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# Structure of the distal human gonadotropin releasing hormone (hGnRH) gene promoter and functional analysis in GT1-7 neuronal cells

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## ABSTRACT

**To assess potential species-specific expression of gonadotropin releasing hormone (GnRH), the distal human (h) GnRH promoter was cloned, characterized and tested in gene transfer studies. The nucleotide sequence of ~3.8 kb of 5′-flanking region was determined. Homology to the rat (r) GnRH sequence was observed in the proximal promoter region between –551 h (–424 r) and the transcriptional start site and within multiple distal promoter regions. In contrast, there was little similarity in the sequences between –1131/–551 h and –1031/–424 r. A deletion panel of 5′-flanking hGnRH promoter constructs was made and tested in transient transfection assays in GnRH-producing mouse GT1-7 neuronal cells. The largest hGnRH promoter construct (–3832/+5 h) exhibited high levels of reporter activity, similar to that observed with the largest rGnRH construct (–3026/+116 r). However, in contrast to the rat gene, deletion of distal promoter sequences of the hGnRH promoter to –1971, –1131 or –551 did not result in a decrease in luciferase reporter activity. Further truncation to –350 resulted in a 3-fold decrease in luciferase activity. There was no preferential use of the putative upstream hGnRH start site in neuronal cells. DNase I protection assays showed unique protection patterns with nuclear extracts from GT1-7 and Gn10 neuronal cells and the hGnRH and rGnRH promoter fragments. These data suggest the presence of different *cis*-acting elements and *trans*-acting factors that mediate species-specific neuronal GnRH expression.**

## INTRODUCTION

Gonadotropin releasing hormone (GnRH) is a hypothalamic releasing factor, expressed in a small subset of hypothalamic neurons, that controls pituitary gonadotropin subunit biosynthesis and thus the reproductive axis (1). Although the gene for human (h) GnRH has been cloned by several groups (2–5), neither sequence information beyond –1131 nor functional characterization of the distal promoter beyond –551 is available. Based on the recent observations by Kepa *et al.* (6–7) and Mellon and co-workers (8–9) that the rat (r) GnRH promoter contains a neuronal-specific enhancer in the region between –1863 and –1571, we wondered whether the hGnRH promoter would be similarly organized. Previous work by Radovick and colleagues (4,10) and Dong *et al.* (5) characterized the activity of the hGnRH promoter to –551 in Gn10 neuronal cells (10,11), derived from a mouse olfactory lobe tumor at the time of migration of GnRH neurons, and JEG-3 choriocarcinoma cell lines (4,5). Since previous work characterizing the rGnRH promoter was performed in GT1-7 neuronal cells (12), derived from a mouse forebrain tumor post migration of the GnRH neurons, we wished to compare the activity of the distal hGnRH and rGnRH promoters using the same neuronal cell type and expression vector system.

We sequenced the distal hGnRH promoter using overlapping sequencing reactions. A comparison of the two genes showed only partial similarity of the distal (–3800/–1500) and proximal (–350/+1) promoter regions, with marked differences in the mid-promoter (–1500/–350) region. In gene transfer studies, the largest hGnRH construct from –3832/+5 h was transcriptionally robust, exhibiting levels similar to that observed with the largest rGnRH construct (–3026/+116 r). In contrast to the rat, the hGnRH promoter maintained high levels of reporter activity with successive 5′ promoter deletions to –551. Only with deletion to –350 did luciferase activity drop significantly. There was no activity of hGnRH constructs containing only the upstream transcriptional start site at –579 in the neuronal cells. In contrast to the ability of distal rat promoter sequences to confer enhancer (5- to 7-fold) activity to a heterologous neutral promoter ($RSV_{180}$), the distal human promoter sequences had little effect (1- to 2-fold). DNase I footprinting of the rGnRH and hGnRH promoters suggested that different *cis*-acting elements bind nuclear proteins from GT1-7 and Gn10 neuronal cells. Thus, there appear to be marked differences in the structural organization of the rGnRH and hGnRH promoters that are reflected in a

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| Primer | Sequence (5′ → 3′) | Position |
|---|---|---|
| H1 | GTGATGAATATTACCAGTTAGC | –3457 to –3436 |
| H2 | GCCAATACTATCTATTGTAG | –3179 to –3158 |
| H3 | CCAAGGAAGAATTACCTTTAAAA | –2972 to –2950 |
| H4 | AAACTGCTTCATCAACATCAAAA | –2678 to –2720 |
| H5 | CAAAACAAAGTTGACTCTTGCC | –2191 to –2470 |
| H6 | GGCAAGAGTCAACTTTGTTTTG | –2469 to –2497 |
| H7 | GGAAGCATCCAGCACAGGAG | –2140 to –2159 |
| H8 | TCACTGGATTTCTTGTAGGTTA | –1854 to –1879 |
| H9 | TCATTAGTCCTATCCAGAGACCA | –1793 to –1767 |
| H10 | GGGAATCGGCAATTTCCCCTCAT | –1304 to –1326 |

**Figure 1.** Localization and sequence of oligonucleotide primers used in characterizing the distal hGnRH promoter. Oligonucleotide primers were synthesized using known sequences to complete the sequence of the distal hGnRH promoter.

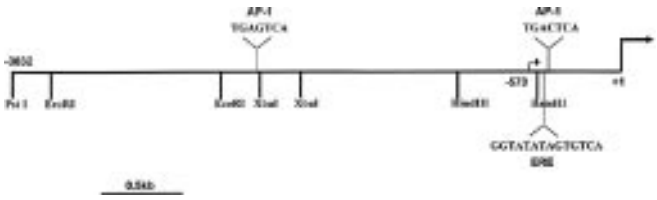

**Figure 2.** hGnRH upstream promoter organization. Mapping and sequencing of the 5′-upstream sequences are described in Materials and Methods. Restriction sites used for cloning of reporter constructs are indicated in their appropriate location. Transcription start sites are indicated by solid arrows at –579 and +1. Potential transcription regulatory sequences are indicated: i.e. AP-1, activator protein 1; ERE, estrogen response element (as characterized in 11).

similar overall activity in neuronal cells, but a unique arrangement of *cis*-acting elements to direct species-specific and neuronal-specific expression of GnRH.

## MATERIALS AND METHODS

### Nucleotide sequence analysis of the distal hGnRH promoter

Sequence analysis was performed using the dideoxynucleotide chain termination procedure with the Sequenase kit (US Biochemicals) (13). DNA fragments containing the distal hGnRH promoter (*Pst*I–*Hin*dIII) (10,11) were subcloned into pGEM vectors. Restriction sites used for localization of synthetic oligonucleotide primers used in sequencing and subcloning are indicated in Figures 1 and 2 respectively. Each DNA segment was sequenced and read from both strands of at least two different reactions.

### Promoter reporter fusion constructs and plasmid constructs

The promoterless vector (pA$_3$LUC) and the pRSV$_{400}$LUC vector were constructed as previously described (6,14). The pA$_3$LUC vector contains a trimerized SV40 polyadenylation signal located upstream of inserted promoter sequences that results in minimal background luciferase activity (15). The largest fragment of the hGnRH construct was used to make a series of deletion constructs using available restriction sites (4,10,11). The rGnRH promoter constructs were as previously described (6,14). The heterologous constructs were made using a 180 bp fragment (–130/+50) of the 3′ long terminal repeat of the Rous sarcoma virus promoter inserted downstream of GnRH promoter fragments (pRSV$_{180}$LUC). pSV40βgal was used as an internal control for transfection efficiency.

### Tissue culture and transfection assay

GT1-7 neuronal cells are an immortalized mouse hypothalamic cell line which synthesizes and secretes abundant GnRH (12) and were kindly provided by P.Mellon (UCSD). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) and 5% fetal calf serum. Medium was supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/µl Fungizone. Transient transfections were performed using electroporation as described (6). Ten micrograms of test plasmid and 5 µg pSV40βgal were transfected into GT1-7 cells and harvested after 16–18 h. Lysates were assayed for luciferase and β-galactosidase to control for transfection efficiency. Resultant luciferase activities were then normalized to the activity of pRSV$_{400}$LUC transfected in parallel to control for cell number across experiments as described (6,14).

### DNase I protection assay

Nuclear extracts from GT1-7 and Gn10 neuronal cells were prepared as previously described (16). Protein concentration was determined using the BioRad kit, then aliquoted and kept frozen at –80°C. DNA probes were prepared using restriction fragments of hGnRH (–551/–350 and –350/+5) and rGnRH (–2012/–1597 and –171/+116) promoters. Each fragment was end-labeled with [α-$^{32}$P]nucleotides (3000 Ci/mmol; NEN) and reverse transcriptase (17). Unincorporated nucleotides were removed using a Sephadex G-50 column. DNase I protection was performed using 1–2 µl purified probe (0.5–1.0 ng or 5–10 000 c.p.m.), 400 ng ssDNA (2 µl), 60 mM KCl, 240 µg GT1-7 or Gn10 nuclear extract and 2.5–1000 ng DNase I. The samples were run on a 6% acrylamide–8 M urea gel, followed by autoradiography (17).

### Statistical analysis

Significant changes in promoter activity between constructs was determined by one way analysis of variance (ANOVA) using the INSTAT program.

## RESULTS

### Nucleotide sequence of the distal 5′-flanking region of the hGnRH gene

Previous analysis of the hGnRH promoter had included 5′-flanking sequence up to only –1131 (3). Thus, we first subcloned fragments of the largest DNA fragment available and used a series of synthetic oligomers to further sequence the distal hGnRH promoter (see Fig. 1). Sequence analysis of overlapping subcloned fragments revealed that the largest fragment contained sequence from –3832 to +5 of the hGnRH promoter (see Fig. 2). Computer analysis of the human GnRH 5′-flanking sequence revealed significantly fewer convenient restriction sites than in the rGnRH promoter. Scanning of the GenBank database of reported consensus sequences for classes of DNA binding proteins revealed two activator protein-1 (AP-1) sites at positions

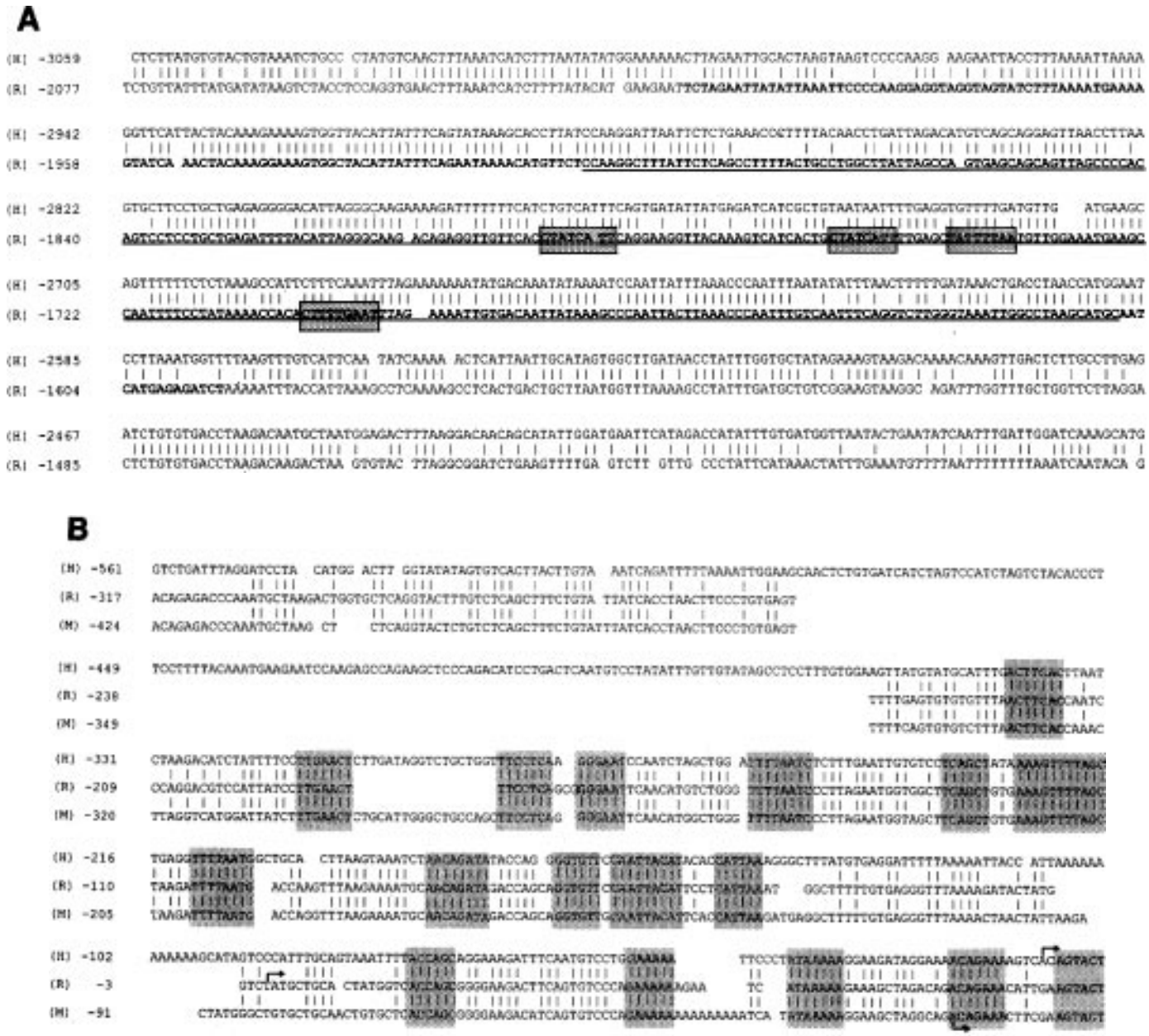


**Figure 3.** A partial nucleotide sequence comparison of the distal 5′-flanking region of the hGnRH and rGnRH genes and the proximal h, r and mouse (m) genes. The program ALIGN was used. Identical nucleotides are indicated by dots and gaps are indicated by hyphens. (**A**) Homology of hGnRH and rGnRH promoters in the region of the rat enhancer. Boxed areas as per Mellon and colleagues (8,9) present in the rat and not human sequence. (**B**) Homology of the proximal promoter region containing the homology among human (h), rat (r) and mouse (m).

–2292 and –472, an imperfect estrogen response element (ERE) at position –534 (11) and several putative DNA binding sites corresponding to members of the POU domain (18) class of DNA binding proteins, including an octamer consensus at –3370. AP-1 consensus sites (6) were present in different regions than previously reported for the rGnRH promoter at –99 (6).

## Sequence homology between the hGnRH and rGnRH promoters

To analyze potential similarities and differences in the sequences of the hGnRH and rGnRH promoters, a sequence alignment (ALIGN) program was utilized. Interestingly, there were three major regions of homology in the distal promoter: –3036 to –2923 h with –2053 to –1940 r (region I); –2766 to –2539 h with –1786 to –1559 r (region II); –1775 to –1552 h with –1311 and –1106 r (region III). Despite homology in the distal promoter sequences, the putative repetitive sequences suggested by Whyte *et al.* (8) to be critical for neuronal-specific expression of the rGnRH promoter were not present in the human sequence (Fig. 3A). Between –1552 and –579, the putative upstream start site in the human promoter (5), there was little similarity with the rat promoter. However, in the proximal promoter (region IV, –343 to

+8 h and –332 to +96 r), there was marked homology similar to the proximal mouse (m) GnRH promoter (see Fig. 3B).

### Promoter activity of the distal hGnRH promoter in GT1-7 neuronal cells

The largest hGnRH promoter fragment was placed into the $pA_3LUC$ vector and tested in a transient transfection assay optimized for rGnRH promoter activity in GT1-7 neuronal cells. $pA_3LUC$ (promoterless vector) exhibited low activity (4–500 light units; LU). In contrast, $pRSV_{400}LUC$, containing a neutral promoter, was dramatically expressed ($7 \times 10^6$ LU). The –3832/+5 hGnRH construct was significantly expressed in the cells (53 000 LU), similarly to the largest rGnRH construct, –3026/+116 (80 000 LU).

### Deletion analysis of the hGnRH promoter in neuronal cells

A series of deletion constructs of the hGnRH promoter was made to assess the *cis*-acting regions that direct hGnRH gene expression in neuronal cells (Fig. 4A). Deletion from –3832 to –1971 resulted in no change in luciferase activity (35 000 LU). Further truncation to –1131 increased reporter activity slightly to 45 000 LU, which was maintained with deletion to –551. Additional deletion to –350 decreased luciferase activity by 70% to 14 000 LU. Interestingly, this region of the hGnRH promoter between –551 and –350 has little similarity to the rat gene. These results with the hGnRH promoter were in marked contrast to results with the rGnRH promoter constructs tested in parallel, containing a neuronal-specific enhancer region between –1863 and –1571 and multiple proximal regions that mediate neuronal expression (see Fig. 4B; 6–9).

Since the human gene has an upstream start site not found in rodent species (5), we created a construct containing the sequence from –1131 to –551 that contains the upstream but not the downstream start site. This construct was not transcriptionally active in the neuronal cells (Fig. 4A). These data confirm the suggestion by Dong *et al.* (5) that the upstream start site (–579) in the hGnRH promoter is not used preferentially for GnRH expression in hypothalamic neuronal cells.

### Inability of the distal hGnRH sequences to confer enhanced activity to a neutral promoter

Since the distal promoter sequences of the hGnRH and rGnRH genes were significantly homologous despite differences in their functional activities, we asked whether distal hGnRH promoter fragments could confer enhancer activity on a heterologous promoter. Various promoter fragments were inserted in front of the neutral promoter $RSV_{180}$ and tested in gene transfer studies. The hGnRH fragments –3832/–2412, –2412/–3832, –2412/–2196 and –2196/–1971 of the hGnRH promoter were able to enhance reporter activity only minimally, 1- to 2-fold above that seen with $pRSV_{180}LUC$ (Fig. 5A). In contrast, the fragment –2012/–1597 of the rGnRH promoter increased the activity of the $RSV_{180}$ promoter 5- to 7-fold in an orientation-independent manner (Fig. 5B). Together, these studies confirmed a species-specific difference in the structural organization of the distal GnRH promoter.

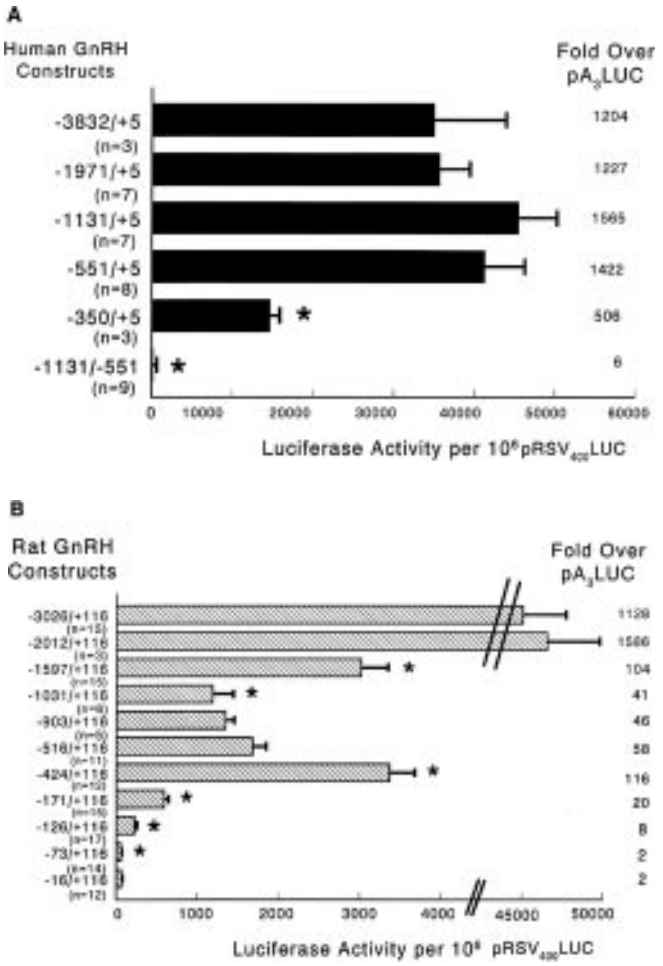


**Figure 4.** Deletion analysis of hGnRH (**A**) and rGnRH (**B**) promoter activity in GT1-7 neuronal cells. Ten micrograms of hGnRH and rGnRH deletion constructs or pRSVLUC and 5 µg pSV40 βgal were co-transfected into GT1-7 cells and cells harvested after 16–18 h. Lysates were assayed for β-galactosidase and luciferase activities. Data are standardized to β-galactosidase activities as a control for transfection efficiency and expressed per $10^6$ light units of $pRSV_{400}LUC$ to control for cell number between experiments. Each bar represents the average ± SEM of luciferase activity obtained from *n* transfections. $*P < 0.05$ of significant change in promoter activity from previous construct.

### DNase I protection assays with extracts from neuronal cells show differences between hGnRH and rGnRH proximal promoter regions

To assess whether the differences in the functionally active *cis*-acting elements of the hGnRH and rGnRH promoters were accompanied by species-specific variation in interactions with *trans*-acting nuclear proteins from neuronal cells, we performed DNase I footprinting of DNA fragments shown to direct neural-specific promoter activity. Nuclear extracts from GT1-7 and Gn10 cells were incubated with –551/–350 and –350/+5 of the hGnRH promoter (Fig. 6A and B) and –2012/–1597 and –117/+116 of the rGnRH promoter (Fig. 7A and B). Extracts from both neuronal cells were tested to assess whether a different complement of *trans*-acting factors was present in the neuronal cell line derived from a tumor in the olfactory lobe during GnRH

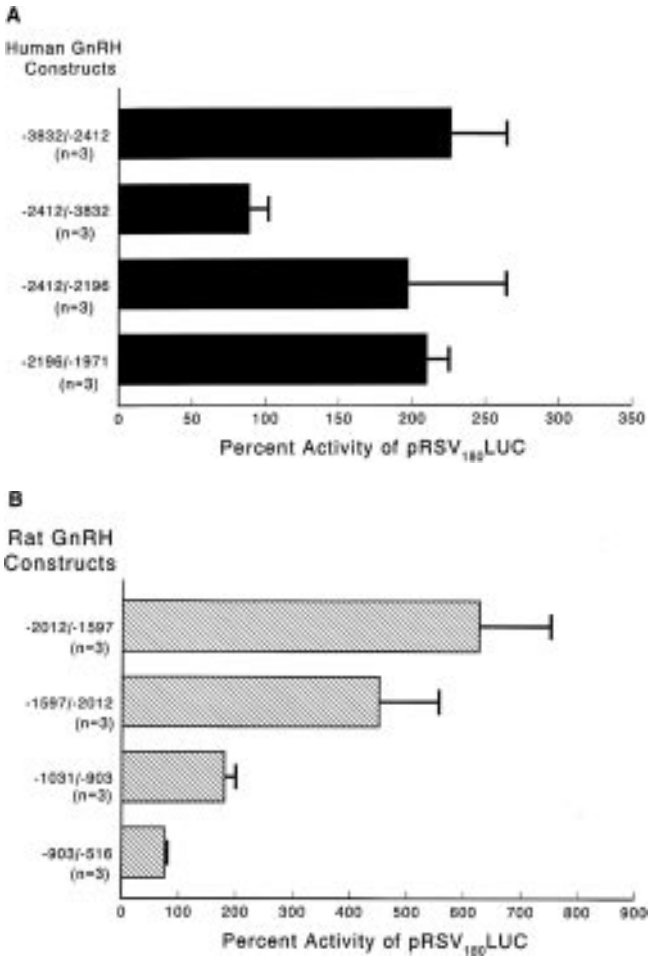


**Figure 5.** Ability of hGnRH (**A**) and rGnRH (**B**) promoter fragments to confer activity to a neutral promoter. Ten micrograms of $pRSV_{180}LUC$ or various test plasmids were co-transfected with 5 μg pSV40 βgal and harvested at 16–18 h. Lysates were assayed for β-galactosidase and luciferase activities. Data are expressed as a percentage of the luciferase activity of $pRSV_{180}LUC$.

neuronal migration (Gn10), which produces low levels of GnRH mRNA and protein, in comparison with a cell line from a forebrain tumor post migration of GnRH neurons (GT1-7), which makes abundant mRNA and protein.

The hGnRH promoter fragment –551/–350 (Fig. 6A) was protected in the region proximal to the upstream start site at –579. In Gn10 cells, this A-T region produced a potential footprint extending from –489 to –508, whereas GT1-7 extract protected a region from –479 to –535. Interestingly, this fragment contains a putative hERE, an imperfect palindrome GGTATATAGTGTC at –534/–521, as compared with the consensus ERE GGTCANNNTGACC described by Radovick and co-workers to be active in Gn10 cells (10).

The proximal promoter fragment –350/+5 h was heavily protected, but to a greater degree with GT1-7 than Gn10 extracts (Fig. 6B). The CAAT motif at –162/–159 h and –53/–50 h as well as the TATA box at–32/–25 h was not footprinted with extracts from Gn10 neuronal cells. These differences in the complement of active transcription factors may explain the markedly different levels of endogenous GnRH production in the GT1-7 compared with the Gn10 neuronal cells.

The DNA fragment containing the rGnRH enhancer region from –1600 to –1950 (Fig. 7A) was heavily protected with extracts from GT1-7 cells, as shown previously by us and others (6,8–9). Extracts from Gn10 cells also footprinted some of the same areas, but several unique protected regions were observed. In particular, the sequences –1764/–1758 r and –1735/–1714 r, shown to be critical components of the rat enhancer (6–9), were footprinted with GT1-7 but not Gn10 extracts. These subtle differences may explain why many have been unable to achieve high level expression of the rGnRH promoter in Gn10 cells by transient gene transfer assays.

The proximal promoter fragment of the rGnRH gene, –171/+116 (Fig. 7B), bound multiple proteins from GT1-7 and Gn10 nuclear extracts. In contrast to the human promoter, both neuronal extracts protected the CAAT box at –56/–52 r and TATA at –19/–15 r. Thus, both species-specific differences and differences in the complement of neuronal proteins between the two cell lines were observed in the footprinting assays.

## DISCUSSION

Although the hGnRH and rodent GnRH genes were cloned in the 1980s, little information is available concerning a species comparison of promoter sequences and functional activity in neuronal cells. Recent studies have characterized the distal rGnRH promoter and shown the presence of a potent neural-specific enhancer between –1863 and –1571 (6–9). To see if similar regions exist in the human gene to mediate neuronal expression, we sequenced >3 kb of the distal hGnRH promoter and tested its activity in GT1-7 neuronal cells.

Sequence comparison showed few regions of significant homology in the distal promoter regions. Despite sequence homology to the region of the rat enhancer between –2000 and –1600, the human promoter sequence lacks the critical boxed repeats suggested to be important by Mellon and co-workers for neuronal-specific expression (8,9) (see Fig. 3A). The mid-promoter regions are dissimilar between the two GnRH genes. Unlike the rat promoter, the human promoter contains a second upstream start site at –579, suggested to be important in GnRH expression in non-neuronal reproductive tissues (5). Perhaps upstream start site-specific control regions in the hGnRH promoter account for the divergent mid-promoter region sequences in comparison with the rat gene.

In gene transfer studies in neuronal cells, the largest hGnRH construct, –3832/+5, exhibited activity similar to the largest rGnRH construct, –3026/+116. Unexpectedly, however, serial truncation of the distal hGnRH promoter restored high reporter activity. This is in sharp contrast to the pattern of activity of rGnRH deletion constructs. Only deletion to –350 of the hGnRH promoter resulted in a significant decrease in luciferase activity. In contrast to the ability of the rGnRH promoter fragment –2012/–1597 to confer enhancer activity to a potent heterologous promoter, various distal human promoter fragments had little effect on the $RSV_{180}$ minimal promoter. Together, these data suggest a species-specific difference in the structural organization of *cis*-acting promoter elements used to confer neural-specific expression.

DNase I footprinting with extracts from GT1-7 and Gn10 neuronal cells and proximal hGnRH and rGnRH promoter fragments confirmed a different pattern of protein–DNA interactions. With the functional data, these studies localize the

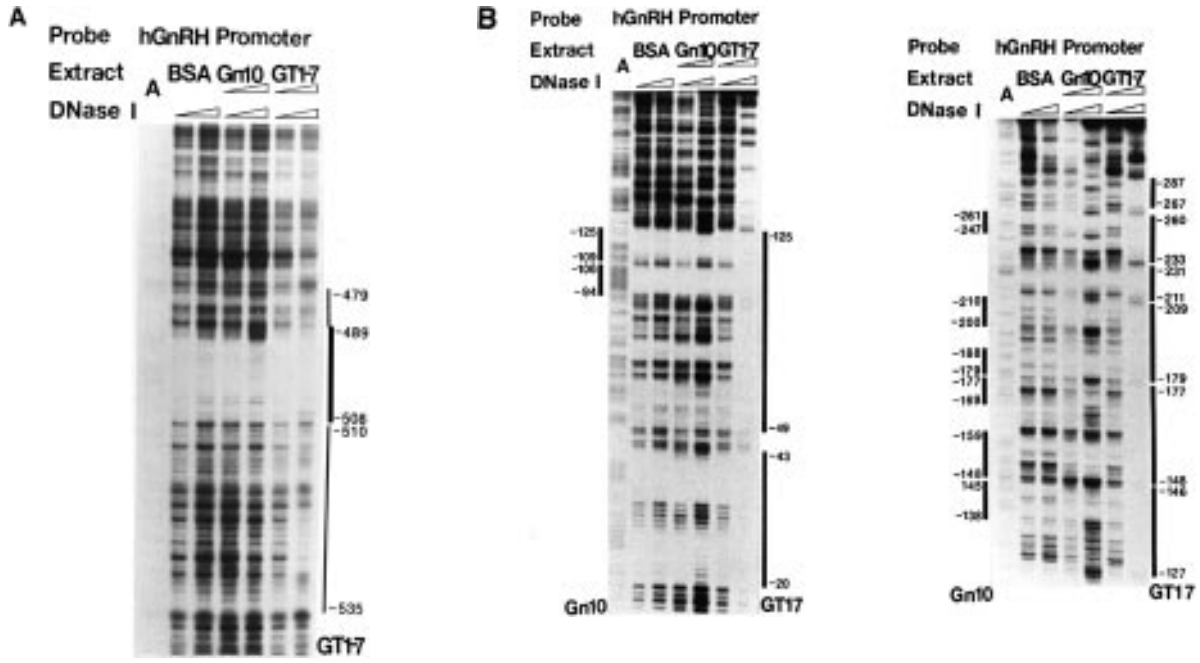


**Figure 6.** DNase I footprinting of the hGnRH promoter regions with GT1-7 and Gn10 neuronal extracts. DNA fragments –551/–350 (**A**) and –350/+5 (**B**) of the hGnRH promoter were end-labeled on the coding (A and B1) or non-coding (B2) strand and incubated with 240µg Gn10 or GT1-7 nuclear extracts in the presence of increasing amounts of DNase I. Lane A represents 'A' Maxam and Gilbert chemical sequencing ladders of the same probe. BSA lanes represent increasing DNase I digestion without nuclear proteins. Solid bars outline regions protected by neuronal proteins. In (A), the bars between areas of nucleotide sequence identify protected regions from Gn10 (heavy bar) and GT1-7 (continuous bar) nuclear extracts. In (B1 and 2), bars between areas of nucleotide sequence identify protected regions from Gn10 (left) and GT1-7 (right) nuclear extracts.

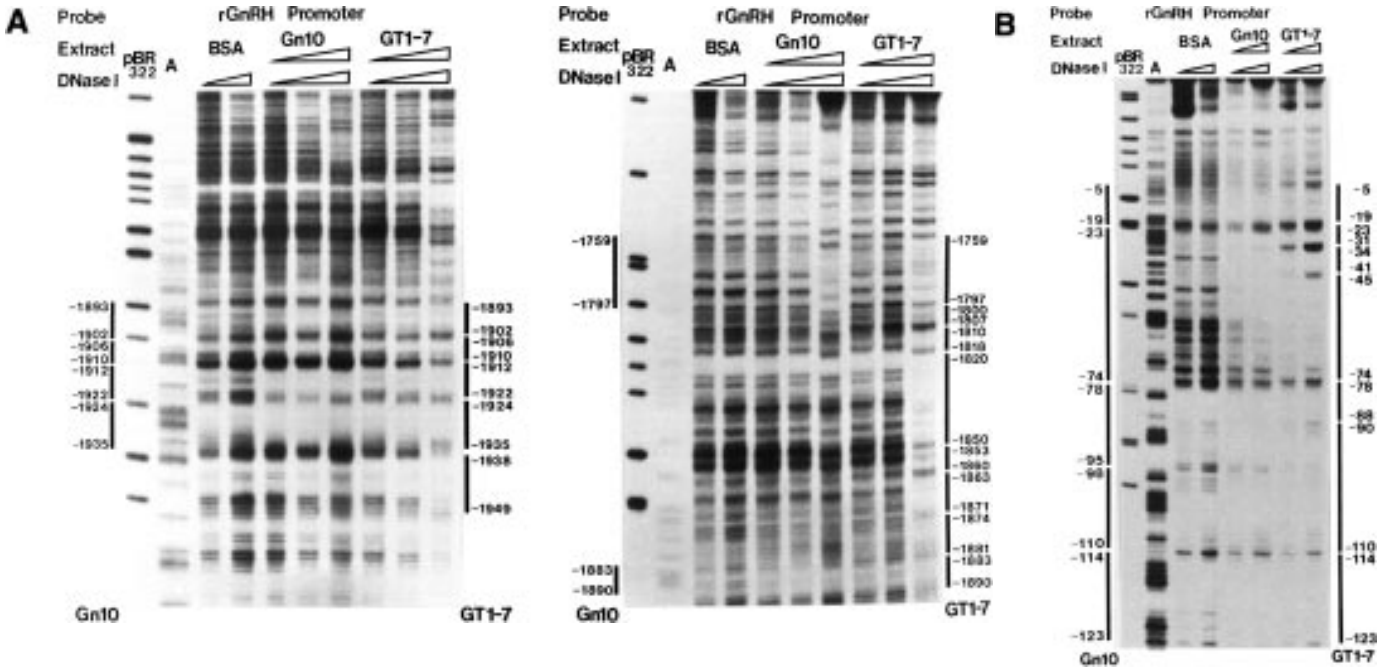


**Figure 7.** DNase I footprinting of the rGNRH promoter regions with GT1-7 and Gn10 neuronal extracts. DNA fragments –2012/–1597 (**A**1 and 2) and –171/+116 (**B**) of the rGnRH promoter were end-labeled on the coding strand and incubated with 240µg Gn10 or GT1-7 nuclear extract in the presence of increasing amounts of DNase I. Lane A represents 'A' Maxam and Gilbert chemical sequencing ladders of the same probe. BSA lanes represent effects of increasing DNase I digestion in the absence of neuronal protein. Bars between areas of nucleotide sequence identify protected regions with Gn10 (left) and GT1-7 (right) nuclear extracts.

neural-specific human GnRH enhancer to –535 and –479. Although gene transfer studies in Gn10 cells previously defined a functional ERE at –534/–521 in the proximal hGnRH promoter (10), no clear protection of this region was observed with Gn10 extracts as was documented with extracts from GT1-7 cells. The low abundance of estrogen receptor (ER) in Gn10 cells (10), the lack of optimal ligand concentration in the preparation of Gn10 extracts and the difficulty of demonstrating DNase I protection by steroid receptors secondary to their rapid on/off rate may explain these findings. The extended protected area produced with GT1-7 extracts most likely contains multiple nuclear proteins, however, none are ER, since our subline of these cells do not contain ER (7).

The intense protection of functionally significant *cis*-acting elements of both the rat and human promoters with GT1-7 in comparison to Gn10 nuclear extracts may reflect the different complement of active transcription factors that mediate the marked difference in the level of endogenous GnRH expression between the two cell lines. Although several protected areas were similar with the two neuronal extracts, unique footprints support the potential different complements of *trans*-acting proteins expressed in the migratory versus post-migratory GnRH neurons. Together, these studies support the hypothesis that neuronal proteins interact in a species-specific manner on the hGnRH and rGnRH promoter to ensure neural-specific expression.

Why there would be species-specific differences in the structural organization of the GnRH gene is unclear. No sequence or functional analysis of the distal mouse GnRH promoter is currently available to ask whether the differences we observed reflect a rat-specific or rodent-specific pattern of organization. Recent studies by Radovick and colleagues have shown the ability of the DNA sequences –3832/+5 and –1131/+5, but not –484/+5, of the hGnRH promoter to target luciferase expression to hypothalamic GnRH neurons in transgenic mice (20). Together with data from the functional assays and DNase I footprinting, these data suggest that sequences between –535 and –479 contribute significantly to the expression of hGnRH *in vivo* and *in vitro*. The characterization of the complement of nuclear proteins binding to this region will be the focus of future investigation.

The structural organization of other hypothalamic releasing hormone genes is currently under active investigation. The corticotrophin releasing factor (CRF) gene contains both proximal and distal transcription initiation sites with tissue-specific differential utilization resulting in multiple mRNAs of variable translational efficiency (21–23). The growth hormone releasing hormone (GHRH) gene has been shown to have two distinct promoter regions to direct hypothalamus- versus placenta-specific expression (24). Cross-species comparisons in functional assays of hypothalamic releasing factor promoters other than GnRH in neuronal cells, however, has not been possible due to the lack of available model systems. Future studies will be necessary to see if species-specific promoter organization exists within other hypothalamic releasing hormone genes.

In summary, these studies identify the locus of neuronal-specific expression of the hGnRH promoter and provide a foundation to fine map the differences in hGnRH and rGnRH promoter activity in neuronal tissues. Future studies are needed to elucidate the ramifications of species-specific differences in the structural organization of the hGnRH and rGnRH promoters.

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