GENES: STRUCTURE AND REGULATION:

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Excision of the First Intron from the Gonadotropin-releasing Hormone (GnRH) Transcript Serves as a Key Regulatory Step for GnRH Biosynthesis*

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The mammalian gonadotropin-releasing hormone (GnRH) gene consists of four short exons (denoted as 1, 2, 3, and 4) and three intervening introns (A, B, and C). Recently, we demonstrated that excision of the first intron (intron A) from the GnRH transcript is regulated in a tissue- and developmental stage-specific fashion and is severely attenuated in hypogonadal (hpg) mouse because of its lack of exonic splicing enhancers (ESE) 3 and 4. In the present study, we examined the influence of intron A on translational efficiency, thereby establishing a post-transcriptional control over GnRH biosynthesis. First, we verified that an intron A-retained GnRH transcript is a splicing variant but not a splicing intermediate. Intron A-retained transcripts can be transported to the cytoplasm in contrast to intron B-containing transcripts, which are restricted to the nucleus. This result implicates the intron A-retained GnRH transcript as a splicing variant; it has a long 5′-untranslated region, as the GnRH prohormone open reading frame (ORF) begins on exon 2. We investigated whether an intron A-retained GnRH transcript can properly initiate translation at the appropriate start codon and found that intron A completely blocks the translation initiation of its downstream reporter ORF both *in vivo* and *in vitro*. The inhibition of translation initiation appears to be due to the presence of a tandem repeat of ATG sequences within intron A. Constructs bearing mutations of ATGs to AAGs restored translation initiation at the downstream start codon; the extent of this restoration correlated with the number of mutated ATGs. Besides the failure in the translation initiation of GnRH-coding region in the intron A-containing variant, the present study also suggests that the interference between mature GnRH mRNA and intron A-retained splicing variant could occur to lower the efficiency of GnRH biosynthesis in the GT1-1-immortalized GnRH-producing cell line. Therefore, our results indicate that the precise and efficient excision of intron A and the joining of adjacent exons may be a critical regulatory step for the post-transcriptional regulation of GnRH biosynthesis.

Gonadotropin-releasing hormone (GnRH)[1] is a hypothalamic neurohormone that plays a pivotal role in the neuroendocrine regulation of mammalian reproduction and sexual development. The majority of GnRH-secreting neurons are located in the preoptic area (POA) of the hypothalamus (1). The mammalian GnRH gene consists of four short exons (denoted as 1, 2, 3, and 4) and three intervening introns (A, B and C). The translation start site of GnRH gene resides on the exon 2. GnRH exon 2 encodes a signal peptide, the GnRH decapeptide, and a part of the GnRH-associated peptide. Exon 3 and 4 encode a remaining part of the GnRH-associated peptide and the 3′-untranslated region (UTR) (2). In GnRH-producing neurons, all three introns are efficiently excised from the primary gene transcript, resulting in a mature GnRH mRNA (3–5). Several extrahypothalamic tissues also express GnRH gene transcripts with a relatively low abundance. It is of interest to note that GnRH RNA species that retain intron A are expressed in human reproductive tissues (6), and the primary transcript appears to be more prevalent than the mature mRNA in rat ovary (7). Our recent findings using an *in vitro* splicing system have shown that the introns B and C are easily excised from the GnRH primary transcript, but intron A is not (8, 9). The attenuation of intron A excision is most likely due to its suboptimal 3′-splice site. Exonic splicing enhancers (ESEs) located in the exon 3 and 4 (denoted as ESE 3 and 4) and a subset of putative transacting factors specific for GnRH-producing cells are thought to be required for the efficient removal of intron A in GnRH neurons (8–10).

In addition to cell type-specific regulation, the excision rate of intron A from the GnRH gene transcript is regulated during sexual maturation in the mouse POA (11). A functional significance of the intron A excision in the regulation of GnRH synthesis is clearly implicated in a nature's knockout hypogonadal (hpg) mouse, where exons 3 and 4 as well as ESE 3 and 4 are truncated. Even though this mutant retains the intact sequence encoding the mature GnRH decapeptide and expresses a detectable amount of GnRH transcript in the hypothalamus (11, 12), no GnRH peptide can be produced resulting in drastic reductions in serum gonadotropin levels and an undeveloped gonad (12, 13). Recently, we found that, in the hpg mouse, the excision rate of GnRH intron A is extremely low even in the POA (11), raising the possibility that intron A can

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[1] The abbreviations used are: GnRH, Gonadotropin-releasing hormone; POA, preoptic area; UTR, untranslated region; ESE, exonic splicing enhancer; hpg, hypogonadal; RT, reverse transcription; CMV, cytomegalovirus; β-gal, β-galactosidase; ORF, open reading frame; uORF, upstream ORF; CHO, Chinese hamster ovary; CTL, control; Luc, luciferase; mIA, mouse GnRH intron A; mE1, mouse GnRH exon 1; mE1IA, mouse GnRH exon 1 and intron A (long 5′-UTR).

TABLE I
Primer sequences for gene cloning and RT-PCR

Name	Sequences
mE1 H3 up[a]	5′-AAGCTTAACTGTGCTCACCAGCGGGGA-3′
mIA H3 up	5′-AAGCTTGTACAGTTCTTTGTTGTTCT-3′
mE1 N1 dn[b]	5′-CCATGGCTGTTTGGATGTGAAAGTCA-3′
mIA N1 dn	5′-CCATGGTAAGGGACATCAAGACACAGA-3′
hE1 H3 up	5′-AAGCTTATAGTCCATTTGCAGTATAAT-3′
hIA H3 up	5′-AAGCTTGTAAAAGGCTTTGTATTATTT-3′
hE1 N1 dn	5′-CCATGGGAATTCCTGTTTAGAGGCAGAGAGCCA-3′
hIA N1 dn	5′-CCATGGCACTATGGTCACCAGCGGGGA-3′
rE1 H3 up	5′-AAGCTTCACTATGGTCACCAGCGGGGA-3′
rIA H3 up	5′-AAGCTTGTAAAATTTTTTGTTTTTTCT-3′
rE1 N1 dn	5′-CCATGGCTGTTTGGATGTGAAAGCCAA-3′
rIA N1 dn	5′-CCATGGTAAGGGACATCAAGACACAGA-3′
mIA 810 up	5′-GCGAAGCTTATTGACTTGGAGGAACT-3′
mIA 810 dn	5′-GCGCCATGGAGTTCCTCCAAGTCAAT-3′
mA 1071 up	5′-GCGAAGCTTTAAGTGCCTTTATCTAGATCA-3′
mA 1071 dn	5′-GGTCCATGGTGATCTAGATAAAGGCACTTA-3′
mA 1385 dn	5′-GAACCATGGCTTCTTTGTGGTAAGGCA-3′
mAΔ3ΔATG up1	5′-AAGCTTCACAAAGAAGTACTACATAAGCCAGAACCA-3′
mAΔ3ΔATG up2	5′-GAAGCTCAAGACAGGTAGAAGTCCCT-3′
mAΔ3ΔATG dn1	5′-CTTGAGCTTCTTAGACTTGGGACA-3′
mAΔ3ΔATG dn2	5′-CCATGGTAAGGGACTTCAAGAC-ACAGA-3′
Mouse GnRH E1 up	5′-GGAAGACATCAGTGTCCCAGA-3′
Mouse GnRH IA up	5′-CACAAAGAAGTACTACATATGCCAGAACCA-3′;
Mouse GnRH IB up	5′-GTGCAAATGGAAACTGTTTT-3
Mouse GnRH E3 dn	5′-AGAGCTCCTCGCAGATCCCTA-3′
Mouse GnRH E4 dn	5′-TGAAATCTACGCTGCTGGGT-3′

[a] up, upstream.
[b] dn, downstream.

affect translation efficiency, thereby establishing post-transcriptional control over GnRH prohormone synthesis.

Thus, it is worthwhile examining the functional relevance of a retained intron A in the regulation of GnRH biosynthesis. First, we investigated whether intron A-retained GnRH transcripts could be transported into the cytoplasm where translation occurs; then, we examined whether an intron A-retained form of GnRH mRNA transcript can properly initiate translation at the start codon downstream to intron A.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Mouse, human, and rat GnRH gene fragments containing exon 1 and/or intron A were amplified from genomic DNA by PCR. Various deletion constructs and point-mutated mouse GnRH intron A fragments were produced from the intron A fragment by PCR. All upper primers contained a *Hin*dIII restriction site at their 5′-end, and all lower primers had an *Nco*I site. All PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI), and sequence identities were confirmed by chain termination sequencing methods. Luciferase reporter plasmids were prepared by inserting each fragment into the pGL3-control vector (Promega) using the *Hin*dIII/*Nco*I sites that reside between an SV40 minimal promoter and the luciferase coding sequence. To avoid the excision of the intron A region in the cells, the last G base of intron A, was deleted. Primer sequences used for cloning are presented in Table I.

Northern Blot Hybridization and Reverse Transcription (RT) PCR—Total RNAs from various tissues and cells were isolated as described previously (8). Nuclear and cytoplasmic RNAs were isolated separately in accordance with a previous report (14) with modifications. Briefly, cytoplasmic RNAs were first fractionated by homogenization of tissues or cells in lysis buffer (0.3 M sucrose, 0.25% sodium deoxycholate, 10 mM Tris-HCl, pH 7.4, 1.5 mM $MgCl_2$, and 0.5% Nonidet P-40) and briefly centrifuged at 3000 × *g* to exclude the nuclear fraction; nuclear RNAs were isolated from the precipitated nucleus after washing out the cytoplasmic contaminant twice with lysis buffer. RNAs were retrieved from each fraction by a single-step acid guanidinium thiocyanate-phenol-chloroform method. For Northern blot hybridization, 30 μg of each RNA were resolved on a 1.2% formaldehyde agarose gel and transferred for 18 h by diffusion blotting to a Nytran filter (pore size, 0.45 μm; Schleicher & Schuell). Complementary RNA probes to GnRH cDNA or intron A were generated using a commercial *in vitro* transcription system (Promega) in the presence of [α-^{32}P]UTP. Hybridization procedures were performed as described previously (8). For RT-PCR analysis, 1 μg (for tissues and non-GnRH-producing cell lines) or 100 ng (for the GT1-1 cell line) of the RNA templates were reverse-transcribed with 200 units of Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) using the manufacturer's instructions. Subsequently, a 3-μl aliquot of each RT sample was subjected to PCR in a 40 μl reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM dNTP, 20 pmol of upstream and downstream primers, and 2.5 units of *Taq* polymerase (PerkinElmer Life Sciences). Ten-microliter aliquots of PCR products were electrophoresed on 2% agarose gels in Tris acetate-EDTA buffer and visualized by ethidium bromide staining. The primer sequences used for RT-PCR experiments are presented in Table I.

Cell Culture and Transient Transfections—All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin/streptomycin, and 10% fetal bovine serum under a humidifying atmosphere containing 5% CO_2 at 37 °C. For transfections, cells were plated in 60-mm dishes and grown to 40–60% confluence for 1–2 days. Cells were washed twice with Dulbecco's phosphate-buffered saline, and the medium was changed to serum- and antibiotics-free Dulbecco's modified Eagle's medium prior to transfection. One microgram of plasmid DNA was transfected using LipofectAMINE PLUS reagent (Invitrogen), and excess DNA complexes were washed out with Dulbecco's phosphate-buffered saline on the following day. After 24 more hours of incubation in regular medium, the cells were harvested and subjected to luciferase assays or RT-PCR.

Luciferase Assay—Each luciferase-reporter construct and a CMV promoter-driven β-galactosidase (β-gal) expression construct were co-transfected. Cell extracts were prepared by incubating cells in 0.3 ml of reporter lysis buffer (Promega) for 15 min at room temperature. After a brief centrifugation, supernatants were stored at −70 °C until assay. β-gal and luciferase assays were performed using commercial enzyme assay kits (Promega) and the β-gal activity was used to normalize for transfection efficiency.

In Vitro Transcription/Translation-coupled Reactions—Mouse GnRH-luciferase fusion genes were subcloned into pGEM-3Z vector (Promega) using *Hin*dIII/*Bam*HI restriction sites. *In vitro* transcription/translation-coupled reactions were performed using a coupled reticulocyte lysate system (Promega) in the presence of SP6 RNA polymerase (Promega), according to manufacturer's instructions. To detect transcribed RNA, [α-^{32}P]UTP was added to the reaction mixture, and labeled RNAs were resolved on a 6% urea-polyacrylamide gel. [^{35}S]methionine was used to label translated peptide, and the reaction products were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel; dried gel was exposed to x-ray film (Fuji, Japan) for 1 day.

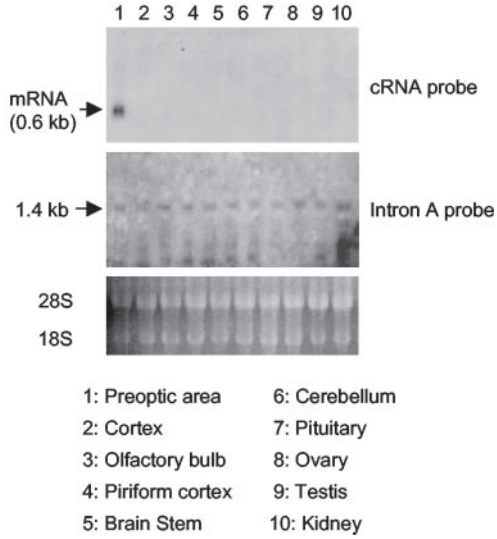

FIG. 1. **Ubiquitous expression of intron A-retained GnRH transcript in rat tissues.** Total RNAs from various rat tissues were resolved on a 1.2% formaldehyde gel and transferred to a Nytran membrane. Hybridizations were performed using ^{32}P-labeled RNA probes complementary to rat GnRH cDNA (*top panel*) or intron A (*middle panel*). Electrophoresed RNA was stained with ethidium bromide and is shown in the *bottom panel*.

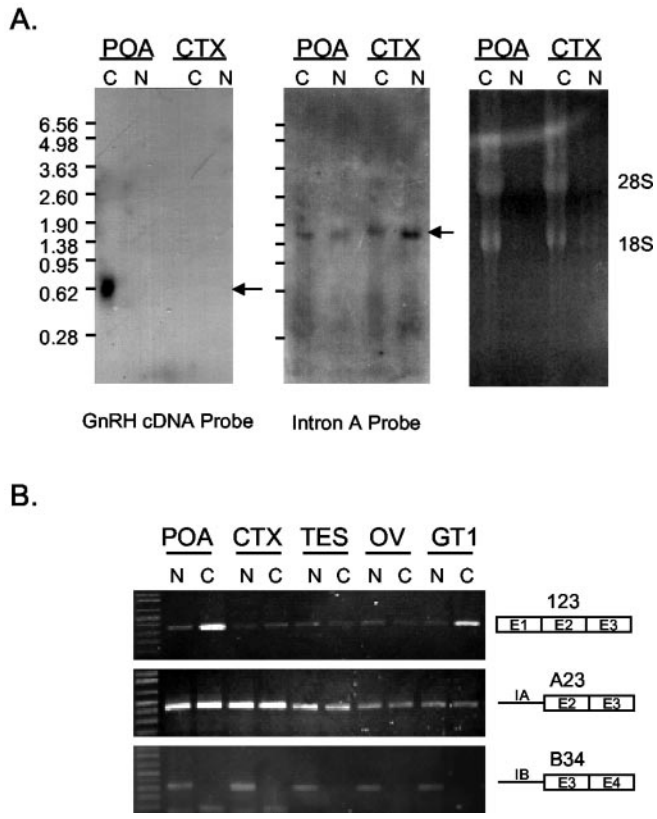

FIG. 2. **Transport of intron A-containing GnRH gene transcripts to the cytoplasm.** *A*, cytoplasmic (*C*) and nuclear (*N*) RNAs separately isolated from the rat POA and cerebral cortex (*CTX*) were resolved on a 1.2% formaldehyde gel and transferred to a Nytran membrane. Hybridization was performed using ^{32}P-labeled RNA probes complementary to rat GnRH cDNA (*left panel*) or intron A (*middle panel*). *B*, cytoplasmic and nuclear RNAs from mouse POA, cerebral cortex (*CTX*), testis (*TES*), ovary (*OV*), and GT1-1 cells (*GT1*) were reverse-transcribed using Moloney murine leukemia virus reverse-transcriptase, and each kind of cDNA was amplified by PCR. PCR products were resolved on 2% agarose gels and visualized by staining with ethidium bromide.

RESULTS

Expression of the Intron A-retained GnRH Transcript in Various Tissues—It is well known that several extrahypothalamic tissues express GnRH transcripts, though with relatively low abundance (6, 7); we have recently demonstrated that the excision of GnRH intron A is severely attenuated in these tissues (8). This finding suggests that the intron A-containing GnRH transcript may be expressed as a splicing variant. To explore this possibility, we examined the expression of intron A-intact GnRH transcripts in several tissues (Fig. 1). Mature GnRH mRNA was predominant in the POA of the hypothalamus, as expected. However, the GnRH transcript, which contains intron A but no other introns, was ubiquitously expressed in all examined tissues. Other GnRH transcripts were barely detectable in POA or extrahypothalamic tissues, indicating that the majority of intron-containing GnRH transcripts retains only the first intron. Interestingly, the intron A-containing GnRH transcript was expressed even in the POA; the expression level was not significantly higher than that of other tissues. These results strongly support the notion that the excision of intron A from the GnRH primary transcript occurs specifically in GnRH-producing cells, and in other cells the intron A-bearing GnRH transcript exists as a major splicing variant.

Export of Intron A-containing GnRH Transcript from the Nucleus—To clarify whether the intron A-containing GnRH transcript is not merely a splicing intermediate but a splicing variant, we examined the translocation of intron A-containing transcripts of the nucleus. Northern blot analysis showed that mature GnRH mRNA exists exclusively in the cytoplasm and not in the nuclear fraction of the rat POA; intron A-retained transcripts were, however, detectable in both cytoplasmic and nuclear fractions of the rat POA as well as the cerebral cortex (CTX) (Fig. 2*A*). We used RT-PCR to examine the cellular localization of GnRH transcripts in various mouse tissues and the GnRH-producing GT1-1 cell line. Again, mature GnRH mRNA was abundant in the cytoplasmic fraction of the mouse POA and GT1-1 cells; we also detected GnRH mRNA in other fractions, but at lower levels. Intron A-retained transcripts were, however, detectable to a similar extent in both cytoplasmic and nuclear fractions (Fig. 2*B*). In contrast to the intron A-containing GnRH transcript, intron B-containing transcripts were found only in the nuclear fraction (Fig. 2*B*). These results confirm the previous finding that intron B is easily excised from the GnRH primary transcript (8) and strongly suggest that the intron A-containing GnRH transcript is a splicing variant and not simply a splicing intermediate of the gene transcript.

Retained GnRH Intron A Blocks the Translation of Downstream Coding Sequences—The intron A-retained transcript may have a long 5′-UTR as the coding region for the GnRH prohormone starts at the second exon (2), and frame-shifted translation over the GnRH coding sequence cannot occur. It is well known that long 5′-UTRs generally lower translation efficiency (15), and our previous report suggests that intron A affects the downstream open reading frame (ORF) translation in the Chinese hamster ovarian cell line (CHO-K1) (11). In this study, we further examined the effect of intron A on the translation of downstream ORF in various cell lines, including both GnRH-producing and non-GnRH-producing cell lines. Three GnRH-luciferase fusion constructs (shown in Fig. 3*A*) were used for transient transfection into several cell lines, such as GT1-1, CHO-K1, mouse fibroblast (NIH-3T3), and human cervical cancer cell (HeLa). When mouse GnRH exon 1 was fused to the 5′-end of the luciferase coding sequence (mE1-Luc), luciferase activities varied from 42 to 108% as compared with those from the control luciferase reporter plasmid (CTL-Luc),

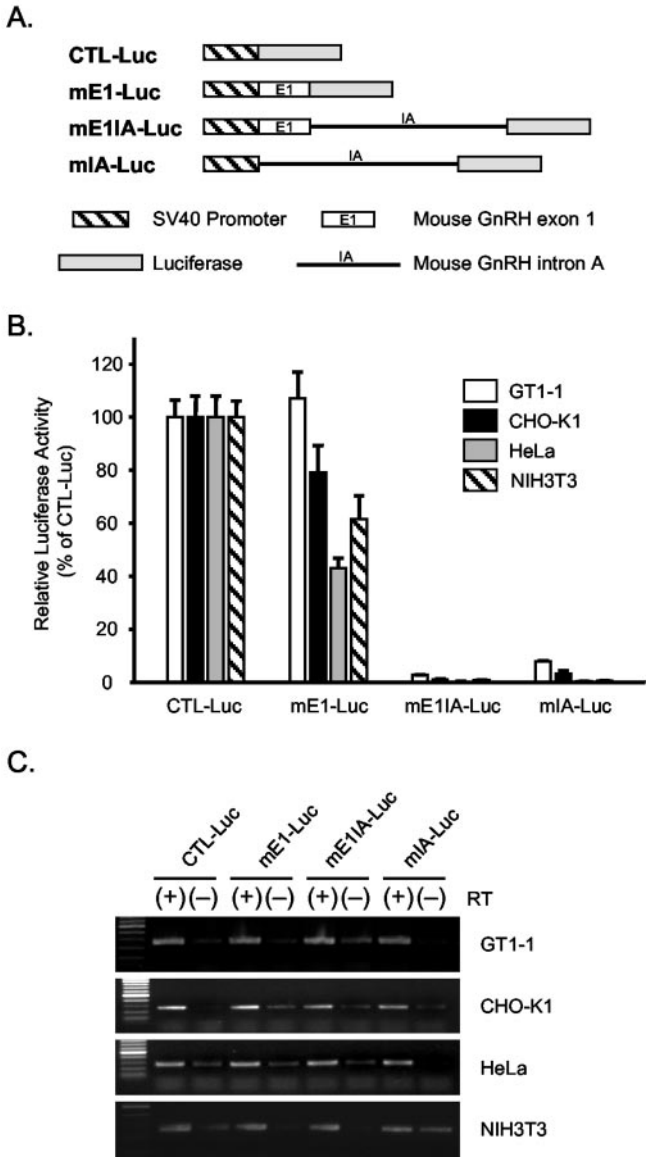

FIG. 3. **Influence of 5′-UTR sequences from mouse GnRH on downstream luciferase activity.** *A*, schematic diagram for GnRH-luciferase fusion gene constructs is shown. *B*, these constructs and the CTL-Luc vectors were transfected into GT1-1, CHO-K1, NIH-3T3, or HeLa cells. Luciferase activities were measured 24 h after transfection and normalized by cotransfected CMV-β-gal activity. Data are shown as mean ± S.E. (n = 6–12). *C*, total RNAs were isolated from transfected cells, and DNA contaminants were removed by incubation with DNase I at 37 °C for 30 min. One microgram of each RNA sample was subjected to RT-PCR analysis (*RT* (+) *lanes*). To assure the removal of plasmid contaminants, the PCR reaction without RT was also performed for each RNA sample (*RT* (−) *lanes*). PCR products were resolved on 2% agarose gels and visualized by staining with ethidium bromide.

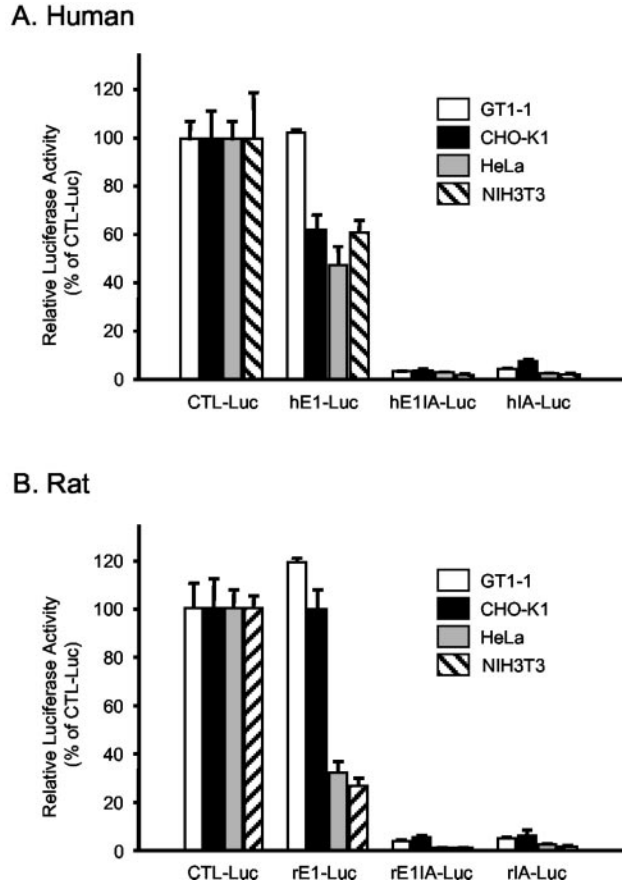

FIG. 4. **Influence of human and rat GnRH intron A on translation efficiency of a downstream luciferase gene.** Control luciferase reporter and the constructs containing human (*A*) or rat (*B*) GnRH exon 1 and/or intron A upstream to luciferase ORF were transfected into GT1-1, CHO-K1, NIH-3T3, or HeLa cells. Luciferase activity was measured 24 h after transfection and normalized by cotransfected CMV-β-gal activity. Data are shown as mean ± S.E. (n = 6).

depending on cell type (Fig. 3*B*). Fusion of the long GnRH 5′-UTR (mouse exon 1 and intron A) to the luciferase ORF (mE1IA-Luc), however, showed a dramatic reduction in luciferase activity; in fact, it was comparable to that of the promoterless negative control vectors. The inhibitory effect of a long 5′-UTR on the luciferase reporter was likely due to the presence of intron A, as judged by the results from the mouse GnRH intron A-luciferase fusion construct (mIA-Luc). To exclude the possibility that mE1IA-Luc and mIA-Luc constructs have a certain defect in transcription, we examined the expression of the GnRH-luciferase fusion mRNA by RT-PCR. Because all constructs can produce luciferase mRNA at significant levels in all cell lines tested (Fig. 3*C*), the intron A-bearing transcripts must have a significant defect in translation.

In addition to the mouse GnRH intron A, the equivalent human and rat GnRH intron also strongly lowered the translation efficiency of downstream ORF (Fig. 4). When the human or rat GnRH exon 1 was fused to luciferase ORF, significant luciferase activities were detected (human, 49–103% and rat, 27–118% of CTL-Luc). Both human and rat GnRH intron A, however, strongly suppressed luciferase activities to the levels of the promoterless reporter. The results indicate that the translational regulation by intron A is well conserved, at least among the human, rat, and mouse, and that intron A-containing GnRH transcripts in these species could scarcely contribute to the synthesis of GnRH prohormone.

To assure the translation inhibition by intron A of the downstream reporter ORF, we performed *in vitro* transcription/translation-coupled reactions. Both SP6 promoter-driven mE1-Luc and mE1IA-Luc RNA transcripts were translated *in vitro* using a reticulocyte lysate system. The mE1-Luc construct yielded a single protein whose molecular mass was ~60 kDa as expected. In contrast to mE1-Luc, mE1IA-Luc could not produce a detectable amount of luciferase protein (Fig. 5). These results clearly indicate that the translation of downstream coding sequence is completely blocked when the mouse GnRH intron A resides upstream.

ATG Sequences in the Mouse GnRH Intron A Region Contribute to the Inhibition of Translation—To determine which regions are involved in the inhibition of translation initiation at downstream start codon, we generated several deletion con-

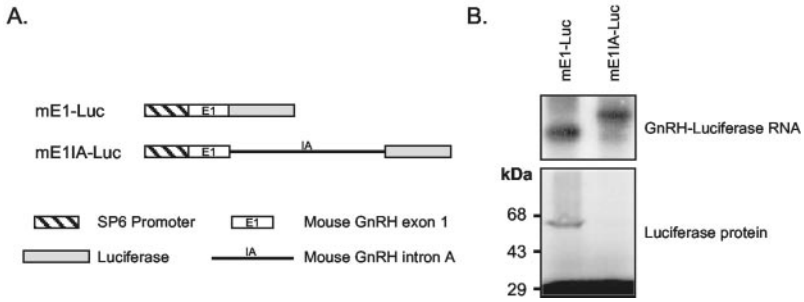

FIG. 5. ***In vitro* transcription/translation-coupled reactions using mouse GnRH exon 1- or exon 1 and intron A-luciferase fusion constructs.** The mE1-Luc or mE1IA-Luc constructs were fused to the SP6 promoter. The schematic diagram for these constructs are shown in *panel A*. RNAs and peptides were synthesized from the plasmids using SP6 RNA polymerase and reticulocyte lysate in the presence of [^{32}P]UTP or [^{35}S]methionine *in vitro*. Synthesized RNAs were resolved on a 6% urea-polyacrylamide gel, and peptides were electrophoresed on a 10% SDS-polyacrylamide gel. Dried gels were exposed to x-ray films for 1 day (*B*).

structs from the mIA-Luc reporter plasmid and transfected these constructs into GT1-1 and CHO-K1 cells. Experiments using serial deletion constructs such as mIA-LucΔ1, Δ2, Δ3, and mIA-LucΔ7 revealed that a short proximal fragment of intron A was sufficient to inhibit luciferase expression in both cell lines (Fig. 6); the region just prior to this short fragment (designated as mIA-LucΔ4) also strongly interfered with luciferase translation. However, luciferase activity was almost normal when the mIA-LucΔ5 construct was used. In CHO-K1 cells, IA-LucΔ6 produced a luciferase activity slightly higher than that of other constructs (Fig. 6). Interestingly, sequence analysis revealed that the intron A of mouse GnRH contains 14 ATG sequences, and at least five of these are putative translation initiation sites; only mIA-LucΔ5 contains no ATG sequence in the fragment. This strongly suggests that ATG sequences in the mouse GnRH intron A play an inhibitory role at the downstream start codon.

To verify this possibility, we introduced point mutations of the mIA-LucΔ3 construct, which contains five ATG sequences. We changed these ATGs to AAG sequences designated as mIA-LucΔ3mut1 and found that it produces luciferase activity comparable with that of mE1-Luc, which lacks intron A. Interestingly, the restoration of luciferase activity correlated well with the number of remaining ATG sequences rather than any particular ATG sequence location. This result strongly suggests that the proximal five ATGs, at least, act on the downstream ORF cooperatively (Fig. 7). In addition to these experiments, we also performed *in vitro* transcription/translation-coupled reactions to verify this prominent role of ATG sequences of intron A and their inhibition of the downstream start codon. In agreement with the transient transfection experiments, the presence of an intact or proximal fragment of intron A strongly inhibited the synthesis of luciferase. The translation of luciferase ORF in the mIA-LucΔ3mut1, however, did produce a significant amount of luciferase protein (Fig. 8). Together, these results strongly indicate that multiple ATG sequences of the GnRH intron A block the appropriate translation initiation at the downstream start codon and may cause the failure in GnRH prohormone synthesis.

Interfering Influence of mE1IA-Luc on the Expression of mE1-Luc in the GT1-1 Cell Line—It can be postulated that mature mRNA and its intron A-retained splicing variant can exist together in the cells, although their relative amounts differ according to the cell- or tissue-types. The notion was partly supported by the finding that various tissues and even the immortalized GT1-1 cell line contained both transcripts (Figs. 1 and 2). We further examined the possible interactions between these two kinds of GnRH transcripts by co-transfection experiment. When mE1-Luc and mE1IA-Luc constructs were co-transfected into GT1-1 cells, increasing amounts of mE1IA-Luc lowered luciferase activities from the same amount of an mE1-Luc construct in a dose-dependent manner. When five times more mE1IA-Luc plasmid was simultaneously introduced with mE1-Luc into GT1-1 cells, the luciferase activities from these cells were significantly reduced to 63% of those from the cells transfected with the same amounts of mE1-Luc and promoterless control luciferase plasmids. mE1IA-Luc could not alter the luciferase activities from control luciferase plasmid. In contrast to GT1-1 cells, mE1IA-Luc did not have any influences on the expression of mE1-Luc fusion construct in NIH3T3 or CHO-K1 cells (Fig. 9). These results suggest that the intron A-retained splicing variant may lower the efficiency of GnRH biosynthesis, at least in GnRH-producing cells.

DISCUSSION

This study demonstrates that intron A-retained GnRH transcripts are expressed ubiquitously in a variety of cell types and can be exported from the nucleus to the cytoplasm. RNA splicing occurs co-transcriptionally only in the nucleus by interactions between splicing factors and the COOH-terminal domain (CTD) of RNA polymerase II (16), and *in situ* hybridization studies using exon- and intron-specific probes clearly demonstrate that intron-containing splicing intermediates from various genes are confined to the nucleus (17–19). Indeed, intron B-containing GnRH transcripts are detectable only in the nucleus, in contrast to intron A-retained transcripts. This result is well in accordance with our previous finding that introns B and C are "consensus" introns and are efficiently excised from the primary transcript (8). This leads us to believe that an intron A-retained GnRH transcript works as a splicing variant form of GnRH transcripts that contains a long 5′-UTR and predominates in non-GnRH-producing tissues.

It should be noted that the GnRH prohormone ORF begins at the second exon; thus, we found it worthwhile to examine whether intron A-retained GnRH transcripts can initiate translation properly. The 5′-leader sequence size in this variant is larger than the average length of eukaryotic 5′-UTRs, 50–100 nucleotides (15). In addition, GnRH intron A contains multiple ATG sequences and putative stop codons, although they cannot cause frame-shifted translation over the GnRH ORF. This strongly suggested that intron A might affect translational efficiency at a downstream start codon. Indeed, GnRH intron A strongly suppressed the translation initiation of the downstream start codon both *in vivo* and *in vitro*, and the elimination of ATG sequences from intron A restored the translation efficiency of the downstream reporter gene, suggesting that translation from AUG codons in intron A is involved in the inhibitory effect on the downstream start codon. Evidence is accumulating that 5′-UTRs have a profound effect on the translational efficiency because of their primary and secondary

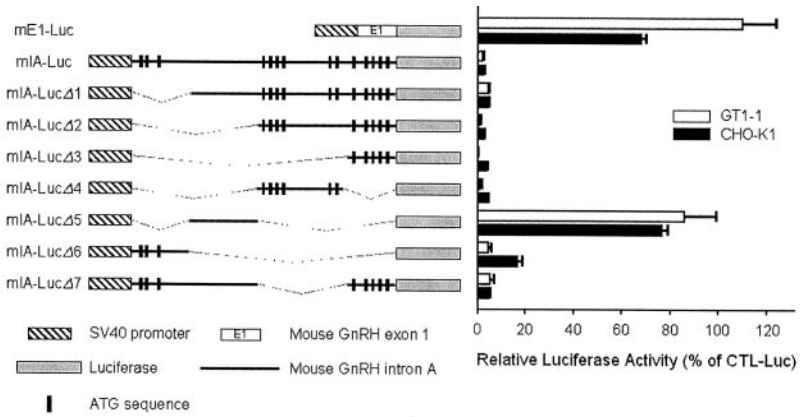

FIG. 6. **Effect of various partial intron A fragments on the downstream luciferase gene translation.** The fusion constructs, consisting of partial GnRH intron A fragments and the luciferase coding region, were cloned as described under "Experimental Procedures." Schematic diagrams for the constructs are shown on the *left*. *Short bars* on intron A represent locations of ATG sequences. Each construct was transiently transfected into GT1-1 or CHO-K1 cells. Luciferase activities were determined 24 h after transfection and normalized by cotransfected CMV-β-gal activity. Data are shown as mean ± S.E. (n = 4).

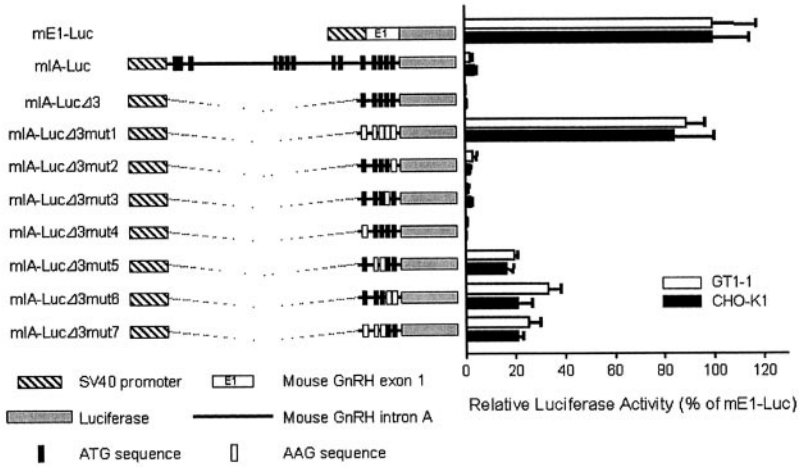

FIG. 7. **Effect of mutating upstream ATGs on translation of the downstream luciferase gene.** Five ATGs of mouse IA-luciferaseΔ3 (*mIA-LucΔ3*) were mutated to AAGs by PCR-based mutagenesis, and the mutations were confirmed by chain termination sequencing. Schematic diagrams for the gene constructs are shown on the *left*. *Short bars* on intron A region represent ATG sequences. CTL-Luc vector, mE1-Luc, mIA-Luc, deletion construct mIA-luciferaseΔ3 (*mIA-LucΔ3*), and mutated IA-luciferaseΔ3s (*mIA-LucΔ3mut1* to *IA-LucΔ3mut7*) were transiently transfected into GT1-1 or CHO-K1 cell lines. Luciferase activity was measured 24 h after transfections, normalized by cotransfected CMV-β-gal activity, and presented as a percentage of the CTL-Luc value. Data are shown as mean ± S.E. (n = 6).

structures. In addition, it is well known that an upstream start codon can modulate translation efficiency. The presence of an upstream AUG sequence (uAUG) and stop codon can generate a short or so-called upstream ORF (uORF). These uORFs usually inhibit translation from downstream start codons, although in some cases they have been reported to be stimulative. As examples, genes such as mammalian S-adenosylmethionine decarboxylase (20), HER-2 (neu, erbB-2) protooncogene (21), suppressor of cytokine signaling 1 protein (22) and CCAAT/enhancer binding protein (23) have been reported to contain inhibitory uORFs, whereas the uORFs in the mouse glucocorticoid receptor (24) and the human androgen receptor (25) are crucial for the translation initiation of a major ORF located downstream.

It is of interest that uORFs are found in less than 10% of known mammalian mRNAs; however, they are strongly biased toward certain classes of genes such as growth factors and their receptors, tumor suppressors, and regulated transcription factors (26), strongly suggesting that translational control by uORFs serves as a fine regulatory mechanism. Although the exact molecular mechanism underlying the translational regulation by uORF is not fully understood, Morris and Geballe (26) have proposed a possible explanation for the inhibitory effect of uORFs on the translation of downstream major ORF from the fact that the uORF itself must be translated to participate in translational regulation and that inhibition is dependent on the sequence and the length of the intercistronic region. In their proposed model (26), a scanning ribosome encounters the initiator AUG of the uORF and initiates translation. Upon reaching the stop codon of the uORF, the carboxyl terminus of the nascent peptide sometimes interacts with part of the translational machinery, depending on the surrounding nucleotide sequences. This interaction is thought to reversibly inhibit either translation termination or a release of the completed peptide, which, in turn, arrests the translating ribosome over the termination codon. The arrested ribosome fails to reinitiate translation and creates a blockade to scanning by additional ribosomes entering at the cap, thus inhibiting translation of the downstream ORF (26). Our results strongly suggest that the possible uORFs generated by a retained intron A could act through this mechanism, inhibiting translation of a downstream ORF.

Along with extrahypothalamic tissues, intron A-retained GnRH transcripts are implicated in the mutant hpg mouse, which has a truncated GnRH gene. Sequence analysis showed that the GnRH gene of the hpg mouse contains the intact

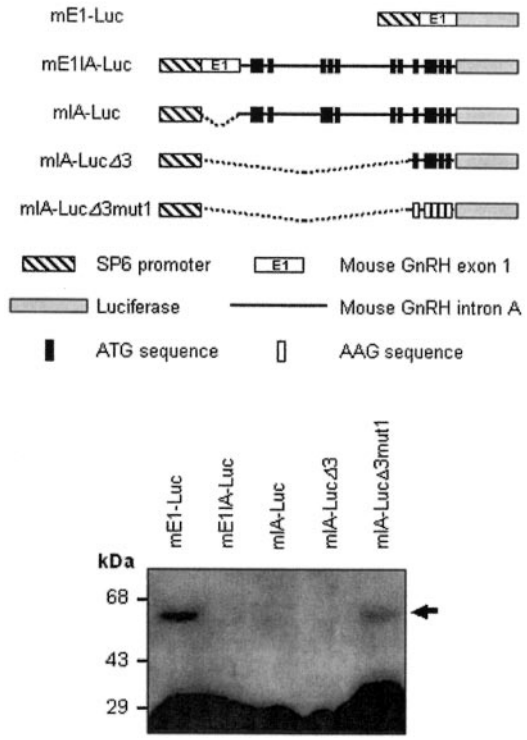

FIG. 8. ***In vitro* transcription/translation-coupled reactions using intact or mutated mouse GnRH-luciferase fusion constructs.** Mouse GnRH-luciferase fusion constructs (mE1-Luc, mE1IA-Luc, mIA-Luc, deletion construct mIA-Luc∆3, and mutated deletion construct mIA-Luc∆3mut1) were fused to the SP6 promoter. A schematic diagram for these constructs are shown in the *top panel*. Luciferase from each construct was synthesized using SP6 RNA polymerase and reticulocyte lysate in the presence of [^{35}S]methionine *in vitro*. Synthesized peptides were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel. Dried gels were exposed to x-ray films for 1 day.

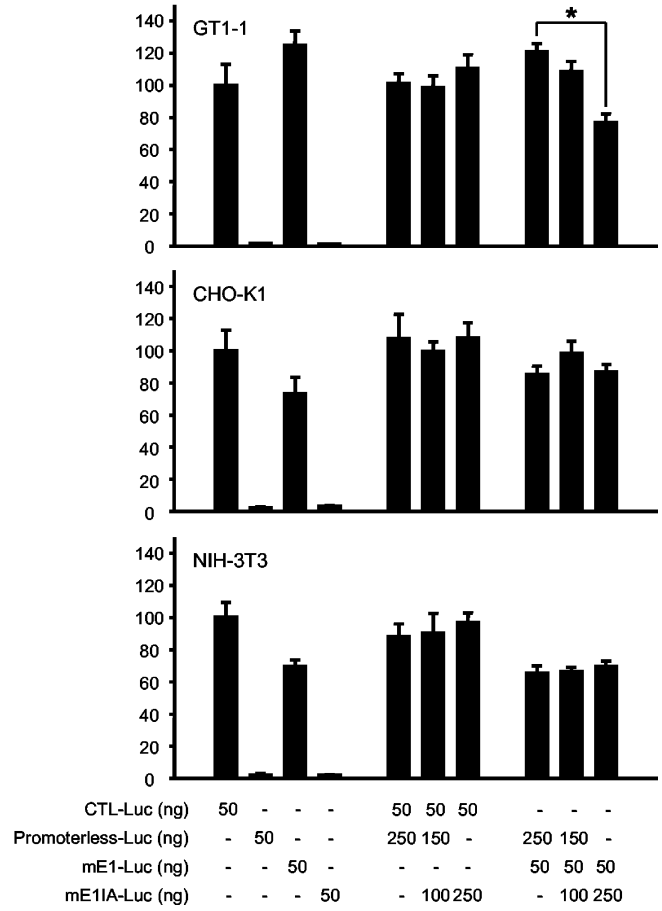

FIG. 9. **Effect of co-transfected mE1IA-Luc on luciferase activity from mE1-Luc.** Various amounts of SV40 promoter-driven CTL-Luc, promoterless-Luc, mE1-Luc, and/or mE1IA-Luc constructs were transfected into GT1-1, CHO-K1 or NIH-3T3 as indicated. Luciferase activities were determined 24 h after transfection and normalized by cotransfected CMV-β-gal activity. Data are shown as mean ± S.E. (n = 8, *, $p < 0.05$).

promoter region and the first two exons but lacks the remaining parts of the gene (12). The previous reports have shown that GnRH transcripts could be detected in the POA of these animals by RT-PCR and *in situ* hybridization even at a lower level than that of normal mice (11, 12). They suggested that transcription could be driven by the GnRH promoter sequence to produce a certain kind of mRNA, which might be a fusion RNA species produced by the joining of the remaining GnRH mRNA sequence with another unidentified sequence. Indeed, this possibility can be postulated from the fact that a part of GnRH promoter is known to be sufficient for the transcription of downstream reporter genes in a subset of hypothalamic neurons (27). It should be noted that, although the GnRH gene of the hpg mouse retains the sequence encoding the GnRH decapeptide and a part of the GnRH-associated peptide, no GnRH decapeptide can be detected in the hypothalamic extract of the animals even by sensitive methods such as high performance liquid chromatography coupled with highly specific anti-GnRH antisera (13). Interestingly, our recent study indicated that a majority of the GnRH transcript in the hpg mouse retained intron A due to an extremely low splicing efficiency for this intron (11). The decreased excision rate of GnRH intron A in the hpg mouse is presumably due to a lack of ESE 3 and 4, which were implicated in our previous report (8, 9). With these previous results, the present study provides a possible explanation for the hypogonadism of the hpg mouse. The failures in the translation initiation of GnRH transcript caused by the retained intron A might be a major cause of the complete lack of GnRH decapeptide in the hypothalamus of the hpg mouse, even though a relatively low, but significant amount of GnRH transcript can be detected in the POA of these mice. As 5′-UTRs are also known to affect mRNA stability (28), something the retained GnRH intron A might also do so. As mentioned above, the hpg mouse has the intact GnRH promoter, but the mRNA level of GnRH in the hpg mouse is much lower than that of the normal mouse (11, 12). This raises the possibility that intron A may be contributing to GnRH gene expression via its mRNA stability. This possibility needs to be explored.

It is also of interest to note that the intron A-containing reporter can lower the expression efficiency of the GnRH E1-Luc constructs in a GT1 cell-specific manner. The result suggests a possible interfering influence of an intron A-retained GnRH variant on the efficient biosynthesis of GnRH. In this regard, it should be noted that the excision rate of GnRH intron A is significantly low in prepubertal mouse POA (11). It showed a gradual increase during postnatal development. This result strongly suggested that the maturation of splicing machinery responsible for GnRH neuron-specific excision of the intron A could occur with sexual maturation. With the previous findings, our present result indicates that the efficient excision of the intron A may contribute to the efficient production of GnRH. An exact molecular mechanism underlying the interference is presently unknown. However, competition between mature GnRH mRNA and its intron A-retained variant to the translation machinery in GnRH-producing cells would be most probable, because these two transcripts contain the same ribosomal entry sequence. As shown in Figs. 3 and 4, the translation efficiency for E1-Luc constructs varied according to cell

type when compared with that of CTL-Luc containing no GnRH UTR sequences. The translation efficiencies of E1-Luc were generally impaired in non-GnRH-producing cell lines, although significant amounts of luciferase can be still produced in these cells. It appears that the translation machinery in GT1 cell is specified to translate GnRH mRNA more efficiently. Similar results were also observed in other genes. For instance, translation efficiencies of heterogeneous neuronal nitric oxide synthase (nNOS) transcripts were significantly affected by cell types. Even in the same cell line, cell differentiation significantly altered the translation efficiency of that transcript. These results strongly suggest that there must be cell type-specific translation machinery that is specified for a subset of gene transcripts (29). Thus, GT1 cell-specific inhibitory influence of E1IA-Luc on the expression of E1-Luc may occur by competitively reducing the translation efficiency of that E1-Luc transcript.

In conclusion, we clearly demonstrate that the GnRH transcript bearing its intron A actually acts as a splicing variant form of the GnRH transcript that is predominant in non-GnRH-producing cells. In contrast to the exon 2-skipped splicing variant of GnRH mRNA, which lacks the coding sequence for the GnRH decapeptide region and its surrounding parts (30), this third variant retains an intact ORF. It cannot, however, be appropriately translated because of the presence of multiple uAUGs on intron A, and it inhibited the expression of the reporter construct mimicking a mature GnRH mRNA in a GT1 cell-specific manner. These results indicate that the precise and efficient excision of intron A and the joining of exons serves as key regulatory steps for the synthesis of GnRH prohormone, thereby contributing to the tissue- and developmental stage-specific expression of this gene product, and also provides a possible molecular mechanism for the absence of functional GnRH biosynthesis in the hypothalamus of the mutant hpg mouse.

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