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Cell-specific expression of the mouse gonadotropin-releasing hormone (GnRH) receptor gene is conferred by elements residing within 500 bp of proximal 5′ flanking region

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Gonadotropin-releasing hormone (GnRH) is a decapeptide produced by the hypothalamus. Upon binding to specific high-affinity receptors on gonadotrope cells of the anterior pituitary gland, GnRH stimulates the synthesis and secretion of LH. In light of the critical role of GnRH in reproduction much effort has been directed toward understanding the regulation of this hormone and its cognate receptor. The recent availability of genomic clones for the GnRH receptor has facilitated research to address the molecular mechanisms underlying regulation of GnRH receptor gene expression. We have expanded the analysis of the promoter for the mouse GnRH receptor gene and report that in addition to transcriptional start sites located within 100 bp of the translation start codon there is a more distal transcriptional start site approximately 200 bp 5′ of the initiation codon. The initiation of transcription from this more distal site was sufficient to confer cell-specific expression on luciferase. Further, transient expression assays of constructs containing progressive 5′ deletions in the GnRH receptor gene promoter reveal the presence of one or more *cis*-acting elements located between −500 and −400 (relative to ATG) necessary for transcriptional activity in the gonadotrope-derived αT3 cell line. Finally, αT3 but not COS-7 cell nuclear extract contained protein(s) that bind to at least two separate motifs contained within the −500 to −400 region. We suggest that activation of GnRH receptor gene expression in the αT3 cell line requires the binding of at least two transcriptional regulatory proteins to basal enhancer elements located within a 100 bp region between −500 to −400 relative to the translation start codon in the mouse GnRH receptor gene.

Keywords: GnRH receptor; gonadotropin-releasing hormone; gene; transcription; gene expression; promoter

Introduction

The primary amino acid sequence of gonadotropin-releasing hormone (GnRH) was first reported in 1971 (Matsuo *et al.*, 1971). In 1985, the cDNA encoding GnRH was first reported (Nikolics *et al.*, 1985). During the intervening years, a clear picture of the key role of this molecule in controlling reproductive function of mammals has emerged (Brinkley, 1981; Clayton & Catt, 1981; Desjardins, 1981; Clarke *et al.*, 1983). The pulsatile discharge of GnRH from hypothalamic neurosecretory cells is obligatory for synthesis and secretion of luteinizing hormone (LH) and, to a lesser extent, follicle-stimulating hormone (FSH) from the anterior pituitary gland (Clayton & Catt, 1981; Clarke *et al.*, 1983). In addition, expression of the genes encoding for both the common α and unique β subunits of the gonadotropins is regulated by GnRH (Hamernik *et al.*, 1986; Mason *et al.*, 1986; Hamernik & Nett, 1988; Gharib *et al.*, 1990).

In light of the critical role of GnRH in controlling reproductive function of mammals much effort has been devoted toward understanding the physiological consequences of regulation of this hormone and its cognate receptor. As is the case for changes in the secretion of GnRH by the hypothalamus, changes in the number of pituitary GnRH receptors have been implicated as an important mechanism underlying the regulation of gonadotropin secretion (Savoy-Moore *et al.*, 1980; Clayton & Catt, 1981; Crowder & Nett, 1984; Nett *et al.*, 1987). Thus, changes in pituitary content and secretion of LH may not only be associated with changes in GnRH availability but also changes in the number of GnRH receptors and, consequently, the responsiveness of the pituitary to a given dose of GnRH (Wise *et al.*, 1984). Since the initial isolation of a cDNA encoding the GnRH receptor (Tsutsumi *et al.*, 1992), several groups have demonstrated changes in the number of GnRH receptors associated with different physiological states or hormonal treatments correlated with concomitant fluxes in steady state concentrations of mRNA for the GnRH receptor (Sealfon *et al.*, 1990; Bauer-Dantoin *et al.*, 1993; Brooks *et al.*, 1993; Kaiser *et al.*, 1993). Thus, it is likely that regulation of expression of the GnRH receptor gene is an important determinant of the concentration of GnRH receptors in the anterior pituitary gland.

To understand the molecular mechanisms underlying regulation of GnRH receptor gene expression we (Campion *et al.*, 1994) and others (Albarracin *et al.*, 1994; Zhou & Sealfon, 1994) have isolated the gene encoding the murine GnRH receptor. This gene spans minimally 25 kbp (kilo-base pairs) and is compised of three exons and two introns. Based on primer extension analysis, several closely associated transcriptional initiation sites have been identified within 100 bp of the translation start codon (Albarracin *et al.*, 1994; Campion *et al.*, 1994). Further, transient expression assays in gonadotrope-derived and non-gonadotrope cell lines indicate the presence of gonadotrope-specific promoter activity located within approximately 1200 bp of proximal 5′ flanking region (Albarracin *et al.*, 1994; Campion *et al.*, 1994). Herein, we have expanded these analyses and suggest that basal activity of the mouse GnRH receptor gene promoter in the gonadotrope-derived αT3 cell line requires the binding of at least two proteins to enhancer elements located within a 100 bp region between −500 and −400 relative to the initiation codon. Further, the initiation of transcription from at least two start sites is necessary for full transcriptional activity of the mouse GnRH receptor gene promoter.

Results

Isolation of the proximal 5′ flanking region of the mouse GnRH receptor gene

A mouse (Balb/c) genomic library constructed in EMBL-3SP6/T7 was screened by phage plaque hybridization using

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radiolabelled mouse GnRH receptor cDNA. Six recombinant phage clones containing GnRH receptor sequences were plaque purified and further characterized by restriction mapping and nucleotide sequencing. Comparison of the nucleotide sequence of the gene with the previously published GnRH receptor cDNA sequence allowed the assignment of intron-exon junctions. Consistent with Zhou & Sealfon (1994), the coding region of the mouse GnRH receptor gene is contained with three exons (all nucleotide sequences are in GenBank accession numbers L33778, L33788 and L33789) spanning approximately 25 kbp.

The 5′ flanking region of the mouse GnRH receptor gene was isolated from a single lambda clone that contained the first exon and approximately 14 kbp of intron 1. Approximately 2 kbp of proximal 5′ flanking region was sequenced and utilized for the promoter studies. With the exception of minor discrepancies, the sequence of the 5′ flanking region (Figure 1) is in agreement with Albarracin *et al.* (1994) who reported approximately 1200 bp of 5′ flanking region from the mouse GnRH receptor gene.

Identification of transcriptional start sites in the mouse GnRH receptor gene

To map the 5′ end of the mouse GnRH receptor gene, primer extension analysis was performed using αT3 mRNA with an exon 1 specific primer (Figure 2; location of the primer is indicated in Figure 1). The 5′ terminus was mapped to an A residue located 62 bp from the initiation codon. An additional primer extended product terminated at a G residue located approximately 90 bp from the ATG initiation codon. Our assignment of the transcriptional start sites by primer extension is in close agreement with those recently reported (Albarracin *et al.*, 1994). Neither 5′ terminus is associated with a consensus TATA element or CCAAT sequence.

To confirm the assignment of the 5′ terminus of the mRNA, S1 nuclease protection analysis was performed using αT3 mRNA (Figure 3) and uniformly labelled probes representing either approximately 1900, 500, 330 or 190 bp of 5′ flanking region. Each S1 probe was generated by digesting the proximal 5′ flanking region with either XhoI (supplied by the cloning vector), NsiI, TaqI or HaeIII followed by asymmetric PCR using an exon 1 specific primer. The location of the primer is indicated in Figure 1 and was directed against the nucleotides encoding the first seven amino acids of the GnRH receptor. As expected, αT3 mRNA protected each probe from S1 digestion in a region corresponding to the 5′ terminus as mapped by primer extension (Figure 3). In addi-

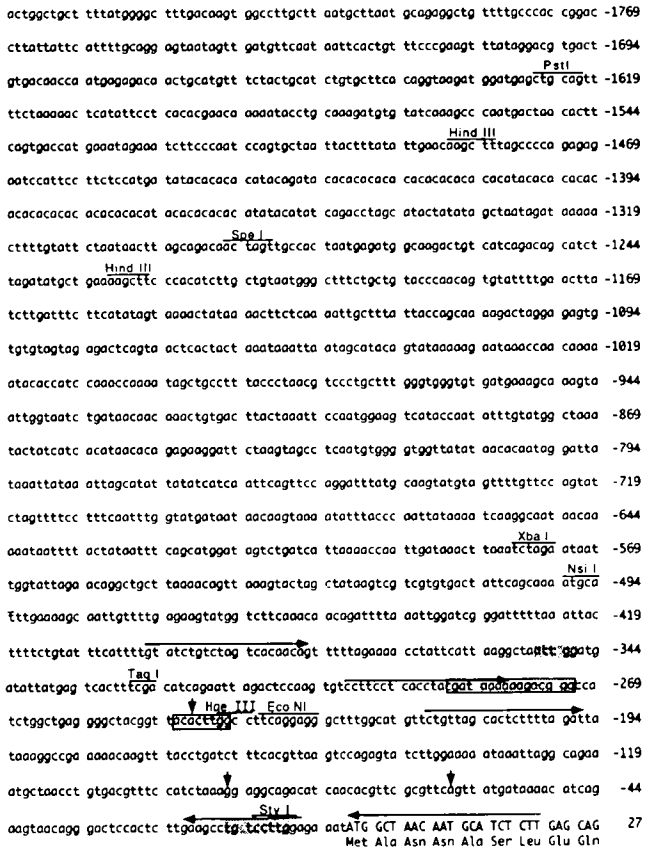

Figure 1 Nucleotide sequence of the mouse GnRH receptor gene 5′ flanking region. Transcriptional start sites are indicated by arrowheads. Restriction enzyme sites used in vector construction and S1 nuclease analysis are indicated. The arrow-line overlying the initial coding sequence indicates the position of the anti-sense primer used in primer extension analysis. The position of sense and anti-sense primers used in vector construction are also indicated. Shaded, boxed sequences are potential 5′ CAP site and TATA homology. Shaded sequences represent potential regulatory elements including the gonadotrope-specific element (GSE) immediately upstream of the translation start codon

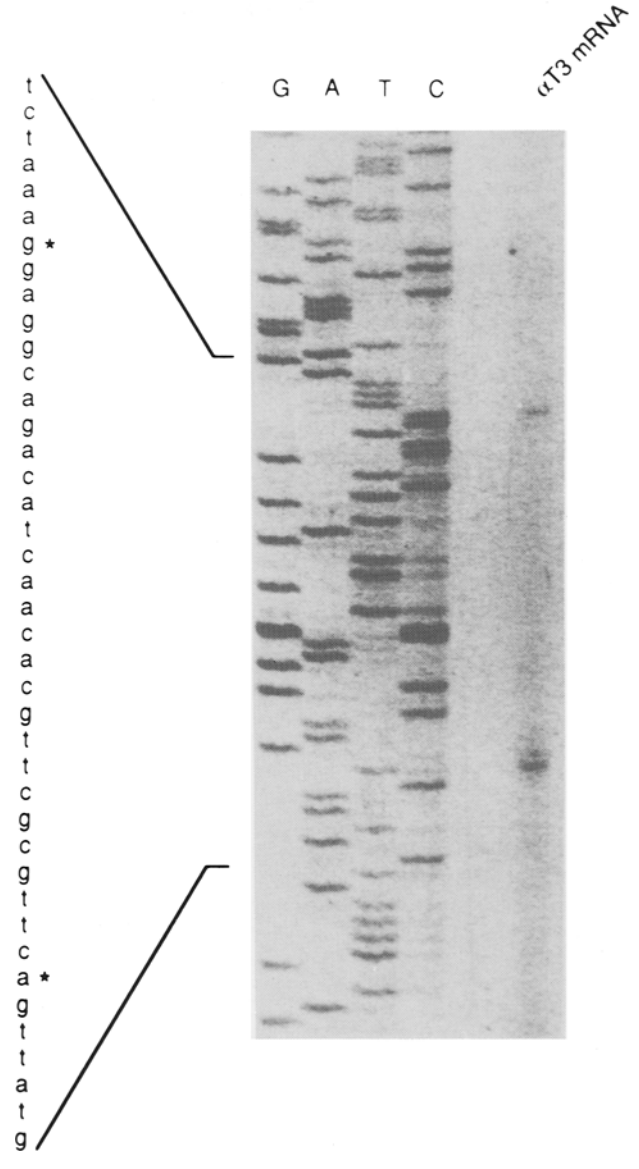

Figure 2 Primer extension analysis of αT3 mRNA. A 21 nucleotide anti-sense primer (see Figure 1) was annealed to 5 μg of αT3 mRNA. After reverse transcription, samples were digested with RNase A and size fractionated by electrophoresis through 8% polyacrylamide – 8 M urea gels. Sequencing ladder was generated by priming a genomic DNA fragment with the same 21 nucleotide anti-sense primer. The sequence on the left is complemented to represent the actual sequence of the sense strand

tion, the complex banding pattern is consistent with the presence of multiple, closely associated transcriptional start sites. Surprisingly, however, the S1 analysis also revealed a protected fragment approximately 200 bp 5′ of the initiation codon suggesting the presence of an additional transcriptional start site more distal to those mapped by primer extension. Each probe extending upstream of −200 protected a fragment of identical size; thus, it seems unlikely that the S1 analysis simply revealed the presence of an additional exon in the mouse GnRH receptor gene. Further, the full length HaeIII probe was fully protected from S1 nuclease digestion in the presence of αT3 mRNA. This is consistent with the assignment of the start site immediately upstream of the HaeIII recognition sequence and placing it approximately 200 bp 5′ of the ATG start codon.

Unlike the proximal transcriptional start sites, the more distal start site we have identified may be TATA associated. Although S1 analysis precludes nucleotide assignment of the 5′ terminus, the start site appears to reside within a consensus CAP sequence and is approximately 30 bp downstream of an element displaying TATA homology (Figure 1).

Transcriptional activity of the mouse GnRH receptor promoter

Upon identification of both proximal and distal transcriptional start sites within the mouse GnRH receptor gene, we next sought to determine if both start sites are necessary for promoter activity. This was tested by transient expression assays in the gonadotrope-derived αT3 cell line (Windle *et al.*, 1990). Expression vectors containing either both the proximal and distal transcriptional start sites or only the more distal transcriptional start site were constructed (Figure 4) and tested for their ability to promote luciferase expression in αT3 cells. Deletion of sequences residing between −1900 and −600 (all numbering is relative to the translation start codon) resulted in an approximately 50% attenuation in luciferase expression (Figure 5). In contrast, deletion of approximately 100 bp between −600 and −500 (XbaI to NsiI) consistently resulted in a 2–3-fold increase in luciferase expression. Thus, we suggest the presence of one or more enhancer elements residing between −1900 and −600 and transcriptional repressor activity located between −600 and −500. Furthermore, while deletion of the proximal promoter between EcoNI (−200) and the translation initiation codon attenuated luciferase expression, the promoter remained functional. In contrast, deletion to −220 completely abrogated promoter activity (data not shown). Thus, we conclude that both proximal and distal transcriptional start sites appear to contribute to full transcriptional activity of the GnRH receptor gene promoter.

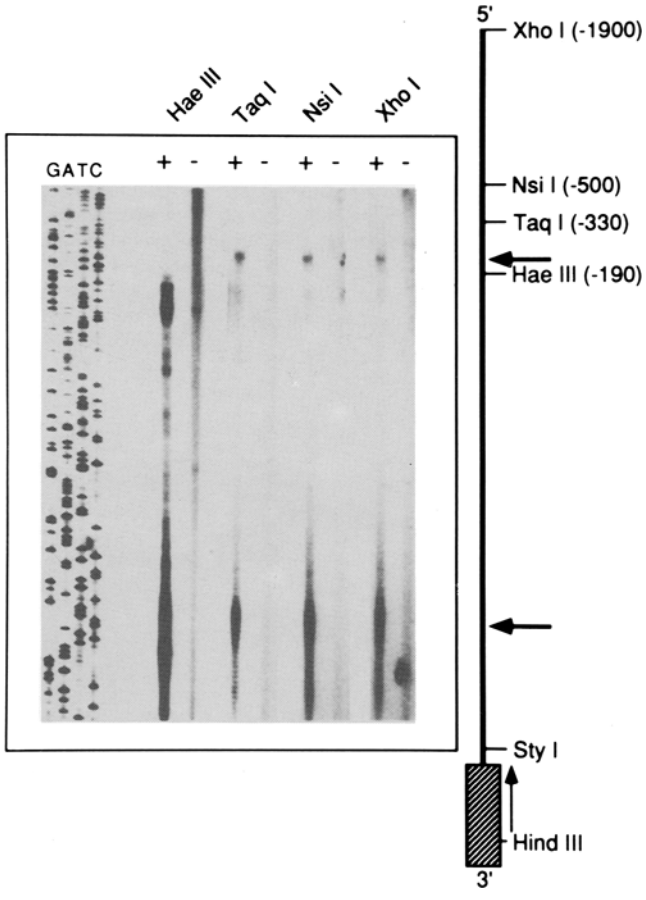

Figure 3 S1 Nuclease analysis of αT3 mRNA. Single-stranded DNA probes were prepared by assymetric PCR using restriction fragments from the mouse GnRH receptor gene. The approximate positions of the restriction enzyme sites and the location of the PCR primer are indicated. Each probe was annealed to 10 μg of αT3 mRNA for 12–16 h and then digested with S1 nuclease. Products of S1 digestion both in the presence (+) and absence (−) of mRNA were resolved by electrophoresis in 6% polyacrylamide-6 M urea gels. The sequencing ladder was generated using the same primer as used in assymetric PCR

Contained within the putative 5′ UTR of the mouse GnRH receptor gene is an 8 bp sequence homologous to the 'gonadotrope-specific element' (GSE) previously described as important for gonadotrope specific expression of the glycoprotein hormone α subunit gene (Horn *et al.*, 1992). Deletion of this element, in combination with the proximal transcriptional start sites, in the −500/−200 construct attenuated activity of the promoter in αT3 cells by approximately 60%. At issue, however, is whether this element plays any role in modulating cell specific activity of the GnRH receptor gene promoter as with the glycoprotein hormone α subunit gene (Horn *et al.*, 1992). Thus, pMGR-1200/-200LUC, pMGR-500/-200LUC and pMGR-500LUC (Figure 4) were examined for cell specific activity by transient expression in gonadotrope-derived (αT3 cells) and non-gonadotrope cell lines (HeLa, GH3, C127 and COS-7) (Figure 6).

Expression of all three GnRH receptor gene promoter vectors was significantly higher in αT3 cells than in any of the other four cell lines examined (Figure 6). Expression of the test vectors was not different from the promoterless luciferase construct in either HeLa, GH3, C127 or COS-7 cells. The absence of expression of the test vectors in the heterologous cell lines was not due to poor transfection efficiency as expression of a co-transfected positive control vector (pSV-LacZ; the SV-40 promoter and enhancer fused 5′ to the coding sequence of β-galactosidase) was equal to, or greater, in the non-gonadotrope cell lines as compared to αT3 cells. Thus, it would not appear that the GSE-homolog located immediately upstream of the ATG start codon is necessary for cell specific activity of the GnRH receptor gene promoter. Further, it would appear that the element(s) conferring this property must reside between −500 and −200 bp relative to the translational start site in the mouse GnRH receptor gene promoter.

To further refine the boundaries of the essential basal promoter elements, we next used PCR amplication to construct a series of 100 bp deletions in the proximal promoter of the mouse GnRH receptor gene. The resulting vectors (pMGR-400LUC, pMGR-300LUC and pMGR-190LUC) (Figure 4) were examined for transcriptional activity by transient expression in αT3 cells. As in the previous experiments, expression of luciferase was significantly higher for pMGR-500LUC than for the promoterless control (Figure 7). However, further deletion of 100 bp residing immediately downstream of the NsiI site at −500 led to an 88% attenuation in luciferase expression (Figure 7). Thus, we suggest the presence of one or more critical, basal-level enhancer element(s) residing between −500 and −400 in the mouse GnRH receptor gene promoter. While not yet directly tested it will be of interest to determine if the basal level enhancer(s)

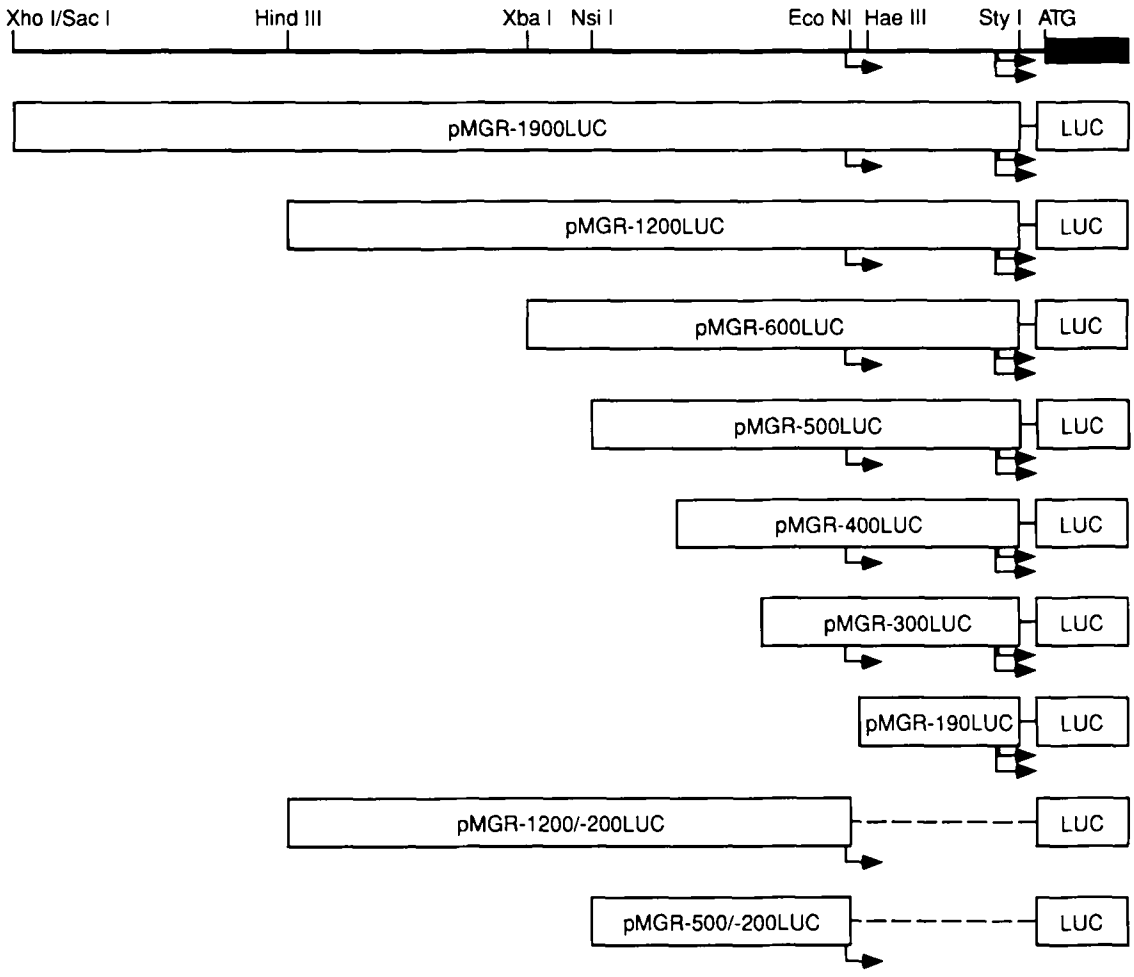

Figure 4 Construction of luciferase expression vectors. Restriction enzyme sites denoted in the 5′ flanking region of the mouse GnRH receptor gene (top) were used to construct progressive 5′ or 3′ deletions. Plasmids pMGR-400, -300 and -190LUC were constructed by PCR amplifications as described in Materials and methods. Each 5′ flanking region was fused to the gene encoding luciferase. Arrows denote the approximate location of transcriptional start sites. Vectors containing both transcriptional start sites terminated at the StyI site immediately upstream of the start site of translation whereas pMGR-1200/-200LUC and pMGR-500/-200LUC terminated at EcoNI approximately 200 bp upstream of the translational start site

located in this region are sufficient for cell-specific activation of the GnRH receptor gene.

Analysis of DNA-binding protein activity in αT3 cell nuclear extracts

Based on the functional data above suggesting the presence of critical enhancer element(s) between −500 and −400, we sought to determine if this same region of DNA could bind one or more proteins specific to αT3 cells. Overlapping oligodeoxyribonucleotides spanning the sequence between −500 and −400 served as either radioactive probes or non-radioactive competitors in gel-mobility shift assays using either αT3 or COS-7 nuclear extracts (Figure 8). Probe 2 (−449 to −404) failed to shift any protein in either αT3 or COS-7 nuclear extracts (data not shown). In contrast, both probe 1 (−484 to −439) and probe 3 (−426 to −365) exhibited binding to protein(s) in αT3 cell extract (Figure 8). Further, both binding activities appear sequence specific. Binding to probe 1 was competed with 100-fold molar excess of unlabeled homologous competitor but not heterologous competitor (probe 3). Similarly, competition for binding to probe 3 was evident only for the homologous competitor (Figure 8). Finally, neither of the sequence specific binding activities were evident in the COS-7 nuclear extract thus raising the possibility that one or both binding activities are important in mediating cell specific expression of the GnRH receptor gene promoter.

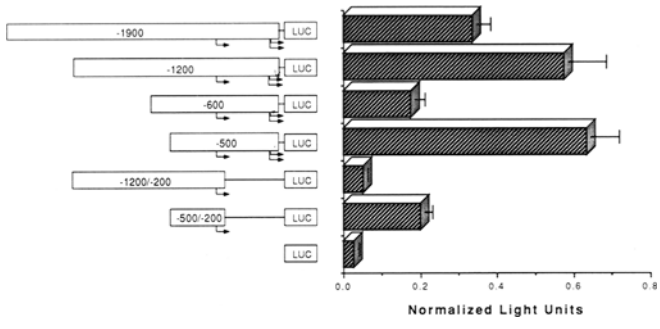

Figure 5 Transient expression assay in αT3 cells. 1.4 μg of the indicated expression vectors (numbering is relative to the translation start site) were co-transfected with 0.5 μg of pSV-LacZ by LipofectAMINE into triplicate wells. At 24 h post-transfection, cells were harvested and cellular lysates assayed for luciferase and β-galactosidase activity. Values are means ± SD of luciferase activity after adjusting for β-galactosidase expression

Discussion

At maturity, the pituitary gland consists of at least five different cell types that secrete six different hormones. Thus, the pituitary gland is an excellent model to study the ontogeny of cell-type specific activation of gene expression. In this

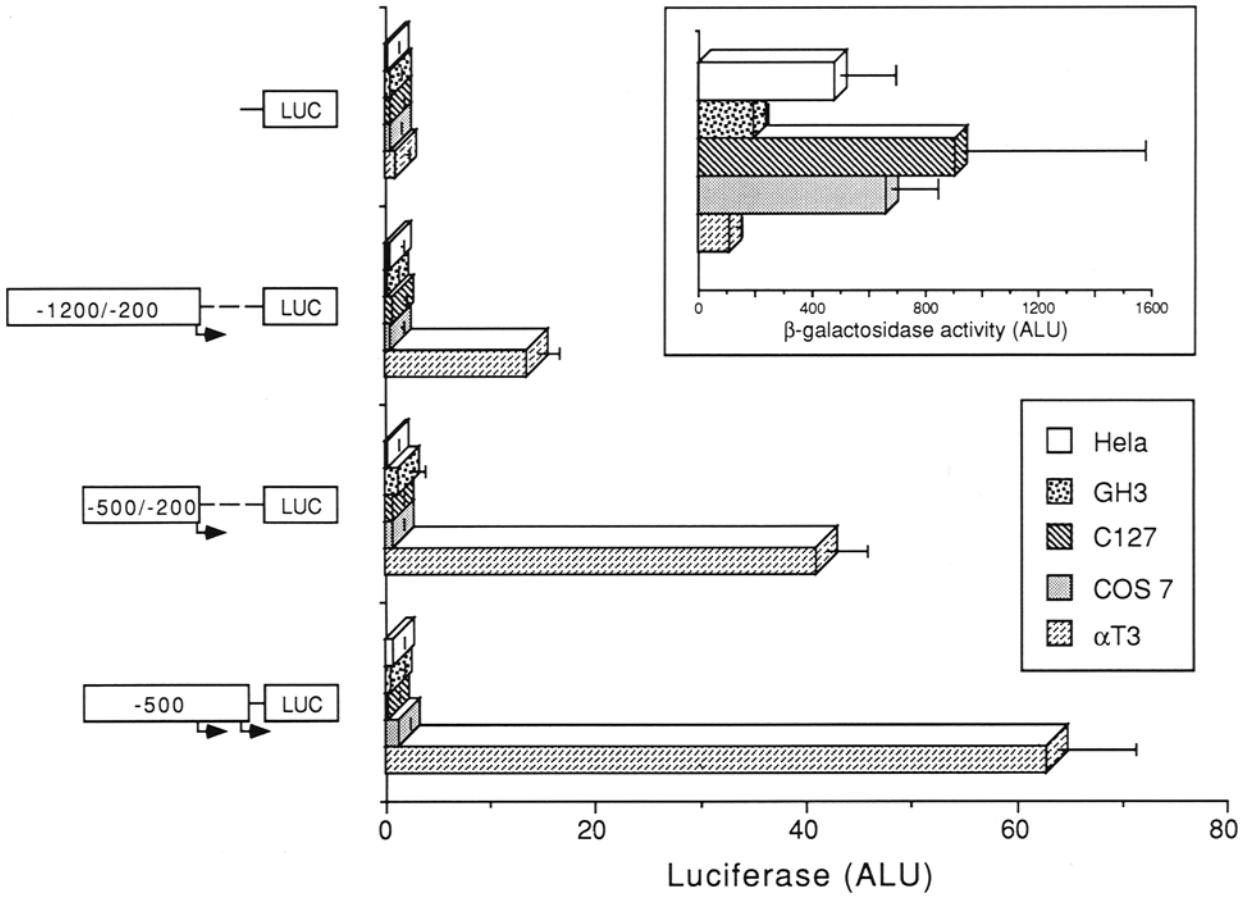

Figure 6 Transient expression assay is gonadotrope-derived (αT3) and non-gonadotrope derived (GH3, C127, COS7, HeLa) cell lines. 1.4 μg of the indicated expression vectors and 0.5 μg of pSV-LacZ were co-transfected by LipofectAMINE into triplicate wells of each cell line. At 24 h post-transfection, cells were harvested and cellular lysates assayed for luciferase and β-galactosidase activity. Values are means ± SD of luciferase activity expressed as arbitrary light units (ALU)/mg protein. Due to differences in activity of the SV40 promoter among the difference the different cell lines (see inset Figure), luciferase values were not adjusted for β-galactosidase activity. Values in inset figure are means ± SD of β-galactosidase activity (ALU/mg protein)

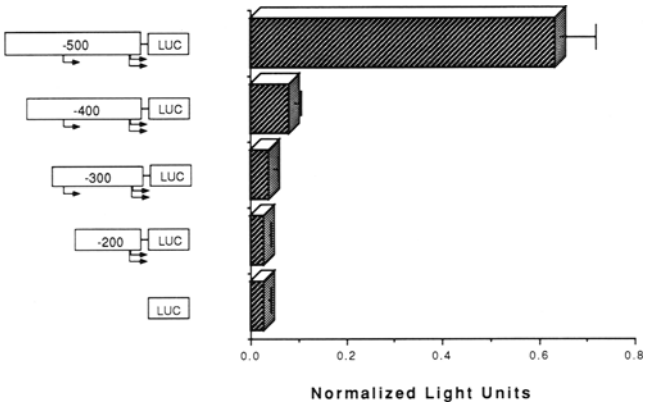

Figure 7 Deletion analysis of the proximal 5′ flanking region of the mouse GnRH receptor gene. 1.4 μg of the indicated expression vectors and 0.5 μg of pSV-LacZ were transfected by LipofectAMINE into triplicate wells of αT3 cells. At 24 h post-transfection, cells were harvested and cellular lysates were assayed for luciferase and β-galactosidase activity. Values are means ± SD of luciferase activity after adjusting for β-galactosidase expression

regard, much has been learned as to the developmental regulation of growth hormone and prolactin gene expression (Theill & Karin, 1993). Unfortunately, our understanding of the phenotypic development of gonadotrope cells and the regulation of their primary gene products, the glycoprotein hormone α subunit, the unique LH and FSH β subunits, and the GnRH receptor, is much less developed. In regard to the β subunit genes and the GnRH receptor, lack of progress in this arena is due to several factors. First, is the absence of a permanent cell line for expression of the β subunit genes. Second, until recently, there were no genomic clones for the GnRH receptor. Thus, to begin to address the molecular mechanisms underlying gonadotrope-specific expression of the GnRH receptor gene, we have cloned this gene from the mouse. In agreement with Zhou & Sealfon (1994), this gene is comprised of three exons and two introns and spans approximately 25 kbp. Several closely associated transcriptional start sites are located within 100 bp of the translation start codon (Campion *et al.*, 1994; Albarracin *et al.*, 1994). Further, approximately 1900 bp of proximal 5′ flanking region from the mouse GnRH receptor gene has been shown to be sufficient to restrict expression of a heterologous reporter gene to a gonadotrope derived cell line. Herein, we have expanded the analysis of the mouse GnRH receptor gene promoter and report several novel results.

First, the proximal promoter for the mouse GnRH receptor gene may be more complicated than we initially thought. We have used a combination of primer extension and S1 analyses to identify at least two transcriptional start sites. The first resides approximately 70 bp 5′ of the translation start codon and actually consists of several clustered initiation sites, none of which appear to be associated with a consensus TATA motif. The second start site, identified by S1 nuclease analysis, resides approximately 200 bp upstream of the start codon and may be TATA associated. Although

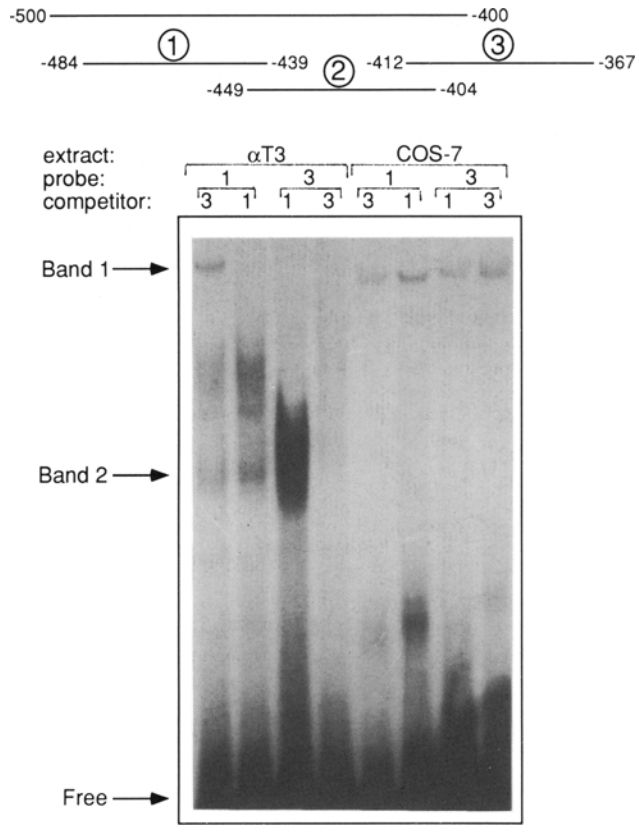

Figure 8 Gel-mobility shift assay using αT3 and COS-7 nuclear extract. Nuclear proteins precipitating at 75% saturation with $(NH_4)_2SO_4$ were incubated with radiolabelled probes indicated at the top of the figure. Competitions for binding were carried out with unlabelled DNA at approximately 100-fold molar excess of labelled probe. Reactions were analysed by electrophoresis through polyacrylamide gels. Band 1 indicates binding activity specific to probe 1 (−484 to −439) and band 2 indicates binding activity specific to probe 3 (−412 to −367)

was not reported by Albarracin *et al.* (1994), it is likely that this discrepancy simply reflects the amount of RNA used in RNase protection in their study (20 μg of total RNA) and S1 analysis in our study (10 μg poly(A)$^+$ RNA). Finally, while initiation of transcription from both the proximal and distal start sites appear to contribute to full transcriptional activity of the mouse GnRH receptor gene promoter, it is important to note that the fidelity of cell-specific expression is retained in expression vectors containing only the more distal transcriptional start site.

The physiological significance of both TATA associated and TATA-less initiation sites in the same gene is not known, however, a similar architecture has been reported for a number of other eukaryotic genes including, most recently, the human GnRH receptor gene promoter (Fan *et al.*, 1995). Ultimately, it would be of interest to determine if start site selection is itself a regulated step in transcription of the GnRH receptor gene. Finally, it is interesting to note that multiple species of GnRH receptor mRNA have been identified in several species (Tsutsumi *et al.*, 1992; Bauer-Dantoin *et al.*, 1993; Brooks *et al.*, 1993; Turzillo *et al.*, 1994). While much of this variation may be due to alternative splicing or length of 3′ untranslated region (Tsutsumi *et al.*, 1992; Zhou & Sealfon, 1994; Fan *et al.*, 1995), start site selection would also account for some heterogeneity in transcript length.

At least one of the predominant tissue sites of expression of the GnRH receptor gene, as with the glycoprotein hormone α subunit gene and the unique LH and FSH β subunit genes, is in gonadotrope cells of the anterior pituitary gland. Thus, a fundamental question in reproductive endocrinology is what are the molecular codes specifying gonadotrope-specific expression. As pointed out above, the lack of *in vitro* homologous expression systems has hampered efforts toward this goal in regard to the LH and FSH β subunit genes. However, the mouse αT3 cell line (Windle *et al.*, 1990), which expresses both α subunit and GnRH receptors has enabled analysis of multiple DNA regulatory elements involved in gonadotrope-specific expression of the α subunit gene (Horn *et al.*, 1992; Schoderbek *et al.*, 1993). Mellon and co-workers used differential footprint and deletion analyses to identify a putative gonadotrope-specific element in the human glycoprotein hormone α subunit gene (Horn *et al.*, 1992). A core motif in the critical element (TGACCTTG) is conserved in the α subunit genes of mice, cattle, rats and horses and appears to bind the nuclear orphan receptor termed steroidogenic factor-1 (SF-1) (Barnhart & Mellon, 1995). Although a sequence homologous to the SF-1 binding site is found in the 5′ UTR of the mouse GnRH receptor gene (TGTCCTTG), our data do not support the notion that this GSE homolog has an important role in mediating cell-specific expression of this gene. Rather, our data are most consistent with localization of the critical cell-specific regulatory elements residing between −500 and −200 relative to the start site of translation. Finally, in contrast to Abarracin *et al.* (1994) we did not observe any significant enhancement of luciferase expression in GH3 cells; however, this difference may be simply methodological, i.e. different expression vectors, different quantities of DNA transfected, different transfection procedures, and, perhaps, some heterogeneity in GH3 cells themselves.

As to more precise localization of the putative regulatory elements, the 5′ deletion analyses are revealing. Removal of approximately 100 bp between −500 and −400 severely attenuates expression and suggests that one or more critical basal level enhancers are contained within this region of the GnRH receptor gene promoter. In fact, based on the gel-mobility shift analysis, we have shown the presence of at least two binding sites in this region. The first is located between −484 and −439 and contains a CCAAT homology (GCAAT) at the immediate 5′ end; however, whether this sequence represents the actual binding site remains to be determined. Otherwise there are no obvious homologies to previously characterized cis-acting elements in this 46 bp region. Similarly, the second binding site, located between −412 and −367, demonstrates no homology with consensus binding sites for any known transcription factors. That the protein or proteins binding to both −484/−439 and −412/−367 regions was identifiable in αT3 cell nuclear extract but not in COS-7 nuclear extract raises the possibility that one or both of these binding activities may be unique to cell lines of gonadotrope origin. Finally, although putative cell-specific enhancer activity may be contained within 500 bp of proximal 5′ flanking region, additional regulatory information residing in the distal promoter may be important in modulating basal transcription of the mouse GnRH receptor gene. In particular, the decrease in luciferase expression upon deletion from −1200 to −600 suggests the presence of one or more enhancer elements located in this 600 bp region. Further, the increased activity of the −500 promoter region as compared to −600 may reflect the removal of a repressor element(s) and raises the possibility of transcriptional derepression as an important mechanism for enhancing basal activity of the GnRH receptor gene promoter.

In summary, we have expanded the preliminary characterization of the proximal promoter of the mouse GnRH receptor gene. Our data suggest the presence of at least two sets of transcriptional initiation sites contained within 200 bp of the translation start codon. Additionally, the element or elements sufficient for cell-specific expression are contained within 300 bp residing between −500 and −200 bp relative to the translation start site. Finally, we suggest the presence of at least two regulatory elements residing between −500

and −400 that are critical for activity of the GnRH receptor gene promoter in αT3 cells. Ultimately, precise definition of the DNA contact points will provide immediate targets for mutagenesis to assess the relative role of each of these potential regulatory elements in mediating the transcriptional activity of the GnRH receptor gene promoter.

Materials and methods

Isolation of the mouse GnRH receptor gene promoter

A mouse (Balb/C) genomic library obtained from CLONTECH (Palo Alto, CA) was screened for GnRH receptor sequences by phage plaque hybridization (Benton & Davis, 1977). Briefly, host E. Coli were infected with limiting dilutions of lambda phage. Plaques were allowed to develop for 12–16 h at 37°C and then absorbed onto nylon membranes. Filters were soaked in 0.5 M NaOH, 1.5 M NaCl for 3–5 min, rinsed in 3 × SSC and allowed to air dry at room temperature. Following u.v. crosslinking, the filters were prehybridized at 42°C for 1–5 h in 50% formamide, 100 μg/ml herring sperm DNA, 0.1% SDS, 5 × SSPE, 5 × Denhardts. Probe was added at 1×10^6 c.p.m./ml and hybridization was allowed to proceed at 42°C for 12–16 h. Filters were washed and exposed to X-ray film overnight. Phage were eluted from agar plugs in 0.5 ml of SM (50 mM Tris pH 7.4, 10 mM $MgCl_2$, 0.1% gelatin) and re-screened as above until all plaques tested positive by hybridization. The probes used were full-length murine cDNA labelled with [α^{32}P]-dCTP using the random primer method, or a 1.8 kb HindIII fragment consisting of sequences residing between the HindIII site located in exon 1 and the HindIII site located at approximately −1200 bp relative to the ATG initiation codon.

Sequence analysis

DNA fragments containing mouse GnRH receptor sequence were sub-cloned into plasmid Bluescript (Stratagene, La Jolla CA). Double-stranded plasmid DNA was alkali denatured and annealed to primers complementary to either the T7 or T3 promoter sequences adjacent to the 5′ or 3′ ends of the multiple cloning site, or gene specific primers. DNA sequences were determined using the chain termination method (Sequenase-United States Biochemical, Cleveland OH) and ^{35}S-dATP. Fragments were size fractionated by electrophoresis in 5–8% polyacrylamide-8M urea gels, fixed in 5% acetic acid, 5% methanol, dried and exposed to X-ray film. Nucleotide sequences were recorded and analysed using the Gene-Works software (Intelligenetics). Potential promoter elements including TATA homologies and CAP sites were identified using the EUKPROM algorithm (PC-Gene, Intelli-Genetics, Mountain View CA).

Primer extension and S1 nuclease analysis

The 5′ termini of the GnRH receptor mRNA were mapped by primer extension analysis and S1 nuclease analysis (Virgin *et al.*, 1985; Sherman *et al.*, 1992). For primer extension, a 21 nucleotide anti-sense primer (5′-AAGAGATGCATTGTTAGCCAT-3′) was radiolabelled at the 5′ terminus using [γ-^{32}P]-ATP and T4 polynucleotide kinase and purified after electrophoresis in 15% polyacrylamide gels. The radiolabelled primer was annealed at 37°C in 1 × reverse transcription buffer (Sherman *et al.*, 1992) for 3 h with 5 μg of αT3 poly(A)$^+$ RNA prepared according to Badley *et al.* (1988). After annealing, extension was carried out using MMLV reverse transcriptase. Single stranded RNA was digested by treatment with RNAse A at 37°C for 30 min. The reaction was extracted with Tris (pH 7.5) saturated phenol: chloroform:isoamyl alcohol (24:24:1), precipitated with ethanol and resuspended in 50% formamide gel-loading buffer. Samples were heated at 95°C for 5 min and size fractionated by electrophoresis through 8% polyacrylamide-8M urea gels followed by autoradiography. The same primer used in primer extension was also used to generate a sequencing ladder from a genomic clone containing exon 1 and associated 5′ flanking region.

The S1 nuclease protection analysis was conducted using the SNP assay from Ambion (Austin TX). Briefly, single stranded probes were generated by assymetric PCR using the identical primer as for primer extension. The template was prepared by linearization of a plasmid containing 1900 bp of proximal 5′ flanking region with either XhoI (supplied by the cloning vector), NsiI, TaqI or HaeIII. Approximately 20 fmol of linearized template was mixed with 25 pmol of primer, 1 × Taq polymerase buffer (Perkin-Elmer Cetus, Norwalk CT), 125 μM each of dATP, dTTP, dGTP, 15 μM of [α^{32}P]-dCTP (3000 Ci/mmol) and 2.5 U of Taq polymerase (Perkin-Elmer Cetus). The mixture was subjected to 30 cycles of amplification at 15 s at 94°C, 15 s at 45°C and 2 min at 72°C. The PCR product was precipiated with 2.5 M ammonium acetate and two volumes of ethanol, allowed to air dry and resuspended in diethylpyrocarbonate treated, sterile water. Approximately 1×10^6 c.p.m. of each probe was lyophilized with 10 μg of poly(A)$^+$ RNA isolated from αT3 cells, resuspended in hybridization buffer (300 mM sodium acetate pH 6.4, 1 mM EDTA, 100 mM sodium citrate pH 6.4, 80% deionized formamide) and allowed to anneal for 12–16 h at 42°C. The DNA-RNA solution was exposed to 50 U of S1 nuclease for 30 min at 37°C. Formamide-EDTA loading buffer was added directly to the samples which were then heated at 95°C for 5 min. Products of S1 digestion were resolved by electrophoresis in 6% polyacrylamide – 7 M urea gels. Also included on the gel was a sequencing ladder generated using the same primer as was used for the assymetric PCR reactions. Gels were dried and exposed to Kodak X-AR film for 3–5 days at −70°C.

Vector construction and transfections

Plasmid pMGR-1900LUC was constructed by isolating a 1900 bp XhoI/StyI fragment from a mouse genomic clone containing exon 1 and associated 5′ flanking region. The XhoI/StyI fragment was blunt-ended using T4 DNA polymerase and ligated into the SmaI site of pGL-2-basic (Promega, Madison, WI) yielding pMGR-1900LUC. The same 1900 bp fragment was also ligated into the SmaI site of pBSK-(Stratagene) yielding pBSK-1900. Plasmid pMGR-1200LUC was made by isolating a 1200 bp HindIII/StyI fragment, blunt ending with T4 DNA polymerase and ligating into SmaI site of pGL-2-basic. Plasmid pMGR-600LUC was made by ligation of a 600 bp fragment from XbaI digestion of pBSK-1900 into the NheI site of pGL2-basic. Plasmid pMGR-600LUC was further digested with SacI and NsiI to release an 100 bp fragment. The remaining vector was blunt-ended with T4 DNA polymerase and re-ligated yielding pMGR-500LUC. Plasmids pMGR-1200/-200LUC and pMGR-500/-200LUC were constructed by complete digestion of either pMGR-1200LUC or pMGR-500LUC with XhoI followed by partial digestion with EcoNI, gel-isolation of the appropriate sized fragment, blunt-ending with T4 DNA polymerase and re-ligation. Vector and promoter identity was confirmed by sequence analysis using primers directed against the 5′ end of luciferase coding sequence and gene-specific primers. Plasmid pMGR-400LUC, pMGR-300LUC and pMGR-190LUC were constructed by PCR amplification of pMGR-1900LUC using an anti-sense primer directed against the 5′UTR of the mouse GnRH receptor gene and sense primers directed against the appropriate region in the 5′ flanking region. The sequences of the sense −400, −300 and −190 primers were: 5′-GTATCTGTCTAGTCACAACAG-3′, 5′-TCCTTCCTCACCTACGATAAA-3′, and 5′-CTGTTAGCACTCTTTTAGAT-3′, respectively. The sequence of the anti-sense primer was 5′-TCCAAGGACAGGCTTCCTCGAG-3′. The correct size of

the resulting PCR products was confirmed by gel electrophoresis and the products were sub-cloned into pCR-II (Invitrogen, San Diego CA). The insert was isolated from pCR-II by digestion with KpnI and XhoI (the XhoI site was constructed as part of the anti-sense primer) and ligated into pGL2-basic digested with KpnI and XhoI. Promoter identity was confirmed by sequencing.

Transfections were carried out using the LipofectAMINE procedure as recommended (GibcoBRL Life Technologies, Gaithersburg MD). Briefly, approximately 6×10^5 cells were plated in 6-well tissue culture plates the day prior to transfection. On the day of transfection, 1.4 μg of the test vector and 0.5 μg of pSV-LacZ were combined with 5 μl of lipofectAMINE reagent in 200 μl of media (high glucose DMEM) without serum or antibiotics. LipofectAMINE and DNA were incubated for 30 min at room temperature, diluted to 1.0 ml with serum-free media and applied to the cells. Incubation of cells with transfection media was continued for approximately 16 h at 37°C. One ml of media containing 10% fetal bovine serum and 10% horse serum was added. After 5–6 h, media was aspirated, cells washed twice with phosphate buffered saline (pH 7.4) and lysed in the wells by addition of 200 μl of lysis buffer (0.25 M glycylglycine pH 7.8, 1 mM dithiothreitol, 15 mM $MgSO_4$, and 1.0% Triton X-100). Cellular lysates were immediately assayed for luciferase activity by addition of 20 μl of lysate to 100 μl of luciferin substrate (Promega) and luminescence was measured using a Turner model TD-20E luminometer. β-galactosidase activity was determined for 50 μl of lysate using the luminescent assay system and substrate from Clontech (Palo Alto CA). Luciferase activity was adjusted for transfection efficiency by dividing the luciferase value by the β-galactosidase value. Within each transfection, each vector was transfected in triplicate wells and all transfections were repeated at least three times using different plasmid preparations. Transfections data were analysed by one-way analysis of variance with vector as the independent variable. If the F-test was significant ($\alpha = 0.05$) then means within a transfection were separated using Duncan's new multiple range test.

Nuclear extracts and gel-mobility shift assays

Nuclear extracts were prepared from αT3 and COS-7 cells according to Dignam *et al.* (1983). Where indicated, extracts were brought to 40% or 75% saturation with $(NH_4)_2SO_4$ and pellets were resuspended in Dignam buffer D (Dignam *et al.*, 1983), extensively dialyzed against this buffer and then stored at −80°C. Protein concentrations were determined by the method of Bradford (Bio-Rad, Hercules CA). Gel-mobility shift assays were performed as described (Bokar *et al.*, 1988). Briefly, binding reactions contained 100,000 c.p.m. of labelled probe (10 nM), 5 μl of nuclear extract (2–4 μg protein) diluted to 10 μl with Dignam buffer D and 4 μg of poly(dI-dC) (Pharmacia, Piscataway NJ). Where competitors were used, they were included at approximately 100 times the molar concentration of labelled probe. Reactions were carried out for 20 min at room temperature and separated on 7% polyacrylamide gels in 25 mM Tris, 250 mM glycine. Gels were pre-run at 100 V for 2 h. After samples were loaded, current was adjusted to 35 mA. Gels were transferred to Whatman paper, dried, and exposed to Kodak X-AR film overnight. The probes used in the gel mobility shif assays were as follows: Probe 1: 5′-GCAATTGTTTTGAGAAGT-ATGGTCTTCAAACAACA-GATTTTAAATTG-3′; Probe 2: 5′-TTTTAAATTGGATCGGGATTTTTAAATTACTTT-TCTGTATTTCATTT-3′; Probe 3: 5′-TATTTCATTTTGT-ATCTGTCTAGTCACAACAGTTTTTAGAAAACCTAT-3′. Probes were labelled after annealling to the complementary strand with [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase.

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