufficient to reverse the effect of the mutation for exon v4 recognition. This result underlines the importance of the C/A-rich elements as CD44

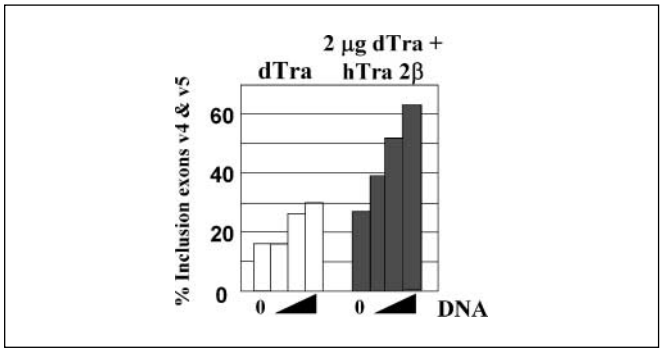

Figure 3. *Drosophila* Tra (*dTra*) does not activate CD44 variable exon splicing. The CD44 reporter from Fig. 2 was cotransfected with expression vectors coding for *Drosophila* Tra (0, 1, 2, or 4 μg) or a mixture of human Tra2-β1 (0, 1, 2, or 4 μg). RT-PCR analysis was done as in Fig. 2. The amount of RNA resulting from inclusion of both exon v4 and exon v5 was quantified as in Fig. 2.

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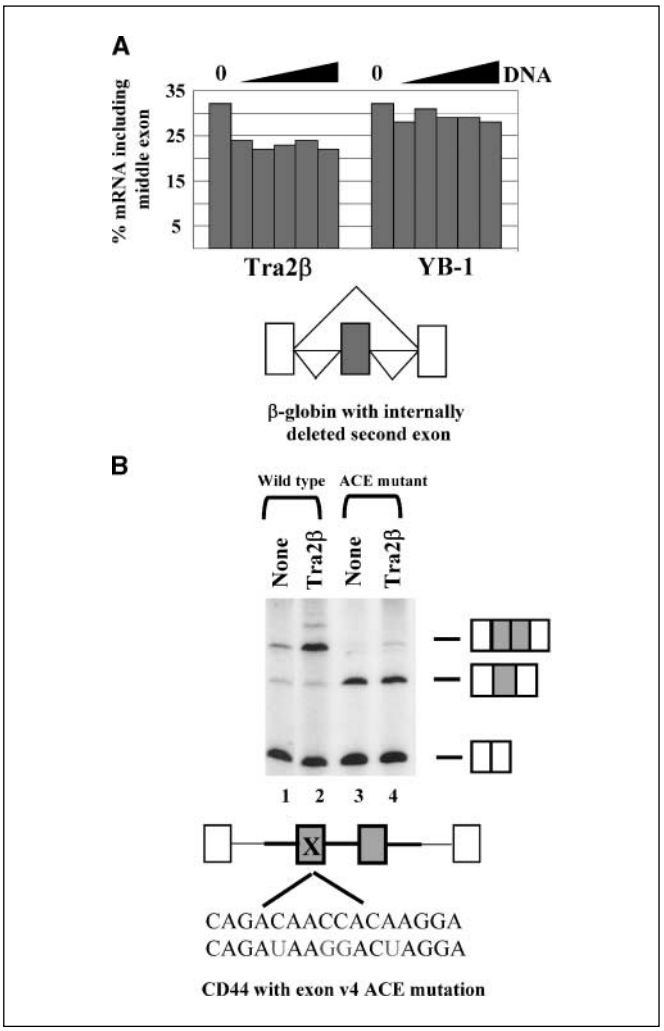

Figure 4. C/A-rich sequences within exon v4 are required for an effect of Tra2 on exon inclusion. *A,* Tra2-β1 did not affect the inclusion of a weak constitutive middle exon derived from the human *β-globin* gene. Increasing amounts of expression vectors coding for Tra2-β1 or YB-1 were cotransfected with the Dup 33 reporter derived from the human β-globin RNA containing a weakened middle exon trough internal deletion (44). The middle exon in this gene is only partially included because of the deletion. *B, lanes 3* and *4,* Tra2 did not activate inclusion of both exon v4 and exon v5 if the C/A-rich sequences within exon v4 were mutated.

enhancer and indicates that Tra2-β1 binds to multiple elements on the CD44 exons v4 and v5.

Effect of other SR proteins on CD44 exons v4 and v5 splicing. We tested other SR proteins for their ability to affect the recognition and inclusion of CD44 variable exons v4 and v5. Although 9G8 and SRp55 did not enhance inclusion significantly (data not shown), SC35 slightly decreased inclusion of the two exons from 19.5 ± 5.8% down to 11.8 ± 3.2% (Fig. 5). SRp20 slightly increased inclusion of both exons up to 32.6 ± 6.6% (Fig. 5), whereas SRp75 had just a small effect on both exon inclusion with 30.4 ± 3.2% but caused pronounced production of a RNA containing only one of the two variable exons from 9.1 ± 1.9% to 24.2 ± 3.3% (Fig. 5). Multiple RNA-binding proteins are observed to bind simultaneously to splicing regulatory signals.

Induction of variable exon inclusion in CD44 is accompanied by Tra2-β1 expression in invasive breast cancer. Thus far, we showed that Tra2-β1 promotes exons v4 and v5 inclusion in experimental cell culture systems. To test whether this effect could be observed in tumor samples, we analyzed human breast cancer tissue.

Expression analysis was done in histologically confirmed breast cancers and corresponding pairs of nonpathologic breast tissue. CD44 alternative splicing was determined by RT-PCR using primers directed against the constitutive exons 5 and 16 of the *CD44* gene (Fig. 6*A*). Tra2-β1 expression in breast cancer development was monitored by Tra2-β-specific RT-PCR (Fig. 6*B* and *C*) and Western blot (Fig. 6*D*). Breast cancer specimen showed an induction of alternative CD44 splicing compared with nonpathologic tissue in accordance to recently published findings (Fig. 6*A*; ref. 16). This induction was accompanied by an increase in Tra2-β expression. We analyzed the amount of Tra2-β1 RNA in 36 breast cancer samples compared with normal breast tissue of the same patient. All values were normalized to 18S RNA. RT-PCR revealed an overexpression of Tra2-β1 RNA in breast cancer samples. The mean of Tra2-β1 RNA in breast cancer was 85.7 ± 23.4% of 18S RNA (mean ± SD) in contrast to 57.7 ± 35.6% in normal breast tissue (Fig. 6*C*). This difference was statistically highly significant ($P < 0.0001$). RT-PCR analysis of SRp40 as a control revealed constant levels in normal and cancerous tissues (data not shown). Western blot results confirmed the induction of Tra2-β1 on the protein level (Fig. 6*D*).

For a more detailed analysis of Tra2-β1 expression in breast cancer, we did additional immunohistochemical analysis of the matched pairs. In accordance with our previous findings (Fig. 7), immunohistochemistry revealed a specific induction of Tra2-β1 in breast cancer. In all specimen investigated, strong nuclear Tra2-β1 expression was restricted to invasive breast cancer tissue (Fig. 7*A* and *B*). Weak or no expression was found in adjacent normal breast tissue of the respective patients (Fig. 7*C*). These findings indicate a specific induction of Tra2-β1 expression in breast cancer development rather than unspecific changes of the splicing machinery.

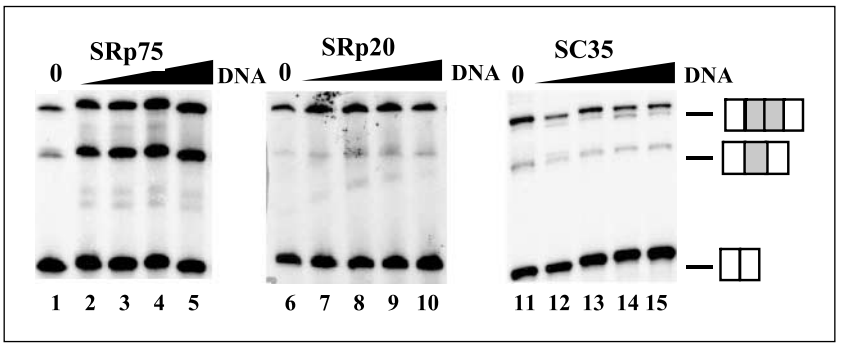

Figure 5. SRp20 and SRp75 have a minimal effect on recognition of CD44 exons v4 and v5. Cotransfections were done using the splicing reporter (Fig. 2) and expression vectors for human SRp20, SRp75, and SC35 (0, 1, 2, 3, or 4 μg).

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Discussion

Changes in alternative splicing of the *CD44* gene are associated with tumor progression and metastasis in breast cancer. A recently published study suggested that specific alterations in the relative concentrations of SR proteins during breast cancer development might be responsible for these effects (16), but functional data were not determined.

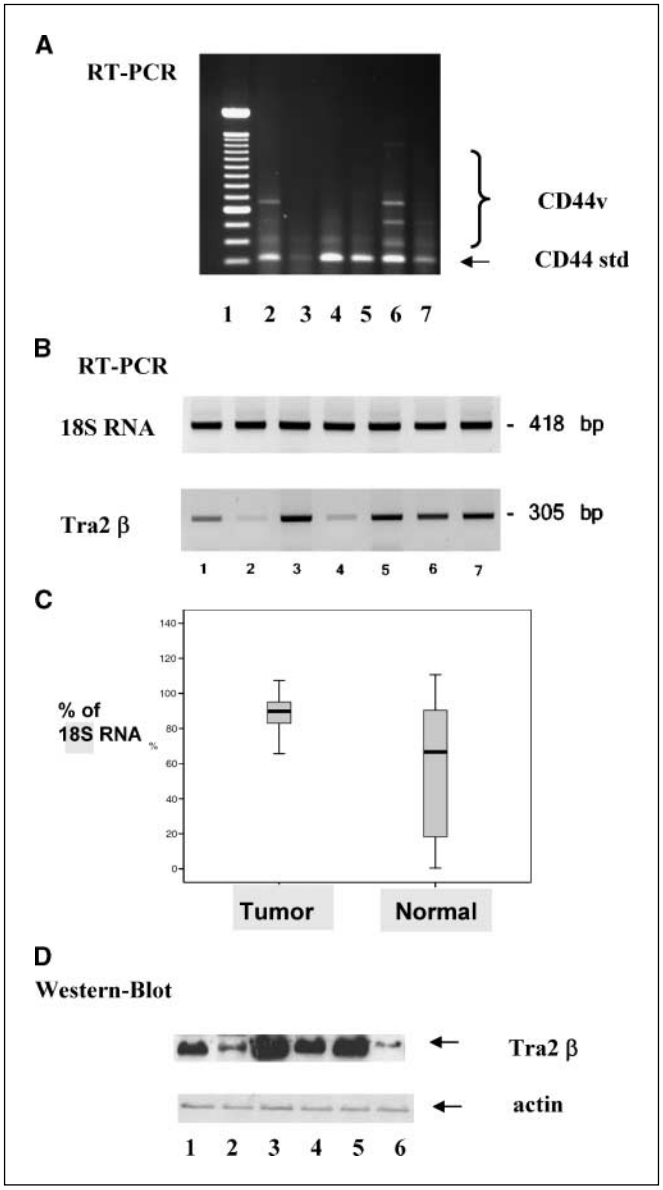

Figure 6. Specific induction of alternative CD44 splicing is accompanied by induction of Tra2-β1 in breast cancer. CD44 splicing and expression of Tra2-β1 in breast cancer and corresponding pairs of normal tissue was determined by low-cycle RT-PCR and Western blot. *A,* RT-PCR analysis of CD44 with equal amounts of total cell RNA (5μg). *Arrow,* constitutive CD44 splicing (*CD44std*); *dashed line,* alternative CD44 splicing. *Lane 1,* marker; *lane 2,* tumor 1; *lane 3,* normal tissue 1; *lane 4,* tumor 2; *lane 5,* normal tissue 2; *lane 6,* tumor 3; *lane 7,* normal tissue 3. *B,* Tra2-β1 RT-PCR analysis with equal amounts of total cell RNA (5 μg). *Lane 1,* tumor 1; *lane 2,* normal tissue 1; *lane 3,* tumor 2; *lane 4,* normal tissue 2; *lane 5,* tumor 3; *lane 6,* normal tissue 3; *lane 7,* positive control (HeLa). *C,* statistical analysis of Tra2-β1 RNA normalized to 18S RNA in 36 breast cancers (tumor) and corresponding normal breast tissue (normal). *Thick lines,* median (50% percentile); *gray boxes,* 25% to 75% percentile; *thin lines,* minimal and maximal value (without rouge results). *D,* Tra2-β protein expression analysis by Western blot with human Tra2-β-specific and actin-specific antibody as control (equal amounts of protein). *Lane 1,* tumor 1; *lane 2,* normal tissue 1; *lane 3,* tumor 2; *lane 4,* normal tissue 2; *lane 5,* tumor 3; *lane 6,* normal tissue 3.

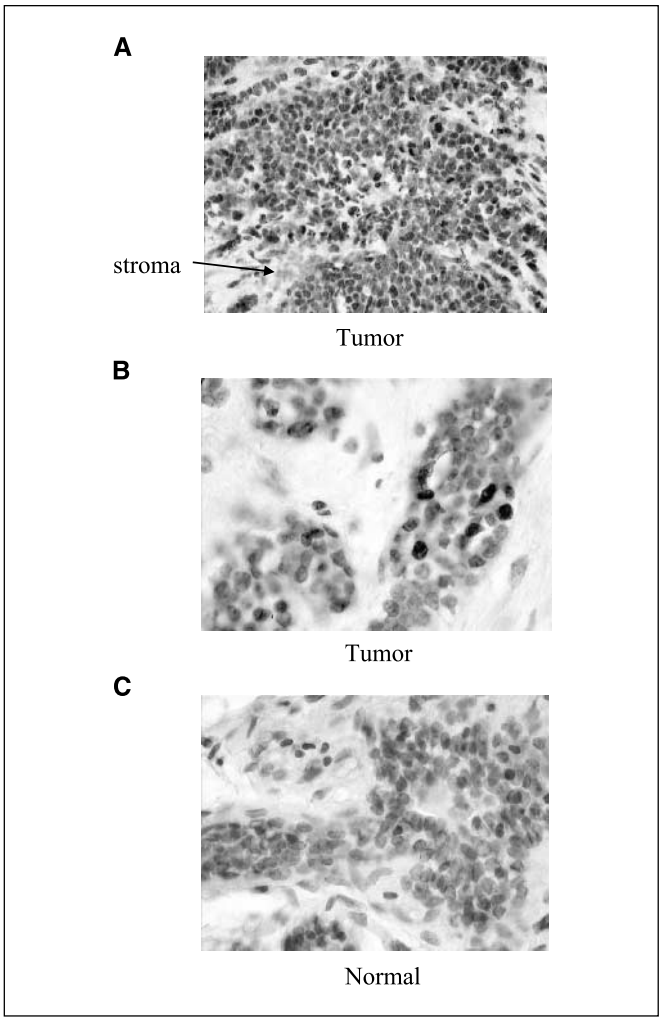

Figure 7. Induction of Tra2-β1 is exclusively restricted to invasive breast cancer cells. Immunohistochemical analysis of Tra2-β1 expression in tumors and normal breast tissue. *A* and *B, dark brown,* breast cancer with a strong specific nuclear expression of Tra2-β1 in invasive breast carcinoma cells. No Tra2-β1 expression is observed in the stromal cells of these breast cancers. *C,* no expression of Tra2-β in corresponding normal breast tissue adjacent to invasive carcinoma is detectable.

dTra2 was the first member of the SR family of splicing factors shown to be required for exon recognition regulated by exonic enhancer sequences (46). Further experiments showed that dTra2, dTra, and several other SR proteins act synergistically to recognize individual exonic enhancer sequences (26–31). In humans, no orthologue for *Drosophila* dTra has been uncovered, suggesting that human Tra2 proteins may bind to RNA with noticeably different binding partners and RNA specificity. *In vitro*, GAAGAA-based enhancers were identified as targets for hTra2-β1 binding, suggesting that hTra2-β1 binds to G/A-rich exon enhancers. Experiments *in vivo* showed that hTra2-β1 binds to a more degenerate sequence GVVGANR, which is partially found in the doublesex gene, where the protein could interact with the dsx-repeat AAAGGACA*AAGGACA*AA, which is rich in CAA sequences (putative Tra2-β1-binding sites are italicized). This enhancer could be considered as a version of C/A-rich elements, which we identified earlier as an exon enhancer sequence involved in the

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specific regulation of alternative CD44 exons v4 and v5 splicing by YB-1 (24).

This background made human Tra2-β1 a potential candidate to regulate alternative CD44 splicing. We observed Tra2-β1-dependent activation of inclusion of two CD44 variable exons that have both C/A-rich and G/A-rich exon enhancer sequences. The ability of both proteins to enhance inclusion, however, was dependent on a single C/A-rich element (CAGACAACCACAAGGA) that resides within exon v4. When this sequence was mutated to CAGAuAAggACuAGGA, inclusion of v4 was eliminated but some level of inclusion of exon v5 remained. Increasing the concentration of Tra2 did not result in increased inclusion of exon v5 in this mutant background despite the presence of the sequence GAAGAA within an enhancer in exon v5. Inclusion of exon v4 was also not restored despite the fact that the created mutation contained two GGA elements similar to the purine-rich enhancer element from the *Drosophila* doublesex exon that binds dTra2. This observation suggests that human Tra2 recognizes C/A-rich exon enhancers rather than G/A-rich exon enhancers in CD44 and agrees with the binding sequence of Tra2-β1 found *in vivo*.

It should be noted that CD44 alternative splicing is often accompanied by inclusion of neighboring variable exons, suggesting interaction between the factors that recognize individual CD44 exons. Exons v4 and v5 may be particularly prone to this type of multiexon recognition. Examination of cytoplasmic RNAs coding for CD44 variable exons has detected not only RNAs that contain both exons v4 and v5 but also RNA species that contain the short intron between them but no other intron (47). Thus, it is possible that exons v4 and v5 are initially recognized as a single large exon followed by removal of the intervening sequence between them. Several reports have also suggested that the levels of CD44 mRNA containing this intron are increased in tumor cells where inclusion of exons v4, v5, and v6 are also increased (48).

Although CD44 splicing was not dependent on *Drosophila* dTra, it is possible that other human proteins play the role of Tra in Tra2-mediated recognition of the CD44 exons, such as hnRNP G (49, 50). Two human proteins that influence exon v4 inclusion, YB-1 and the RNA helicase p72, were thus far identified (24, 44). Like Tra2, YB-1 binds to C/A-rich sequences. Therefore, it seems possible that the interaction of Tra2-Tra-SR proteins could be replaced with a Tra2-YB-1-p72 interaction on CD44 exon v4.

The statistically significant induction of Tra2-β1 expression levels in invasive breast cancer, in contrast to their corresponding pairs of normal tissue controls, is in accordance to our findings of differential SR protein expression changes in a mouse model of breast cancer development (16). These findings were conclusive on the RNA as well as protein level and, here, especially morphologically as determined by immunohistochemistry. These results are accompanied by an induction of alternative CD44 splicing in the same paradigm.

In additional cell culture experiments, we analyzed the potential Tra2-β1 effect on endogenous CD44. In parallel to findings with YB-1, these experiments could not detect an overall induction of alternative splicing of the endogenous *CD44* gene (data not shown). YB-1 and Tra2-β1 are, to date, the strongest splicing activators of CD44. The missing effects on endogenous CD44 might be due to the large internal cassette of variable CD44 exons with large numbers of silencing *cis*- and *trans*-acting elements that are deleted in our reporter constructs.

Besides *CD44*, several other genes show altered RNA processing during breast cancer development, including hormone receptors and genes involved in apoptosis (51–53). Because splicing changes in these proteins may have a pronounced effect on cellular function, it is important to understand the role of alternative processing in breast cancer. Specific splicing factor expression patterns might be responsible for the metastatic phenotype of certain tumors and thereby represent new targets for intervention in subgroups of patients at high risk.

With respect to our functional data, our findings suggest a specific involvement of human Tra2-β1 in the recognition of CD44 variable exons and a potential influence in regulating the expression of CD44 isoforms involved in breast cancer progression and metastasis.

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