Cooperative Actions of Tra2α with 9G8 and SRp30c in the RNA Splicing of the Gonadotropin-releasing Hormone Gene Transcript

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In earlier studies, we demonstrated that excision of the first intron (intron A) from the gonadotropin-releasing hormone (GnRH) transcript is highly cell type- and developmental stage-specific. The removal of GnRH intron A requires exonic splicing enhancers on exons 3 and 4 (ESE3 and ESE4, respectively). Tra2α, a serine/arginine-rich (SR)-like protein, specifically binds to ESE4, whereas 9G8 binds to an element in exon 3 and strongly enhances the excision of GnRH intron A in the presence of minimal amount of other nuclear components. Interestingly, Tra2α can interact with either 9G8 or SRp30c, whereas no interaction between 9G8 and SRp30c is observed. Tra2α has an additive effect on the RNA binding of these proteins. Overexpression or knock-down of these three proteins in cultured cells further suggests their essential role in intron A excision activities, and their presence in GnRH neurons of the mouse preoptic area further strengthens this possibility. Together, these results indicate that interaction of Tra2α with 9G8 and SRp30c appears to be crucial for ESE-dependent GnRH pre-mRNA splicing, allowing efficient generation of mature mRNA in GnRH-producing cells.

Gonadotropin-releasing hormone (GnRH) is a pivotal hypothalamic neurohormone controlling mammalian reproduction and sexual development. The mammalian GnRH gene consists of four short exons and three intervening introns (1, 2). In GnRH-producing cells, all three introns can be efficiently excised from the primary transcript (~4,300 bases), resulting in a mature mRNA consisting of ~560 bases (1). A majority of GnRH-producing neurons reside in the preoptic area (POA) of the hypothalamus. GnRH transcripts, however, are also found in extrahypothalamic areas of the brain and in a variety of peripheral tissues. Interestingly, a greater portion of GnRH transcripts contain the first intron (intron A) in these tissues (3). Previous studies demonstrated that intron A, in contrast to introns B and C, could not be easily removed because of its suboptimal 3′ splice site (3, 4). Moreover, we also recently clarified that the presence of tandem repeats of the ATG sequence and putative upstream open reading frames within intron A completely blocked the translation initiation of downstream GnRH coding region in the intron A-retained GnRH transcripts (5). Hence, precise and efficient removal of intron A is likely to play a crucial role in cell type-specific GnRH gene regulation, and it is of importance to elucidate how intron A of the GnRH primary transcript overcomes such intrinsic defects for production of a mature mRNA in GnRH-producing neurons.

In general, the sequences specifying a splice site are weakly conserved and often surrounded by many cryptic splice sites that cannot be detected by a basic splicing system. Moreover, many pre-mRNAs in metazoan cells contain weak or alternative splice sites that must be recognized in a regulated fashion to generate specific mRNA species (6). Therefore, additional cis-elements within a pre-mRNA could be important for a precise recognition of the splice site. One form of these elements is the exonic splicing enhancer (ESE), which usually consists of a purine-rich sequence. In this context, we previously demonstrated the presence of functional ESEs in GnRH exons 3 and 4, denoted as ESE3 and ESE4, respectively (3, 4). We also showed the physiological significance of the ESEs in intron A excision using the hypogonadal mouse, a native mutant lacking both exons 3 and 4 of the GnRH gene (7).

Because ESEs are essential for alternative splicing, it is easily postulated that the presence of trans-acting splicing factor(s) acting on ESE3 and ESE4 might be prerequisite for producing mature GnRH mRNA in GnRH neurons. Indeed, the addition of nuclear extract (NE) from immortalized GnRH-producing GT1–1 cells efficiently enhanced the excision rate of GnRH intron A in an ESEs-dependent manner, whereas NE from non-GnRH-producing cells failed to do so (8). The most probable trans-acting factors are a family of proteins containing a conserved domain rich in alternating arginine and serine residues. The serine/arginine-rich (SR) proteins and their related members have a modular structure composed of an RNA recognition motif and a conserved domain rich in alternating arginine and serine residues (9). SR proteins are known to play an important role in the regulation of both constitutive and enhancer-dependent alternative RNA splicing (10–12). They appear to be functionally redundant in constitutive RNA splicing and thus can complement each other. It is, however, observed that individual SR proteins have their own functions in the regulation of tissue- and developmental stage-specific alternative RNA splicing (13, 14). Because our previous studies showed that a subset of SR proteins was highly enriched in GT1 NE, we prepared several kinds of recombinant SR and
SR-like proteins (8). Using an in vitro RNA-protein binding assay and subsequent functional studies with these recombinant proteins, we clarified that Tra2α, a mammalian homolog of Drosophila transformer-2 (Tra2) (15) specifically binds to GnRH ESE4 (8).

The fact that Tra2α effectively promotes the splicing of GnRH intron A only in the presence of nuclear components such as HeLa NE or an SR protein-enriched fraction suggests a requirement of additional nuclear co-factors for efficient splicing of intron A. Moreover, a protein fraction precipitated in the presence of 40–50% ammonium sulfate (ASP40–50) from GT1 NE elicits the strongest intron A removing activity, although this fraction does not have ESE4-binding proteins (8). These intriguing findings prompted us to identify additional SR proteins involved in GnRH pre-mRNA splicing. In this study, we demonstrate cooperative interactions among the trans-acting SR proteins Tra2α, 9G8, and SRp30c in ESE-dependent GnRH pre-mRNA splicing.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—All of the materials for cell culture were obtained from Invitrogen. Other chemicals, if not specified, were purchased from Sigma. For antibodies: anti-GST and anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal anti-GnRH from Abcam (Cambridge, MA) and monoclonal anti-GnRH antibody were from Chemicon (Temecula, CA); and anti-Tra2 and anti-9G8 were kind gifts of Drs. S. Stamm (University of Erlangen, Germany) and J. Stevenin (IGBMC, CNRS/INSERM/ULP, France). Affinity-purified anti-SRp30c antibody was raised by immunizing a rabbit with purified GST-SRP30c fusion protein. Oligonucleotides for PCR—The primers used in this study are as follows: E1-up, 5ʹ-GGAAGACATCGTTCCCAAGA-3ʹ; IA-up, 5ʹ-AACTCACCAAGAGTACTATATGGCAAGAAGG-3ʹ; E2-dn, 5ʹ-CTCTTGAGAAAGAATCAACCAATG-3ʹ; E3-up, 5ʹ-AAGACA-TGGGCAAAGGAGGTGCTCA-3ʹ; E3-dn, 5ʹ-AGAGCTTCTCGCAGATCCTCA-3ʹ; E4-up, 5ʹ-GAAAGATCGTGAGGAGGAAGA-3ʹ; E4-dn, 5ʹ-GGAAATCTAGCGTCTGGTTGTT-3ʹ; EGFP-dn, 5ʹ-AACCTGGGCGGTATCGTC-3ʹ; E3A-up, 5ʹ-AGGAGCAAGGAGGTATGCA-3ʹ; E3B-dn, 5ʹ-TAGAGGTTGAGGCGGCACCA-3ʹ; E3C-dn, 5ʹ-GGATGCTGTTGGTTCTGC-3ʹ; and E3D-up, 5ʹ-CAGTGGGCC-GCGTACCC-3ʹ.

Glutathione S-Transferase-tagged SR Proteins—GST-tagged SR proteins, Tra2α, 9G8, SRp30c, SRp20, SRp40, and SRp55, cloned in baculovirus transfer vector (BD Bioscience, Palo Alto, CA) were used to construct recombinant baculoviruses (8). The fusion proteins were expressed in S99 cells. GST-SR proteins were affinity-purified with Glutathione-Sepharose 4 Fast Flow according to the manufacturer’s instructions (Amersham Biosciences).

Plasmids—The reporter DNAs for in vitro splicing assays (pΔΔA234, pΔAcc34, and pΔAc64) and template constructs for ESE3 and ESE4 (pESE3 and pESE4) were described in previous reports (4, 8). DNA constructs for binding assays were obtained by PCR using pΔΔA234 as a template and cloned into the pGEM-T Easy vector (Promega, Madison, WI; denoted as pΔE2, pE4, pE3E4, pE3A, pE3B, pE3C, pE3D, and pE3E4). As a reporter plasmid used for in vivo splicing assays, GnRH 1A234 DNA was obtained by RT-PCR from mouse hypothalamic RNA with E1-up and E4-up primers and cloned into the pEGFP-N1 (BD Bioscience), thus generating pΔΔA234-GFP.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was carried out as previously described (8). RNA-protein binding reaction was performed in a 20 μl of reaction mixture containing 0.4 mM ATP, 20 mM creatine phosphate, 3 mM MgCl₂, 20 units of ribonuclease inhibitor (RNAsin), 5 μg yeast tRNA, 32P-labeled RNA probe, and the indicated GST-SR recombinant protein for 30 min at 30 °C. In case of supershift and competition experiments, 200 ng of anti-GST antibody (Santa Cruz Biotechnology) or 200-fold molar excess cold competitor was added and incubated for another 30 min. After incubation, 6 μl of glycerol was added to each reaction mixture, which was immediately separated on a 5% nondenaturing polyacrylamide gel. Thereafter, the gel was dried and exposed to x-ray film (Fuji) at −70 °C for 1 day.

In Vitro Splicing Assay—The in vitro splicing reactions were performed as described previously (3). Briefly, the gel-purified radiolabeled RNA substrates produced by in vitro transcription was subjected to the splicing reaction in 25 μl of reaction mixture containing 5 mM HEPES (pH 7.9), 20 mM creatine phosphate, 0.4 mM ATP, 0.6% polyvinylalcohol, 3 mM MgCl₂, and 20 units of RNAsin supplemented with 50 μg of HeLa NE or cytoplasmic S100 extract at 30 °C for 2 h. After phenol/chloroform extraction and subsequent ethanol precipitation, the RNAs were separated on a 6% denaturing polyacrylamide gel containing 8 M urea. UV Cross-linking Assay—RNA-protein binding reaction was performed in a reaction mixture with 0.4 mM ATP, 20 mM creatine phosphate, 3 mM MgCl₂, 20 units of RNAsin, 5 μg of yeast tRNA, 32P-labeled RNA probe, and purified proteins for 30 min at 30 °C according to a previously described method with minor modifications (8). UV cross-linking was performed on ice, 4.5 cm away from a UV source at 1.2 J (Stratagene, La Jolla, CA). Each sample was then incubated with 200 units of RNase T1 and 200 units of RNase A for 10 min at 37 °C. The resulting RNA-protein complexes were denatured and resolved by SDS-PAGE. The gels were dried and autoradiographed at −70 °C for 1 day.

GST Pull-down Assay—Tra2α, 9G8, and SRp30c were subcloned into pCMV-Tag3 (Stratagene) for expression as Myc-tagged forms. The expression vector for Myc-Tra2α, Myc-9G8, or Myc-SRp30c was transfected into CHO-K1 cells using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours after transfection, the cells were lysed with modified radiolabeled RNA-protein binding assay buffer containing 150 mM NaCl, 20 mM Tris (pH 7.6), 10 mM Na₃P₂O₇, 2 mM Na₂VO₅, 0.5 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40. Cleared cell lysates were treated with RNase A (0.5 mg/ml) for 10 min at 37 °C. RNase-treated samples were then incubated with 5 μg of purified GST, GST-Tra2α, GST-9G8, or GST-SRp30c protein for 1 h at 4 °C, followed by pull-down with Glutathione-Sepharose 4 Fast Flow (Amersham Biosciences). After three washes with radiolabeled RNA-protein interaction assay buffer, pulled-down protein complexes were subjected to Western blot analysis.

Western Blot Analysis—For immunoblotting, proteins were resolved on 12% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking with 10% nonfat milk in Tris-buffered saline (10 mM Tris, pH 7.5, 150 mM NaCl) with 0.3% Tween 20 (TBS-T), primary antibody (both anti-Myc and anti-GST) were used at a final dilution of 1:4000 was applied to the membrane and incubated for 1 h at room temperature. Thereafter, the blots were washed four times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. After four washes with TBS-T, the immunoreactive bands were visualized by Amersham Biosciences ECL reagents following the provided instructions.

Yeast Two-hybrid Assay—The full-length cDNA fragments for mouse Tra2α, 9G8, and SRp30c were fused in-frame to the DNA-binding domain (BD) of pBD-GAL4 (Stratagene) and the activation domain of the pACT2 vector (BD Bioscience). Transformants of Y187 strain yeast cells expressing rDNA from pACT2-BD, 9G8-BD, or SRp30c-BD plasmid were mated to a set of AH109 strain colonies, which expressed Tra2α, 9G8, or SRp30c fused to the Gal4 activation domain according to the manufac-
turer’s instructions. The resulting diploids were selected by cultivation at 30 °C for 6–12 days on minimal synthetic dropout medium lacking Trp, Leu, His, and Ade but including 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-gal).

**Total RNA Isolation and Northern Blot Hybridization**—RNA isolation and Northern blot hybridization were performed as described previously with minor modifications (3). Briefly, total RNA was isolated by a single step acid-guanidinium-phenol-chloroform extraction method. Twenty μg of total RNA was denatured, resolved on 1.2% formaldehyde denaturing agarose gels, and transferred to a nylon membrane (Schleicher & Schuell). The resulting membranes were prehybridized at 42 °C for 2 h and then hybridized for 16–24 h with cDNA probes for Tra2, 9G8, and SRp30c. After autoradiography, the membranes were stripped and rehybridized with a cDNA probe for 18 S rRNA to ensure equal amounts of loading of RNA samples.

**RT-PCR for in Vivo Splicing Assay**—NIH3T3 or GT1–1 cells were transiently transfected with 10 ng of reporter plasmids p1A234-GFP expressing pre-mRNA substrates. NIH3T3 cells were co-transfected with the indicated Myc-tagged SR protein-expressing plasmids. GT1–1 cells were simultaneously transfected with antisense oligodeoxynucleotides (AS-ODN) with the reporter construct. Total RNAs were prepared from the transfected cells and treated with 10 units of DNase (Invitrogen) for 1 h at 37 °C to avoid possible DNA contamination. DNase-treated RNAs were reverse-transcribed using a commercial reverse transcription kit (Promega), and the resulting cDNA was amplified with E1-up, IA-up, and GFP-dn primers by PCR as previously described (8). Sequences for AS-ODNs are as follows: CTL-ODN (no homology with any mouse genes in GenBank™) (16), 5′-AGTCCTA-CTCGGTACGTATGCAGC-3′; AS-Tra2, 5′-TCTGCTCGCCGC-TGTGCTCAG-3′; AS-9G8, 5′-GGGAACTGGTGCTGGTAAAG-3′; and AS-SRp30c, 5′-GTCCGCCCAGCCCGACGACATC-3′.

**Immunohistochemical Analysis**—Male mice (ICR, 15 weeks of age) were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS), and the brains were post-fixed in the same solution for 24 h. The brains were then cryoprotected in 30% sucrose and sectioned (40 μm); subsequent immunostaining was performed by the free floating method. Brain sections containing POA were washed with PBS and blocked with 5% goat serum and 0.1% Triton X-100 in PBS for 30 min. The following primary antibodies were applied overnight at 4 °C: anti-GnRH, 1:1000; anti-Tra2, 1:1000; anti-SRp30c, 1:3000. After six washes with PBS, the appropriate secondary antibodies conjugated with fluorescent dyes (Jackson ImmunoResearch Laboratories) were applied for 30 min. Subsequently the sections were washed, mounted, and observed under a fluorescence microscope (Carl Zeiss).

**Statistics**—The splicing efficiencies (ratio of spliced product to unspliced substrate) observed in in vitro and in vivo splicing assays were statistically evaluated by Student’s t test. Statistical significance was set at p < 0.05.

**FIGURE 1.** Binding of SRp30c to ESE3 and ESE4. A, a schematic diagram and sequences of ESE3 (gray box) and ESE4 (black box). B, EMSA using GST-tagged purified SR proteins (Tra2α, 9G8, SRp30c, SRp55, SRp20, and SRp40; 300 ng of each protein in a 20 μl reaction mixture) was performed with a radiolabeled ESE3 or ESE4 RNA probe as indicated below each panel. C, supershift experiments were carried out in the presence of anti-GST antibody (200 ng). The arrowheads indicate the specific binding of Tra2α or SRp30c to ESEs, and arrows indicate the supershifted tertiary complexes. The RNA probe and purified SR protein (SRP) used in the reaction are indicated below each panel. D, competition experiments on ESE3 and ESE4 RNA probes were performed in the presence of 200-fold molar excess cold competitor. E, in vitro splicing assay on GnRH intron A with purified Tra2α and SRp30c proteins at indicated concentrations. The reactions were performed in the presence of 20 μg of HeLa NE for 2 h. Unspliced and spliced RNA species are indicated as diagrams on the right side.
RESULTS

SRp30c Directly Binds Both ESE3 and ESE4 and Enhances Intron A Excision—The recognition of a suboptimal splice site by the 3′/H11032 spliceosome complex can be enhanced by the help of ESEs and their trans-acting partners (10, 17). Several SR proteins and their related members are known to regulate the excision of either weak or strong introns by direct interaction with ESEs (18–20). In an earlier study, we demonstrated that Tra2/H9251 is involved in the enhanced excision of GnRH intron A by a direct interaction with ESE4. This enhanced splicing activity, however, requires additional nuclear components (8). Thus, the question of what additional SR proteins are required can be asked.

To approach this question, we examined the interaction between ESEs and purified GST-tagged recombinant SR proteins. EMSA revealed that only SRp30c of six tested SR proteins was able to bind both ESE3 and ESE4, whereas Tra2/H9251 bound only ESE4 (Fig. 1, A and B). Anti-GST antibody-evoked supershift in EMSA further confirms these specific bindings (Fig. 1 C). Binding of SRp30c with a radiolabeled ESE3 RNA probe was inhibited by both 200-fold molar excess of unlabeled ESE3 and ESE4 RNA and vice versa (Fig. 1 D), suggesting that SRp30c might recognize both ESEs via the same RNA-binding domain. SRp30c, in addition to its binding activities, apparently enhanced GnRH pre-mRNA splicing in the HeLa NE-supplemented in vitro splicing assay system. This intron A removing activity is comparable with that of Tra2/H9251 (Fig. 1 E).

9G8 Complements S100 Fraction to Remove Intron A—One of the important features of SR proteins is that they can complement splicing-deficient cytoplasmic S100 extract, which lacks SR proteins but does contain minimal amounts of essential splicing factors for constitutive RNA splicing including snRNPs, hnRNPs, and debranching enzymes (21, 22). We next compared intron A removal abilities of purified SR proteins in the presence of cytoplasmic S100 extract using three different splicing reporter

FIGURE 2. 9G8 strongly promotes splicing of GnRH pre-mRNA in vitro. A, schematic diagrams for the GnRH splicing substrates. The sizes of unspliced pre-mRNA and its spliced products are shown on the right side of the diagram. B, in vitro splicing reactions were performed in the presence of cytoplasmic S100 extract. Tra2α, SRp40, 9G8, SRp20, SRp55, or SRp30c was applied to the splicing reaction mixture as indicated. Unspliced and spliced RNA species are presented on the right side of the panels. Arrowheads indicate spliced products. C, the splicing efficiencies of three RNA constructs by 9G8 are presented as the mean ± S.E. (n = 4; **, p < 0.01 versus S100-only group for each reporter). The ratio of spliced products to unspliced substrates was used as an index for splicing efficiency.

FIGURE 3. 9G8 interacts with GnRH exon 3 in the presence of S100 extract. A, a scheme of RNA probes used for EMSA. The numerals in parentheses following E3A, E3B, E3C, and E3D represent the sizes of each probe as the number of nucleotides. B, EMSA using 9G8 (300 nM) was carried out with various exonic RNA probes (IAE2, E3, and E4) of GnRH pre-mRNA in the absence or presence of 20 μg S100 extract. C, EMSA using 9G8 (300 nM) was carried out with several partial exon 3 RNA probes (E3A–E3D) in the presence of S100 extract. The arrowheads indicate the specific binding of 9G8 to the probes.
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FIGURE 4. Protein-protein interactions among the SR proteins. A, Myc-tagged Tra2α, 9G8, or SRp30c was overexpressed in CHO-K1 cells. RNase-treated cell lysates were pulled down with GST-tagged Tra2α, 9G8, SRp30c, or GST (5 µg each) as indicated. Pulled-down protein complexes were analyzed by immunoblot analyses using anti-Myc or anti-GST antibodies. B, yeast two-hybrid assays on the SR proteins. Yeast cells expressing one of three SR proteins fused to GAL4-BD (DNA-binding domain) were mated with the other cells expressing activation domain (AD)-fused Tra2α, 9G8, or SRp30c as described under "Experimental Procedures." Protein-protein interactions were examined by monitoring α-galactosidase activities.

constructs (Fig. 2A). 9G8 exhibited an apparent S100 complementing activity on 1ΔA234 and 1ΔAcc34 in a dose-dependent manner when compared with those of other SR proteins; it had, however, a negligible intron A removing activity for the 1ΔAcc34 construct lacking exons 2 and 3 (Fig. 2, B and C), suggesting that 9G8 enhances excision of intron A by an interaction with exon 3 but not exon 4. In addition to 9G8, SRp55 exerted a weak S100 complementation activity. However, we only focused on 9G8 in the present study, because SRp55 could not bind to GnRH pre-mRNA, and the functional relevance of this protein in GnRH-producing cells was negligible (data not shown).

The fact that 9G8 did not bind to either ESE3 nor ESE4 suggests an additional binding site(s) for it in the exonic sequence. To address this possibility, we employed RNA probes, such as exon 2 with the distal region of intron A (IAE2), and all of exon 3 with ESE3 (E3) and exon 4 (E4) for RNA-protein binding assays with purified GST-9G8 (Fig. 3A). 9G8 bound to the E3 RNA probe only in the presence of S100 extract (Fig. 3B). To find the exact binding site of 9G8 in exon 3, we designed several truncated exon 3 RNA probes (E3A–E3D) as shown in Fig. 3A; E3A and E3D probes bound to 9G8, indicating that it interacted with the distal region of exon 3 by the help of a certain factor(s) in cytoplasmic S100 extract (Fig. 3C).

Protein-Protein Interactions between Tra2α, 9G8, and SRp30c—Specific protein-protein interactions among SR proteins are known to be important for functional splicesome formation (19, 23). These interactions may lead to the recruitment and stabilization of essential splicing factors on the splice site (20). Because Tra2α, 9G8, and SRp30c contact GnRH pre-mRNA, the molecular interactions between these SR proteins were examined using GST pull-down and yeast two-hybrid assays. Cell extracts from CHO-K1 cells expressing Myc-tagged Tra2α, 9G8, or SRp30c were pulled down with purified GST-tagged Tra2α, 9G8, SRp30c, or GST after treatment with RNase to avoid a possible bridging effect of RNA substrates. Subsequent immunoblot analyses revealed that GST-Tra2α interacted with all of the three Myc-tagged SR proteins and vice versa (Fig. 4A). Yeast two-hybrid assays confirmed direct interactions of Tra2α with 9G8 and SRp30c. Further, Tra2α showed a strong self-interaction, indicating putative dimerization or oligomerization of this protein on its substrates (Fig. 4B). However, 9G8 and SRp30c did not interact with each other, and neither of them elicited self-interactions.

Cooperative Actions of Tra2α with 9G8 and SRp30c on Cis-element Binding and GnRH Pre-mRNA Splicing in Vivo—Because Tra2α strongly interacts with 9G8 and SRp30c, we used a UV cross-linking assay to determine whether such physical interactions promote the binding of 9G8 and SRp30c on their target cis-element. The RNA probes E3D, ESE4, and E34, which contains the distal region of exon 3, and ESE4 were used (Fig. 5A). 9G8 bound to E34 and E3D RNA probes in the presence of the S100 extract and/or Tra2α. Interestingly, the presence of both S100 extract and Tra2α greatly enhanced the cross-linking of 9G8 to the E34 and E3D probes. Binding of 9G8 to E34 in the presence of Tra2α (Fig. 5A, fourth and eighth lanes of the top panel) suggests that Tra2α promotes 9G8 to recognize its binding site, presumably through direct protein-protein interaction. Both Tra2α and SRp30c bind to ESE4, raising the possibility that the interaction between these proteins affects their recognition and binding activities with ESE4. We tested this possibility using ESE3 and ESE4 RNA probes. Increasing SRp30c concentrations with a constant amount of Tra2α enhanced the binding activity of Tra2α to the ESE4 probe and vice versa (Fig. 5B, top panels). For ESE3, however, Tra2α did not exert any influence on the binding activity of SRp30c (Fig. 5B, bottom panels). This finding suggests a synergistic effect of Tra2α and SRp30c on ESE4 binding activity through a direct interaction between these two proteins.

Whether this cooperative binding influences excision of intron A was examined using in vitro RNA splicing assays. Increasing the amount of Tra2α greatly enhanced 9G8-evoked splicing of the 1ΔA234 reporter in a dose-dependent manner. Because 9G8 complements S100 extract and exhibits a strong intron A removing activity, it was expected that increasing 9G8 concentration would enhance the splicing of 1ΔA234 (Fig. 5C, top panels). The synergistic effect of Tra2α and SRp30c was, however, negligible, likely because of their inability to complement the cytoplasmic S100 extract (Fig. 5C, bottom panels).

Tra2α, 9G8, and SRp30c Promote the Removal of Intron A in Vivo—To examine the role of SR proteins in the excision of mouse GnRH intron A in vivo, we adopted a competitive RT-PCR analysis with three primers as described previously (7). To distinguish the RNA species of the transfected reporter construct from endogenous GnRH transcripts, we used a GFP-tagged mouse GnRH intron A-containing reporter construct (p1A234–GFP) and a specific primer complementary to GFP (GFP-dn; Fig. 6A). As shown in Fig. 6B, E1-up and 1A-up primers had different amplification efficiencies according to the relative amounts of the two cDNA species. A standard curve was constructed based on the observed ratio of 1234–GFP to 1A234–GFP PCR products as a function of a given ratio of 1234–GFP to 1A234–GFP cDNAs and showed a linear regression coefficient of 0.971.
Expression levels of Tra2α, 9G8, and SRp30c in GnRH-producing cells (GT1–1 cells) and non-GnRH-producing cells (NIH3T3 cells) were determined using Northern blot analysis; Tra2α, 9G8, and SRp30c mRNAs were much higher in GT1–1 cells than in NIH3T3 cells (Fig. 6C). We also examined the effects of SR protein overexpression on GnRH pre-mRNA splicing in non-GnRH-producing NIH3T3 cells by
transfecting SR protein-overexpressing plasmid constructs with a p1A234-GFP reporter. Overexpression of Tra2α, 9G8, and/or SRp30c increased the excision efficiency of intron A (Fig. 6D). Interestingly, only co-transfection of 9G8 and SRp30c exhibited a significantly additive effect on removing intron A (Fig. 6D, lane 8), despite additive actions between Tra2α and 9G8 or SRp30c in the proceeding in vitro studies. Expression of transfected SR proteins was confirmed by immunoblot analysis (Fig. 6E). These results indicate that intron A can be efficiently removed from the GnRH primary transcript even in non-GnRH-producing cells when Tra2α, 9G8, and SRp30c are sufficiently expressed.

The effects of suppression of these SR proteins on GnRH intron A excision in GT1–1 cells was examined by co-transfection with antisense oligodeoxynucleotides (AS-ODNs). To test the ability of AS-ODNs to block SR protein expression, CHO-K1 cells were co-transfected with three kinds of Myc-tagged SR protein-overexpressing plasmids, together with AS-Tra2α, AS-9G8, AS-SRp30c-ODN, or CTL-ODN. Western blot analyses showed that each SR protein expression level was efficiently and specifically blocked by application of 3 μg of each AS-ODN (Fig. 7A). Introduction of each AS-ODN with the p1A234-GFP reporter gene into GT1–1 cells significantly suppressed the excision rate of GnRH intron A (Fig. 7B). Because depletion of only one of these SR and SR-like proteins is sufficient to inhibit intron A excision in GnRH-producing cells, co-expression of these SR proteins is a likely prerequisite for the efficient GnRH pre-mRNA splicing in GnRH-producing cells in vivo.

Expression of Tra2α, 9G8, and SRp30c in GnRH-immunoreactive Neurons in the Mouse Brain—Once the cooperative actions of Tra2α, 9G8, and SRp30c in the regulation of GnRH pre-mRNA splicing in vitro was clear, our next step was to examine the presence of these proteins in GnRH neurons in vivo. We addressed this issue by immunohistochemical analyses in POA of the mouse hypothalamus, where GnRH-producing neurons are the most abundant. GnRH immunoreactivity was found in the cytoplasm of several neurons from the mouse POA, which showed a scattered distribution as reported in previous studies (24). Tra2α, 9G8, or SRp30c immunoreactivity was apparent as nuclear speckles distributed in a subset of cells including GnRH-immunoreactive neurons of the same slice (Fig. 8), showing that they are expressed in GnRH neurons and suggesting the participation of these proteins in the regulation of GnRH pre-mRNA splicing in vivo.

DISCUSSION

In an extension of previous studies showing an essential role for ESEs and Tra2α in the regulation of GnRH pre-mRNA splicing (3, 4, 8), this study demonstrates the involvement of SRp30c and 9G8 in this regulation through interaction with Tra2α. Presumably, 9G8 recognizes its binding site through an interaction with Tra2α, eventually enhancing GnRH pre-mRNA splicing. SRp30c and Tra2α may cooperate with each other, leading to a synergistic increase in binding to ESE4. Abundant expression of these proteins in cultured GT1–1 cells and GnRH neurons in the mouse POA and the substantial decrease in GnRH pre-mRNA splicing activity in cells, in which expression of one of these proteins is suppressed, indicate that the cooperative actions of these proteins are pivotal for the ESE-dependent GnRH pre-mRNA splicing. GnRH ESE4 is too far away from the 3′ splice site of the intron A (238 nucleotides downstream) to enhance the excision of the intron by the help of splicing machinery of non-GnRH-producing cells like HeLa, NIH3T3, and KK1 cells (3, 8). It has been shown that reducing the length of the spacer between the intron A and ESE4 to 115 nucleotides greatly enhanced the intron A excision in the HeLa NE-based in vitro splicing.
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The present study also demonstrates that SRp30c is capable of binding both ESE3 and ESE4. Competition experiments (shown in Fig. 1D) strongly suggest that different SRp30c molecules can individually bind to either ESE3 or ESE4 rather than simultaneously interact with both sequences. Furthermore, direct protein-protein interaction between Tra2α and SRp30c and the additive influence in the recognition of ESE4 by these two proteins are also shown. These results are very consistent with the previous report showing facilitation of regulated survival motor neuron (SMN) exon inclusion by direct association of SRp30c with human Tra2B (33). Although there is also evidence showing a functional antagonism between SRp30c and Tra2α in the regulation of alternative RNA splicing of Tau exon 10 by competition in their RNA binding activities (34), this was evidently not the case in the GnRH pre-mRNA. Earlier, we showed that ESE4 is composed of three adjacent clusters of purine-rich elements and a hairpin sequence and that Tra2α could directly interact with only the proximal two purine-rich segments (4, 8), strongly suggesting that associated Tra2α and SRp30c cooperated in the recognition of ESE4 by occupying adjacent, but not overlapped, binding elements (Fig. 9). Therefore, ESE4-bound SRp30c is likely to facilitate and/or stabilize an ESE4/Tra2α/SR protein complex, rather than directly stimulate spliceosome formation, based on the results showing the additive effect of Tra2α and SRp30c on ESE4 binding but not on S100 complementation.

A possible role for SRp30c in association with ESE3 and its interaction with Tra2α is still unclear. Because SRp30c fails to reconstitute S100 extract for all three of the GnRH pre-mRNA reporter constructs, it is unlikely that this protein, by itself, can initiate spliceosome formation on ESE4, even though the HeLa NE-based in vitro splicing system showed that ESE3 was sufficient for significant intron A excision. Indeed, intron A removal activity of ESE3 is less potent than that evoked by ESE4 (4). ESE4-bound SRp30c may only serve to facilitate ESE4-evoked splicing enhancement. However, SRp30c was also required for the excision of intron A in GnRH-producing GT1–1 cells as revealed by AS-ODN experiment and showed a synergistic effect with 9G8 in the in vivo splicing system using non-GnRH-producing NIH3T3 cells.

It is of importance that one of the most important roles of SR proteins in enhancing downstream ESE-dependent splicing is to stimulate spliceosome assembly through molecular interaction with the 35-kDa subunit of U2 auxiliary factor (U2AF) (28–30). Considering that the polypyrimidine tract in GnRH intron A has many purine bases (3), which lower the binding affinity of U2AF (31, 32), and only 9G8 successfully reconstitutes S100 extract to remove the intron, 9G8 is most likely to be a specific and efficient mediator to recruit U2AF onto this suboptimal polypyrimidine. Its specificity to GnRH pre-mRNA is also strongly suggested by the observation that the splicing-promoting effect of 9G8 on the β-globin pre-mRNA substrate with a strong intron was not as effective as other tested SR proteins in the presence of either HeLa NE or the S100 extract (8). Despite a strong intron A removing activity, there is no consensus binding sequence for 9G8 as revealed by SELEX (9) on the distal region of the exon 3. Alternatively, lack of high affinity binding sequence can be the most probable reason for the requirement of S100 fraction or Tra2α for 9G8 to efficiently recognize its binding site on the GnRH pre-mRNA. In this context, it is noteworthy that recruitment of mammalian 9G8 or Drosophila RBP1 on dsxRE by Tra/Tra2 was proposed as a plausible explanation for dsxRE/Tra/Tra2-dependent enhancement of splicing (19). Similarly, 9G8 could be recruited by Tra2α onto exon 3 of GnRH pre-mRNA as shown in Fig. 5A and required exon 3 for its intron A removal activity. Therefore, the recruitment of 9G8 by Tra2α, and thereby facilitation of 3’ spliceosome formation, seems to be prerequisite or at least one of the most crucial means for the efficient removal of intron A in GnRH-producing neurons (Fig. 9).

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FIGURE 9. A possible model for enhanced splicing of GnRH pre-mRNA by Tra2α, 9G8, and SRp30c. Efficient splicing of GnRH pre-mRNA can be mediated by the cooperative action of these three proteins. Tra2α specifically binds to ESE4 and can recruit 9G8 to the distal region of exon 3. Each SRp30c directly binds to ESE3 or ESE4 individually. The interaction between Tra2α and SRp30c on ESE4 may enhance and stabilize the association of these proteins with GnRH pre-mRNA. These associations may promote basic splicing machinery including U2AF to recognize the suboptimal 3’ splice site and lead to the excision of GnRH intron A.

system (3). Similar distance-dependent actions of splicing enhancers were also found in previous reports elsewhere (10, 25, 26). For example, Tra2α, a Drosophila melanogaster homolog of mammalian Tra2α and β, has been shown to efficiently remove the weak intron by acting on repeat elements of doublesex-pre-mRNA (dsxRE), located 300 nucleotides downstream of the regulated 3’ splice site in the presence of its binding partner Tra and a certain SR protein (10, 26). Although these elements are inactive at this distance in the absence of Tra/Tra2, the enhancers become functional by forming a stable complex with the proteins in their presence (19, 26). However, dsxRE became apparently Tra2α-independent, when the distance between the target intron and dsxRE was reduced to less than 150 nucleotides. In this regard, Tra2α can be a plausible candidate accounting for the ESE4-dependent excision of GnRH intron A. As shown in Fig. 7, inhibition of Tra2α gene expression with AS-ODN strongly reduced the excision efficiency of intron A, even in GT1–1 cells, supporting the notion of a pivotal role of cis-acting element downstream of the regulated 3’ splice site in the GnRH pre-mRNA. In this context, it is noteworthy that recruitment of mammalian 9G8 or Drosophila RBP1 on dsxRE by Tra/Tra2 was proposed as a plausible explanation for dsxRE/Tra/Tra2-dependent enhancement of splicing (19). Similarly, 9G8 could be recruited by Tra2α onto exon 3 of GnRH pre-mRNA as shown in Fig. 5A and required exon 3 for its intron A removal activity. Therefore, the recruitment of 9G8 by Tra2α, and thereby facilitation of 3’ spliceosome formation, seems to be prerequisite or at least one of the most crucial means for the efficient removal of intron A in GnRH-producing neurons (Fig. 9).

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strongly suggesting that it has a distinct role in the ESE4/Tra2α/9G8-evoked facilitation of GnRH pre-mRNA splicing. It should be noted that there has been a growing body of evidence showing diverse roles for SR protein other than initiating spliceosome formation by recruiting U2AF to the 3′ splice site. For example, ESE-bound SR proteins can contribute to an enhanced intron excision by interaction with splicing regulator(s) other than U2AF in certain cases (35, 36), and it was recently found that they interact with the branch point sequence to enhance ESE-dependent splicing (37). Also of interest, ESE/SR protein-evoked enhancement of pre-mRNA splicing can be achieved by antagonistically reducing the influence of an inhibitory factor (38–40). A prior report demonstrated that there is a putative inhibitory factor(s) involving the excision of GnRH intron A even in GnRH-producing cells (8), leading to an assumption that SRp30c bound to ESE3 might counteract with an inhibitory regulator in GnRH neurons. Nevertheless, the exact role of ESE3-bound SRp30c needs further elucidation.

Initially, we identified 9G8 and SRp30c as pivotal mediators of ESE4/Tra2α-dependent GnRH pre-mRNA splicing using in vitro splicing assays and RNA-protein binding analyses. However, several lines of evidence in the present study strongly suggest a physiological significance of these SR proteins in vivo. First, the expression levels of these SR and SR-related proteins showed strong correlations with intron A removal activity in GnRH-producing and non-GnRH-producing cells, and overexpression of one of the proteins was sufficient to significantly increase intron A excision from a transfected splicing reporter construct as shown in Fig. 6, although they failed to exhibit as apparently additive effects as those shown in in vitro splicing and RNA-protein binding assays. Second, blockage of one of these three proteins in GT1−1 cells efficiently inhibited excision of intron A, indicating their requirement and cooperative actions in these cells. Third, GnRH-producing neurons in the mouse POA possess nucleus-localized and speckles-associated Tra2, 9G8, and SRp30c immunoreactivity. Recently, nuclear speckles-associated SR proteins following multiple phosphorylation on the conserved domain rich in alternating arginine and serine residues were demonstrated to be active in their splicing activities (41–44), strongly suggesting that Tra2α, 9G8, and SRp30c are functional in mouse POA GnRH-producing neurons. In this regard, it is worthwhile to note that our previous study proposed a maturation of splicing machinery responsible for the efficient removal of GnRH intron A during postnatal development in mice (7). It is therefore plausible that expression and/or post-translational activation of these proteins may underlie the increased efficiency of intron A removal in mouse POA during sexual maturation. This possibility should also be further elucidated.

In conclusion, because intron A excision directly affects transcript coding capacity (5), efficient and precise processing of GnRH pre-mRNA is crucial for cell type-specific GnRH production, thus allowing GnRH neurons to exert their functions. Therefore, the requirement for cooperative actions of Tra2α with 9G8 and SRp30c on ESEs in the GnRH gene transcript as demonstrated in the present study may provide a novel insight into GnRH gene regulation, although the involvement of another GnRH neuron-specific splicing regulator(s) different from SR proteins cannot be still ruled out. Furthermore, as a model system, the present study on GnRH pre-mRNA also implies the crucial role of mammalian Tra2 by recruiting and cooperating with a subset of SR proteins, in overcoming an intrinsic defect of a weak intron through ESEs far away from a 3′ splice site.