Oxidative stress-induced alternative splicing of \textit{transformer 2}\(\beta\) (\textit{SFRS10}) and \textit{CD44} pre-mRNAs in gastric epithelial cells

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\textbf{Takeo K, Kawai T, Nishida K, Masuda K, Teshima-Kondo S, Tanahashi T, Rokutan K.} Oxidative stress-induced alternative splicing of \textit{transformer 2}\(\beta\) (\textit{SFRS10}) and \textit{CD44} pre-mRNAs in gastric epithelial cells. \textit{Am J Physiol Cell Physiol} 297: C330–C338, 2009. First published May 13, 2009; doi:10.1152/ajpcell.00009.2009.—The \textit{tra2}\(\beta\) gene encoding an alternative splicing regulator, \textit{transformer 2}\(\beta\) (\textit{Tra2}\(\beta\)), generates five alternative splice variant transcripts (\textit{tra2}\(\beta\)1–5). Functionally active, full-length \textit{Tra2}\(\beta\) is encoded by \textit{tra2}\(\beta\)1 isoform. Expression and physiological significance of the other isoforms, particularly \textit{tra2}\(\beta\)4, are not fully understood. Rat gastric mucosa constitutively expressed \textit{tra2}\(\beta\)1 isoform and specifically generated \textit{tra2}\(\beta\)4 isoform that includes premature termination codon-containing exon 2, when exposed to restraint and water immersion stress. Treatment of a gastric cancer cell line (AGS) with arsenite (100 \(\mu\)M) preferentially generated \textit{tra2}\(\beta\)4 isoform and caused translocation of \textit{Tra2}\(\beta\) from the nucleus to the cytoplasm in association with enhanced phosphorylation during the initial 4–6 h (acute phase). Following the acute phase, AGS cells continued upregulated \textit{tra2}\(\beta\)1 mRNA expression, and higher amounts of \textit{Tra2}\(\beta\) were reaccumulated in their nuclei. Treatment with small interference RNAs targeting \textit{up-frameshift-1} or transfection of a plasmid containing \textit{tra2}\(\beta\)1 cDNA did not induce \textit{tra2}\(\beta\)4 isoform expression and did not modify the arsenite-induced expression of this isoform, suggesting that neither the nonsense-mediated mRNA decay nor the autoregulatory control participated in the \textit{tra2}\(\beta\)1 isoform generation. Knockdown of \textit{Tra2}\(\beta\) facilitated skipping of the central variable region of the \textit{CD44} gene and suppressed cell growth. In contrast, overexpression of \textit{Tra2}\(\beta\) stimulated combinatorial inclusion of multiple variable exons in the region and cell growth. The similar skipping and inclusion of the variable region were observed in arsenite-treated cells. Our results suggest that \textit{Tra2}\(\beta\) may regulate cellular oxidative response by changing alternative splicing of distinct genes including \textit{CD44}, arsenite; splicing regulator; \textit{tra2}\(\beta\)4 isoform; premature termination codon; cell growth.

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\textbf{ALTERNATIVE SPlicing is a process by which exons are either included or excluded from a single pre-mRNA, resulting in the synthesis of functionally diverse protein isoforms from an identical gene (3). Thus, the process expands the coding capacity of the genome and also regulates the gene expression program (15, 16). Exons are defined by three major \textit{cis}-elements, the 5'- and 3'-splice sites and the branch point (14). In addition, exonic or intronic splicing enhancer/silencer elements regulate exon recognition, which are recognized by \textit{trans}-acting factors, such as a family of serine/arginine (SR)-rich proteins and heterogeneous nuclear ribonucleoproteins (11). The complex interplay between \textit{trans}-acting factors and \textit{cis}-acting elements regulates alternative splice site utilization in a developmental stage-, sex-, or tissue-specific manner and in response to surrounding microenvironment (26).

A SR-like protein, \textit{transformer 2}\(\beta\) (\textit{Tra2}\(\beta\)), was discovered as a novel RNA-binding protein (RA301) in rat astrocytes exposed to hypoxia/reoxygenation (17) and then molecularly identified to be a mammalian homolog of \textit{Drosophila} \textit{Tra2} (2). \textit{Drosophila} \textit{Tra2} regulates sex-specific alternative splicing of pre-mRNAs from several genes crucial for sexual differentiation (1). In mammals, there are two mammalian homologues: \textit{Tra2}\(\alpha\) (8) and \textit{Tra2}\(\beta\) (2). \textit{Tra2}\(\beta\) is now known as a sequence-specific pre-mRNA splicing enhancer that binds to the purine-rich exonic enhancer sequence in a concentration-dependent manner. Several pathological conditions, such as hypoxia (17), silicosis (24), arteriosclerosis (30), and breast cancer (33), have been shown to increase \textit{Tra2}\(\beta\), and this protein is now recognized as one of the important splicing regulators that are involved in the pathogenesis of several diseases including cancer (26, 33). To date, \textit{Tra2}\(\beta\) has been shown to regulate alternative splicing of pre-mRNAs from distinct genes including \textit{CD44} (33), \textit{liver scavenger receptor class B} (\textit{SRB}) (34), \textit{tau} (10), \textit{homeodomain-interacting kinase 3} (\textit{HipK3}) (31), \textit{fibroblast growth factor receptor 2} (\textit{FGFR2}) (6), \textit{glutamate receptor subunit B} (\textit{GluR-B}) (6), \textit{calcitonin/calcitonin gene-related peptide} (\textit{CGRP}) (29), and \textit{survival motor neuron 2} (\textit{SMN2}) (13).

The \textit{tra2}\(\beta\) gene (\textit{SFRS10}) is composed of 10 exons and 9 introns, and generates 5 transcripts (\textit{tra2}\(\beta\)1–5) by alternative splicing (see Fig. 1C) (19). Functional, full-length \textit{Tra2}\(\beta\) protein is encoded by \textit{tra2}\(\beta\)1 isoform containing all exons except for exon 2 that has multiple premature termination codons (PTCs). \textit{tra2}\(\beta\)4 isoform is composed of all 10 exons including exon 2. There are three other shorter mRNA variants. \textit{tra2}\(\beta\)2 isoform consists of exon 1 and 2b. \textit{tra2}\(\beta\)3 joins exon 1 to 4 and uses all other downstream exons. \textit{tra2}\(\beta\)5 isoform contains exons 3–10. The \textit{tra2}\(\beta\)3 splice variant is expressed predominantly in the brain, liver, and testis, and weakly in the kidney, and its expression is developmentally regulated in a tissue- and temporal-specific pattern (5). Expression and functions of a minor transcript \textit{tra2}\(\beta\)4 are not fully understood. \textit{Tra2}\(\beta\) regulates alternative splice site selection in a concentration-dependent manner; therefore, \textit{Tra2}\(\beta\) must be maintained at a proper level. Stoilov et al. (27) have recently shown that human \textit{tra2}\(\beta\) pre-mRNA utilizes its own exon 2 for a negative feedback loop. Excess amounts of \textit{Tra2}\(\beta\) bind to four enhancers present in exon 2 and stimulate inclusion of exon 2, resulting in the generation of \textit{tra2}\(\beta\)4 variant that cannot be...
translated. As a consequence, Tra2β synthesis is effectively switched off (27).

In this study, we showed that acute psychological stress specifically stimulated generation of tra2β4 isomorph in rat gastric mucosa. Exposure of a gastric cancer cell line (AGS) to arsenite also transiently stimulated preferential generation of tra2β4 isomorph, followed by upregulation of tra2β1 mRNA expression. We suggest here that enhanced expression and alternative splicing of the tra2β gene may constitute a part of complex cellular responses to oxidative stress.

MATERIALS AND METHODS

Animals and cells. All rats were treated in accordance with the Guiding Principles in the Care and Use of Animals of the American Physiological Society, and all procedures were approved by the Animal Care Committee of the University of Tokushima. Male Wister strain rats weighing ~200 g were purchased from Japan SLC (Shizuoka, Japan). They were subjected to restraint and water immersion stress as described previously (12). Before (0 h) and 0.5, 1, 2, and 4 h after starting the stress, the rats were anesthetized with diethyl ether and killed. Their stomachs were removed immediately and subjected to analyses. A human gastric cancer cell line (AGS) was cultured in RPMI supplemented with 10% FBS and antibiotics at 37°C in 5% CO2.

Cloning and identification of tra2β isoforms. Total RNA was prepared from rat gastric mucosa or AGS cells by homogenizing in a mixture of acid guanidium-isothiocynate-phenol-chloroform (Isogen; Nippon Gene, Tokyo, Japan) and an adaptor primer (5′-CACCCATGCTGACTGTCAGC-3′ (reverse)). RT-PCR was done using SYBR Green Master Mix (Applied Biosystems) and the Applied Biosystems 7500 real-time RT-PCR System.

For reverse transcription (RT)-PCR, 1 μg of isolated RNA was reverse transcribed with oligo(dT)16 (16 mer) primer and MLV reverse transcriptase (Promega, Madison, WI). The following primer sets were used to amplify the open reading frame (ORF) of both human and rat tra2β mRNAs, 5′-ATGAGCGCAAGGCGGA-3′ (forward) and 5′-TATAAGGCGAAGGCTGAG-3′ (reverse). PCR was run with Taq DNA polymerase (Promega) for 30 cycles (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min). The amplified products were ligated into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and subjected to sequencing (model ABI 377; Applied Biosystems, Foster city, CA). To determine the size of 3′-untranslated region (3′-UTR) of rat or human tra2β mRNA, the total RNA was reverse transcribed using an adaptor-conjugated oligo dT primer (5′-TGGAGAGATAGCAGCGCCGCCCGAGGTTTGGGTTTTTTTGT-3′), and PCR was then done using a primer designed for the 3′-end of ORF (5′-TCTACCTCTCGTGGACTCCAGTCATAGTATAACGC-3′ and an adaptor primer (5′-TGGAGAGATAGCAGCGCCGCCCGAGGTTTGGGTTTTTTTGT-3′).

Real-time RT-PCR and Northern hybridization. Amounts of mRNAs for tra2β1 together with tra2β4 were measured by quantitative real-time RT-PCR using a primer set designed to amplify tra2β exons 3–4. A primer set specific for tra2β exon 2 was used to measure tra2β2 mRNA level. These primer sets are listed in Table 1. Real-time RT-PCR was done using SYBR Green Master Mix (Applied Biosystems) and the Applied Biosystems 7500 real-time RT-PCR System.

Glyceralddehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control for normalization. Amounts of CD44 exon 5, standard CD44 transcript joining exon 5 to exon 15, and individual exons 6–14 in the central variable region were measured by quantitative real-time RT-PCR using the primer sets listed in Table 1. GAPDH mRNA was also used as an internal control for normalization.

Expression of tra2β transcripts was also measured by Northern blot analysis. Both human and rat tra2β1 ORF cDNAs prepared as described above were used as probes. A cDNA probe for rat tra2β exon 2 was prepared by digestion of the rat tra2β4 cDNA with Tru9I. The prepared cDNA probes were labeled with [α-32P]dCTP using random primers and the Amersham Megaprime DNA labeling system (GE Healthcare, Buckinghamshire, UK). Samples of 20 μg of total RNA were separated in a 1% agarose gel containing 0.6 M formaldehyde and transferred to a nylon membrane filter. After hybridization, the membrane was hybridized at 60°C with the [α-32P]-labeled probe overnight. After washing, bound probes were analyzed by BAS 1500 Image Analyzer (FUJIFILM, Tokyo, Japan).

Preparation of cell protein extracts. AGS cells were lysed with RIPA buffer, composed of 10 mM Tris-HCl (pH 7.4), 1% NP-40, 1 mM EDTA, 0.1% SDS, 150 mM NaCl, and protease inhibitor cocktail (Roche Applied Science Japan, Tokyo, Japan). The extracted proteins (20 μl lane) were separated by SDS-PAGE in a 12% polyacrylamide gel and immunized rabbits with synthetic peptides of the amino acid residues 1–12 of Tra2β. The extracted proteins (20 μg protein per lane) were separated by SDS-PAGE in a 12% polyacrylamide gel and

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Table 1. List of primer sets used in quantitative real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>tra2β1 plus</td>
<td>5′-GGAAGGTCTGCTACGAGATCCC-3′ (exon 3 forward)</td>
</tr>
<tr>
<td>tra2β4</td>
<td>5′-CAGCTGGAGATTTCTCAGAGG-3′ (exon 4 reverse)</td>
</tr>
<tr>
<td>tra2β4</td>
<td>5′-AGGAAATTGGCAGAGGCTG-3′ (exon 2 forward)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-GCCACCATGCGCTGAGACA-3′ (forward)</td>
</tr>
<tr>
<td>CD44 exon 5</td>
<td>5′-TGAGGGCATTCTCCATGACAGC-3′ (reverse)</td>
</tr>
<tr>
<td>Standard CD44</td>
<td>5′-TCTCTAGAGATTGAGGAC-3′ (reverse)</td>
</tr>
<tr>
<td>CD44 exon 6</td>
<td>5′-TTTCTGAGAACGACCTGTCACAGG-3′ (forward)</td>
</tr>
<tr>
<td>(v1and v2)</td>
<td>5′-CAGGCAATTGTTTGGTGTTG-3′ (reverse)</td>
</tr>
<tr>
<td>CD44 exon 7</td>
<td>5′-TGATCAGCTTCAAAATACCTGACTCACG-3′ (forward)</td>
</tr>
<tr>
<td>(v3)</td>
<td>5′-GTCGGCTGAGAATAATGGTCTAC-3′ (reverse)</td>
</tr>
<tr>
<td>CD44 exon 8</td>
<td>5′-TTTCTACACGCACAGGCGG-3′ (forward)</td>
</tr>
<tr>
<td>(v4)</td>
<td>5′-GTCAGCTTCAAAATACCTGACTCACG-3′ (forward)</td>
</tr>
<tr>
<td>CD44 exon 9</td>
<td>5′-TTTCTGAGAACGACCTGTCACAGG-3′ (forward)</td>
</tr>
<tr>
<td>(v5)</td>
<td>5′-CAGGCAATTGTTTGGTGTTG-3′ (reverse)</td>
</tr>
<tr>
<td>CD44 exon 10</td>
<td>5′-TTTCTGAGAACGACCTGTCACAGG-3′ (forward)</td>
</tr>
<tr>
<td>(v6)</td>
<td>5′-CAGGCAATTGTTTGGTGTTG-3′ (reverse)</td>
</tr>
<tr>
<td>CD44 exon 11</td>
<td>5′-CAGGCAATTGTTTGGTGTTG-3′ (forward)</td>
</tr>
<tr>
<td>(v7)</td>
<td>5′-GTCGGCTGAGAATAATGGTCTAC-3′ (reverse)</td>
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<tr>
<td>CD44 exon 12</td>
<td>5′-TTTCTGAGAACGACCTGTCACAGG-3′ (forward)</td>
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<tr>
<td>(v8)</td>
<td>5′-GTCAGCTTCAAAATACCTGACTCACG-3′ (forward)</td>
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<tr>
<td>CD44 exon 13</td>
<td>5′-TTTCTGAGAACGACCTGTCACAGG-3′ (forward)</td>
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<tr>
<td>(v9)</td>
<td>5′-GTCAGCTTCAAAATACCTGACTCACG-3′ (forward)</td>
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<td>CD44 exon 14</td>
<td>5′-TTTCTGAGAACGACCTGTCACAGG-3′ (forward)</td>
</tr>
<tr>
<td>(v10)</td>
<td>5′-GTCAGCTTCAAAATACCTGACTCACG-3′ (forward)</td>
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v1–v10, variable exons 1–10.

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transferred to a polyvinylidene difluoride membrane. After blocking nonspecific binding sites with 4% purified milk casein, the membrane was incubated with a 1:1,000 dilution of the antiserum at 4°C overnight. The bound antibodies were detected using an enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ). To dephosphorylate Tra2β, cytosolic proteins were prepared from arsenite-exposed cells without the phosphatase inhibitor cocktail and then treated with bacterial alkaline phosphatase as previously described (23). The purities of nuclear and cytosolic fractionations were assessed by Western blotting using appropriate dilutions of antibodies against β-tubulin (1:200; Santa Cruz Biotechnology) and histone H3 (1:200; Santa Cruz Biotechnology), respectively. An anti-β-actin antibody (1:5,000; Sigma Aldrich, St. Louis, MO) was used to assess amounts of samples loaded.

Overexpression of Tra2β and immunofluorocytochemistry. The cDNA encoding the human tra2β1 ORF was amplified by PCR and ligated into pCR 2.1-TOPO vector. Hemagglutinin (HA) tag was added to the NH2 terminus of full-length Tra2β by using primers encoding the HA tag sequence (in italics below) following the Kozak sequence (underlined below), respectively: 5′-primer (5′-TTAAGCTTGGATCCGCCACCATGACCCAGATCTGCAGAAC-3′) and 3′-primer (5′-GCCAGTGTGATGGATATCTGCAGA-3′), and the pCR 2.1-TOPO plasmid including tra2β1 ORF as a template. The amplified product was cloned into the mammalian expression vector pcR 3.1 according to the instruction manual (Invitrogen) and sequenced to be the corresponding cDNA. AGS cells were transfected with 2 μg of the plasmid encoding the HA-tagged tra2β1 cDNA for 24 h and subjected to immunofluorocytochemistry using an anti-HA-FITC antibody (1:240; Sigma Aldrich) and propidium iodide (PI) as previously described (28). Subcellular localization of HA-tagged Tra2β was examined by confocal laser scanning-light microscopy (model FV 1000; Olympus, Tokyo, Japan). FITC and PI were excited at 488 and 633 nm, respectively.

RNA interference. We used a small interference RNA (siRNAs; Hs_SFRS10_6 HP validated siRNA, SI02653504) (Qiagen, Chatsworth, CA) to knock down exon 3-containing tra2β mRNAs (tra2β1 and tra2β4). A siRNA (AllStars, Qiagen) was used as a negative control siRNA. To inhibit the nonsense-mediated mRNA decay (NMD) system, siRNAs targeting different sites of up-frameshift-1 (UPF1); 5′-AGUUUGAAUAGCAGAUUGCUCUC-3′ (UPF1-1 siRNA; Invitrogen) and 5′-UAACACUCUCACCAAAUAGGUGUC-3′ (UPF1-2 siRNA; Invitrogen); were introduced into AGS cells. The Stealth RNAi negative control (Invitrogen) was used as a control siRNA. Lipofectamine RNAiMAX (Invitrogen) was used to transfect above siRNAs into AGS cells for the indicated time according to the manufacturer’s protocol.

Assessment of cell growth. Cell growth was assessed by the bromodeoxyuridine (BrdU) incorporation method using a Cell Proliferation ELISA kit (Roche) according to the manufacturer’s protocol. Twenty-four hours after transfection with a plasmid encoding tra2β1 cDNA or 48 h after treatment with siRNA targeting tra2β exon 3, BrdU was added and incubated for 12 h.

RESULTS

Expression of tra2β mRNAs in rat gastric mucosa. Northern hybridization with a cDNA probe for the rat tra2β1 ORF showed that rat gastric mucosa constitutively expressed 1.7- and 2.2-kb tra2β transcripts (Fig. 1A). Restraint and water immersion stress increased the 2.2-kb transcript level, while the stress rather attenuated the 1.7-kb mRNA expression within 4 h and inversely stimulated expression of the 2.5-kb transcript that was not present in gastric mucosa of unstressed rats (Fig. 1A).

Amplification of the entire ORF of tra2β mRNA by RT-PCR produced only one transcript (indicated as “b”) in gastric mucosa of unstressed rats, while a longer product (indicated as “a”) was additionally detected 4 h after exposure to the stress (Fig. 1B). Cloning and nucleotide sequencing revealed that the product “b” was the rat tra2β ORF. The product “a” consisted of the tra2β ORF and 276 nucleotides inserted at nucleotide 36 of the ORF. The 276-nucleotide sequence was 100% identical to exon 2 of the rat tra2β gene (19). Thus, the 2.5-kb mRNA was the tra2β isoform. Rat tra2β exon 10 contains at least three poly-adenylation sites (17, 24). RT-PCR was done to determine the 3′-UTR sizes. As shown in Fig. 1D, gastric mucosa of unstressed rats expressed tra2β mRNA containing three different sizes of the 3′-UTR (indicated as “c”, “d,” and “e”). According to their lengths, transcription of the 1.7-kb tra2β1 mRNA appeared to be stopped at either the first (corresponding to band “c”) or the second poly-adenylation site (“d”). The 2.2-kb tra2β1 mRNA transcription was likely to be terminated at the third poly-adenylation site (“e”). The acute stress relatively decreased the usage of the first or the second poly-adenylation site for the transcription (Fig. 1D), leading to the decline of the 1.7-kb tra2β1 mRNA level (Fig. 1A, B), and the third poly-adenylation site was preferentially used in response to the stress (Fig. 1D). We also confirmed by Northern blotting with the exon 2 cDNA probe that the 2.5-kb transcript was the exon 2-containing tra2β4 mRNA (Fig. 1E). This tra2β4 isoform level time dependently increased in response to the stress. Considering the size of tra2β4 (2.5-kb) and the sequence of its ORF region, tra2β4 was likely to preferentially use the third poly-adenylation site.

Expression of tra2β isoforms in AGS cells. Next, we investigated alternative splicing of tra2β pre-mRNA in a human gastric cancer cell line (AGS). Northern hybridization with the human tra2β1 ORF cDNA probe showed that AGS cells constitutively expressed 1.7- and 2.2-kb tra2β mRNAs (Fig. 2A) similar to rat gastric mucosa (Fig. 1). Exposure to 100 μM arsenite newly induced a 2.5-kb transcript within 1 h, and its signal disappeared within 6 h (Fig. 2A). Arsenite also increased the 2.2-kb and 1.7-kb tra2β mRNAs. Particularly, the 1.7-kb transcript level started to increase within 2 h and continued to increase up to 10 h. Any signals corresponding to the other isoforms were not detected by Northern hybridization.

As shown in Fig. 2B, amplification of the entire ORF of human tra2β1 mRNA by RT-PCR showed that AGS cells constitutively expressed one major transcript (indicated as “b”) and a longer transcript (indicated as “a”) whose level was roughly calculated to be 3 ± 1% (means ± SD, n = 6) of the major transcript level by densitometry of RT-PCR data. Arsenite increased both transcript levels (Fig. 2B). The transcripts “a” and “b” were purified, cloned, and sequenced to be human tra2β4 and tra2β1 mRNAs, respectively. Human tra2β exon 10 possesses 2 poly-adenylation sites. According to their 3′-UTR lengths (Fig. 2C), the transcription of 1.7- and 2.2-kb tra2β1 mRNAs appeared to be terminated at the first and the second poly-adenylation sites, respectively. In contrast to the rat tra2β gene, human tra2β exon 10 was likely to preferentially use the first poly-adenylation site under oxidative stress (Fig. 2C), which was concordant with the sustained increase in the 1.7-kb tra2β1 mRNA level (Fig. 2A).
The amounts of 2.5-kb \textit{tra2β4} and \textit{tra2β1} (1.7-kb and 2.2-kb transcripts) mRNAs detected by Northern hybridization were quantified by densitometry (Fig. 2D). Each value is expressed as fold change, compared with the \textit{tra2β1} mRNA level in untreated control cells. AGS cells constitutively expressed \textit{tra2β4} mRNA below 10% of the \textit{tra2β1} mRNA level. Arsenite stimulated \textit{tra2β1} and more preferentially \textit{tra2β4} mRNA expression, and \textit{tra2β4} mRNA level accounted for around 40% of \textit{tra2β1} mRNA level at 4 h (Fig. 2D). The time-dependent changes in the amounts of exons 3–4-containing \textit{tra2β4} transcripts (\textit{tra2β1} and \textit{tra2β4} isoforms) and \textit{tra2β4} isoform were precisely examined by real-time RT-PCR (Fig. 2E). Real-time PCR more clearly showed that arsenite rapidly and transiently stimulated \textit{tra2β4} isoform generation with a peak at 2 h, and \textit{tra2β4} isoform production appeared to be gradually increased after the \textit{tra2β4} isoform generation was subsided (Fig. 2E).

\textbf{Arsenite-stimulated generation of \textit{tra2β4} isoform in AGS cells.} The \textit{tra2β4} isoform includes PTC-containing exon 2. This isoform is thought to be actively decomposed through the NMD system (18). We introduced siRNAs targeting different sites of \textit{UPF1} and examined whether NMD participated in the aberrant expression of \textit{tra2β4} isoform. The two different \textit{UPF1} siRNAs effectively knocked down \textit{UPF1} mRNA level below 10% of that in control siRNA-treated cells, while the knockdown did not stimulate \textit{tra2β4} isoform expression (time 0 in Fig. 3A) and did not modify the arsenite-induced upregulation of the expression (Fig. 3A). It has been shown that excess amounts of Tra2β bind to four enhancers present in exon 2 and stimulate inclusion of exon 2 (27). We also tested whether this autoregulatory mechanism was involved in the \textit{tra2β4} isoform generation. For this purpose, a plasmid encoding the \textit{tra2β1} ORF cDNA was transfected into AGS cells. Immunohistochemistry showed that 70% to 75% of the transfected cells expressed HA-tagged Tra2β protein in their nuclei, and amounts of Tra2β estimated by Western blotting were increased by 35 ± 3% (means ± SD, n = 3) (Fig. 3B). However, the overexpression itself did not up-regulate \textit{tra2β4} isoform expression (time 0 in Fig. 3C). Moreover, AGS cells overexpressing Tra2β protein generated \textit{tra2β4} isoform in response to arsenite, similarly as control siRNA-treated cells did (Fig. 3C).
Expression of Tra2β protein in AGS cells. We examined the effect of arsenite on functional, full-length Tra2β protein. First, we checked the specificity of the antiserum used. As shown in Fig. 4A, the antiserum predominantly recognized 38- and 24-kDa proteins. Introduction of a siRNA targeting tra2β exon 3 decreased tra2β mRNA level to <5% of that in control siRNA-treated cells. The exon 3 siRNA, but not control siRNA, selectively removed the 38-kDa immunoreactive band. In addition, an excess amount of the synthetic antigen polypeptide absorbed the immunoreactivity of this protein (shown by an arrow in Fig. 4A), showing that the 38-kDa protein was Tra2β. Densitometry demonstrated that the tra2β exon 3 siRNA reduced Tra2β protein level to 20% of that in control siRNA-treated cells (Fig. 4B).

As shown in Fig. 4C, arsenite treatment transiently reduced Tra2β level at 4 h, and then the level was significantly increased at 10 and 12 h.

Subcellular distribution of Tra2β in AGS cells. When amount of Tra2β was measured in nuclear and cytosolic fractions prepared with the phosphatase inhibitor cocktail, the anti-Tra2β serum recognized Tra2β with two different molecular sizes in the nuclear fractions of untreated cells (Fig. 5A). Arsenite initiated translocation of Tra2β from the nucleus to the cytoplasm (Fig. 5A). In this case, Tra2β with slightly larger sizes was additionally recovered in cytosolic fractions (Fig. 5A). Treatment of the cytosolic fraction, which was prepared 4 h after exposure to arsenite, with bacterial alkaline phosphatase eliminated Tra2β with higher molecular masses (Fig. 5A, right). Within 6 h after exposure to arsenite, Tra2β was reaccumulated in the nuclei and a higher amount of Tra2β was detected in the nuclear fraction at 10 h (Fig. 5A, left). Our antibody did not recognize the protein band with a higher molecular mass, reported as a hyperphosphorylated Tra2β by Daoud et al. (7). However, arsenite caused a transient translocation of Tra2β from the nucleus to the cytoplasm in association with enhanced phosphorylation.

AGS cells were transfected with a plasmid encoding HA-tagged tra2β ORF cDNA, and the subcellular distribution was monitored by confocal laser-scanning microscopy after immunofluorescence staining with an anti-HA antibody. HA-tagged Tra2β was present in the nucleus of untreated control cells. As similarly observed in the fractionation experiments (Fig. 5A), HA-tagged Tra2β also moved from the nucleus to cytoplasm, followed by reaccumulation of higher amounts of Tra2β in nuclei at 6 and 10 h (Fig. 5B).

Regulation of alternative splicing of CD44 by Tra2β. We also examined whether the transient decline and reincrease of nuclear Tra2β with arsenite actually changed alternative splicing of its target pre-mRNAs. For this purpose, we focused on the CD44 gene that is known to Tra2β-dependently change its splicing pattern (33). Theoretically, the human CD44 gene is able to produce several functional mRNAs through the combinatorial inclusion of one or multiple in-frame alternative exons in the central variable region (9, 22). We measured the amount of each variable exon (v-exon) by quantitative real-time RT-PCR (Fig. 6A). After treatment with arsenite, inclusion of v-exons was somewhat reduced 2 h after treatment with arsenite, while at 6 h and thereafter, the inclusion of v-exons, particularly v4 and v5, was facilitated in a time-dependent manner (Fig. 6A).

Using Tra2β-overexpressing cells (Fig. 3B) and Tra2β-knocked down cells (Fig. 4B), we examined whether changes in Tra2β level modified the alternative splicing of CD44 pre-mRNA, similarly as observed in arsenite-treated cells. In this case, we also measured levels of the standard isoform (sdt CD44) that is composed of two constant regions (exons 1 to 5 and exons 15 to 17) (see Fig. 6B, inset). Although the entire alternative splicing patterns have not yet completely been addressed, we measured exon 5 levels to estimate the levels of all CD44 isoforms (diagram in Fig. 6B). As shown in Fig. 6B, knockdown of Tra2β significantly suppressed expression of exon 5-containing CD44 transcripts in total in association with ~50% reduction of each v-exon-containing transcript levels. However, the standard CD44 transcript levels remained unchanged, suggesting that the loss of Tra2β protein may preferentially suppress the inclusion of v-exons in the central variable region. In contrast, the transfection with the plasmid encoding tra2β cDNA did not change amounts...
of all CD44 transcripts in total, when estimated by measuring the amount of exon 5-containing transcripts, while Tra2B overexpression decreased the standard CD44 transcript level to 10% of that in mock-transfected cells and concomitantly increased amounts of all v-exons. At the same time, there were significant variations in the inclusion of each v-exon, suggesting that the increase in Tra2 protein may stimulate the combinatorial inclusion of v-exons.

Regulation of cell growth by Tra2B. Finally, we examined the effect of Tra2B on cell growth. The Tra2B-knockdown down cells (Fig. 4B) significantly suppressed cell growth estimated by BrdU incorporation, compared with that of control siRNA-treated cells (Fig. 6C). In contrast, the Tra2B-overexpressing cells (Fig. 3B) significantly facilitated BrdU incorporation by ~20% (Fig. 6C).

DISCUSSION

In this study, we showed that acute psychological stress preferentially stimulated generation of alternatively spliced tra2β4 isoform. This was not restricted to rat gastric mucosa.

Generation of tra2β4 isoform was also observed in the mouse rectum after isolation stress (unpublished observations). In response to arsenite, other types of cells, including another gastric cancer cell line (KATO III), colon cancer cell lines (T84 and HCT116), and Hela cells, generated tra2β4 isoform (data not shown). In addition to arsenite, hydrogen peroxide similarly induced tra2β4 mRNA expression in AGS cells and

Fig. 3. Effects of treatment with up-frameshift-1 (UPF1) small interference RNA (siRNAs) or transfection of a tra2β1 ORF-encoding plasmid on arsenite-induced induction of tra2β4 mRNA expression. A: after treatment of AGS cells with 10 nM of two different siRNAs targeting UPF1, UPF1-1 siRNA (●) and UPF1-2 siRNA (○), or 10 nM control siRNA (□) for 24 h, amounts of tra2β4 mRNA were measured by real-time RT-PCR before (0 h) and at the indicated hour after treatment with 100 μM arsenite and normalized for amount of GAPDH mRNA. Values are expressed as fold changes (means ± SD, n = 3). B: after AGS cells were transfected with a plasmid encoding hemagglutinin (HA)-tagged tra2β1 ORF for 24 h, levels of Tra2β protein were measured by Western blot analysis using β-actin as a loading control, as described in Fig. 4. The data obtained from three independent experiments were quantitated by densitometry and are expressed as fold changes (means ± SD, n = 3) compared with those of mock-transfected control cells. *P < 0.05 by ANOVA and Scheffe’s test. C: after transfection of AGS cells with the plasmid encoding HA-tagged tra2β1 ORF cDNA (●) or mock for 24 h (○), the amounts of tra2β4 mRNA were measured before (0 h) and at the indicated hour after treatment with 100 μM arsenite. Values were normalized for amount of GAPDH mRNA and are expressed as fold changes (means ± SD, n = 6).
the other cell lines (data not shown). These findings suggest that oxidative stress may facilitate the inclusion of tra2β exon 2. In fact, AGS cells preferentially and transiently generated tra2β4 isoform in response to arsenite and then gradually increased tra2β1 mRNA after the tra2β4 generation was subsided.

Sequence-based analyses have predicted that ~35% of mammalian alternative splicing events produce PTC-containing splice variants that are targeted by NMD (18). The stress-inducible tra2β4 isoform includes exon 2 that contains multiple PTCs; therefore, this variant should be actively decomposed through NMD. However, it has been suggested that PTC-containing splice variants are generally produced at uniformly low levels across diverse mammalian cells, independently of the action of NMD, and most PTC-introducing alternative splicing events are not under positive selection by NMD (20). In agreement with this notion, we found that NMD was not involved in the stress-initiated production of tra2β4 variant, since inhibition of NMD with UPF1 siRNAs did not increase tra2β4 isoform expression and did not affect the arsenite-induced generation of this isoform. Another possible mechanism for tra2β4 isoform expression was explained by the negative feedback loop (27). Excess amounts of Tra2β bind to four enhancers present in the tra2β exon 2 and stimulate inclusion of exon 2, suggesting that Tra2β autoregulates its concentration by changing alternative splicing of its own pre-mRNA (27). However, overexpression of Tra2β by transfection of tra2β ORF cDNA did not result in upregulation of tra2β4 mRNA generation and did not modify its expression after treatment with arsenite. Moreover, the appearance of tra2β4 isoform was rather roughly coincided with the translocation of Tra2β from the nucleus to the cytoplasm. Then, tra2β1 mRNA and its protein were gradually increased at 8–12 h after an addition of arsenite, whereas tra2β4 mRNA was not generated in this phase. These results suggest that the autoregulatory system may not be involved in the arsenite-stimulated generation of tra2β4 isoform.

The tra2β gene has two canonical AUG codons in exons 1 and 4. Theoretically, four Tra2β isoforms are translatable (see Fig. 1C). Tra2β3 protein lacking the first RS domain was shown to be expressed in several tissues (27), while it was reported that any truncated proteins were not translatable from tra2β1 and tra2β4 mRNAs (5, 27). At present, any functions of truncated Tra2β and Tra2β3 proteins have not been clarified. AGS cells and rat gastric mucosa did not express detectable amounts of tra2β2, tra2β3, and tra2β5 transcripts. On the basis of these findings and information, we focused on the role of functionally active, full-length Tra2β in AGS cells. For this purpose, we focused on the CD44 gene that encodes a ubiquitously expressed surface molecule. CD44 pre-mRNA undergoes extensive alternative splicing of multiple v-exons, and these various isoforms have been suggested to participate in tumor progression and metastasis (4, 25). According to the splicing behavior, CD44 exons are compartmentalized into four regions: two constant regions consisting of exons 1 to 5 and exons 15 to 17, which are subject to general standard splicing; a region composed of exons 18 and 19, which shows an alternate use of a short or long cytoplasmic tail, respec-
tively; and a central region that spans exons 6a to 14, also known as v-exons (v1 to v10) (9, 22). It was reported that Tra2β/H9252 was specifically induced and enhanced the inclusion of v4 and v5 in breast cancer (33).

During the acute phase (within 4–6 h) after exposure to arsenite, Tra2β/H9252 synthesis might be switched off due to the preferential expression of tra2β isoform. Western blot analysis showed a transient decline of Tra2β/H9252 protein in this phase. Moreover, nuclear Tra2β/H9252 was substantially decreased due to the translocation into the cytoplasm. In the late phase (at 8 h and thereafter), higher amounts of Tra2β reaccumulated in nuclei in association with the increased expression of tra2β isoform. Coincident with these time-dependent changes, inclusion of v-exons in the central variable region of CD44 was somewhat suppressed during the early phase, while in association with reincrease in Tra2β protein in the late phase, combinatorial inclusion of v-exons, particularly v4 and v5, was enhanced.

In addition to nuclear concentration of Tra2β, splice site selection by Tra2β is regulated by sequestration by interacting proteins (31, 32). Arsenite changes a variety of functional pathways, which may change Tra2β-independent alternative splicing and may modify other splicing regulators that interact with Tra2β. To more directly assess the function of Tra2β, we prepared the Tra2β-overexpressing and knocked down cells, and examined alterna-

Fig. 6. Regulation of alternative splicing of CD44 pre-mRNA and cell growth by Tra2β. A: before (0 h) and at the indicated hour after treatment with 100 μM arsenite, variable exons (v1–v10) in the central variable region of CD44 pre-mRNA were measured by real-time RT-PCR using respective primer sets (Table 1) and GAPDH mRNA as an endogenous quantity control. Structure of the CD44 gene is shown in the inset. Exons are indicated as square boxes with arabic numbers. Exons in the central variable region are also shown as v1–v10. Introns are shown as vertical lines. Values are expressed as fold changes (means ± SD, n = 4) relative to respective levels in untreated control cells. *Significantly different compared with untreated control cells (P < 0.05 by ANOVA and Scheffé’s test). B: primer sets designed to measure the standard (std) CD44 transcript or CD44 exon 5 to measure all CD44 isoforms are indicated in the inset. After transfection with a plasmid encoding HA-tagged tra2β1 or mock alone, or treatment with control siRNA or tra2β exon 3 siRNA, amounts of CD44 exon 5, standard CD44 transcript joining exon 5 to exon 15, and each variable exon (v1–v10) in the central variable region were measured by quantitative real-time RT-PCR using GAPDH mRNA as an endogenous quantity control. Values are expressed as fold changes (means ± SD, n = 3) compared with respective levels in mock-transfected cells or in control siRNA-treated cells. *Significantly different compared with respective values in control siRNA-treated or mock-transfected cells (P < 0.05 by ANOVA and Scheffé’s test). C: AGS cells were treated with control siRNA or tra2β exon 3 siRNA for 48 h or they were transfected with the plasmid encoding HA-tagged tra2β1 cDNA (tra2β1) or mock for 24 h. These cells were then incubated with bromodeoxyuridine (BrdU) for 12 h, and amount of BrdU incorporated into the cells was measured. Values are expressed as fold changes (means ± SD) in three independent experiments. *Significantly different compared with those of control siRNA-treated cells or mock-transfected cells (P < 0.05 by ANOVA and Scheffé’s test).
tions of splicing patterns of CD44 pre-mRNA. Measurement of each v-exon in these cells indicated that elevation of Tra2β level was likely to stimulate the combinatorial inclusion of multiple in-frame alternative exons including v4 and v5. In contrast, knockdown of Tra2β significantly facilitated skipping of the central variable region. It has been suggested that appearance of v-exon-containing CD44 transcripts are associated with accelerated cell growth (21). In fact, our experiments with tra2β1-transfected cells and Tra2β-knocked down cells suggest that Tra2β-mediated regulation of the inclusion of CD44 v-exons may be associated with cell growth. The present study shows that oxidative stress specifically stimulates the inclusion of PTC-containing exon 2 of tra2β pre-mRNA. Resultant tra2β transcript may be used to switch off Tra2β synthesis. After generation of tra2β transcript was sub-sided, tra2β isoform expression was preferentially upregulated. The time-dependent changes in alternative splicing of tra2β pre-mRNA were closely associated with changes in alternative splicing of CD44 pre-mRNA and mitogenetic property of AGS cells. Our results suggest that alternative splicing of the tra2β gene may be involved in the complex cellular response to oxidative stress. Further study is needed to fully elucidate the precise molecular mechanism for transactivation of the tra2β gene and its alternative splicing under oxidative stress.

REFERENCES