45. R. W. Byrne and A. Whiten, *Anim. Behav.* **33**, 669 (1985).
46. D. Cheney and R. Seyfarth, *Behaviour* **94**, 150 (1985).
47. B. O. McGonigle and M. Chalmers, *Nature (London)* **267**, 694 (1977); D. J. Gillan, *J. Exp. Psychol. Anim. Behav. Processes* 7, 150 (1981).
48. R. Seyfarth, *Anim. Behav.* **24**, 917 (1976).
49. S. Essock-Vitale and R. Seyfarth, in *Primate Societies* (*1*), pp. 452–461.
50. J. Dohl, *Z. Tierpsychol.* **23**, 77 (1966); *ibid.* **25**, 89 (1968).
51. P. Rozin, in *Progress in Psychology*, J. N. Sprague and A. N. Epstein, Eds. (Academic Press, New York, 1976), vol. 6, pp. 245–280.
52. D. Cheney and R. Seyfarth, *Philos. Trans. R. Soc. London Ser. B* **308**, 187 (1985).
53. C. D. Busse, *Am. Nat.* **112**, 767 (1978); S. C. Strum, in *Omnivorous Primates*, R. S. O. Harding and G. Teleki, Eds. (Columbia Univ. Press, New York, 1981), pp. 255–302.
54. T. H. Clutton-Brock and P. H. Harvey, *J. Zool. London* **190**, 309 (1980); K. Milton, *Am. Anthropol.* **83**, 534 (1981).
55. A. H. Harcourt, *Behav. Ecol. Sociobiol.* **5**, 39 (1979); *Anim. Behav.* **27**, 251 (1979); *ibid.*, p. 325; A. H. Harcourt and K. J. Stewart, *ibid.* **29**, 206 (1981); D. Fossey, *Gorillas in the Mist* (Houghton Mifflin, Boston, 1983); D. P. Watts, *Anim. Behav.* **33**, 72 (*1985*).
56. Author order was determined alphabetically. We thank N. Cantor, R. Smuts, and J. Watanabe for useful comments on the manuscript. Many of the ideas expressed here profited from discussions with V. Dasser, S. Essock-Vitale, L. Fairbanks, R. Hinde, H. Kummer, P. Marler, D. Premack, and R. Wrangham. Several publications cited were initiated or completed while we were fellows at the Center for Advanced Study in the Behavioral Sciences, Stanford, CA, and we thank the center staff for invaluable support. Supported by the Sloan Foundation, the Exxon Educational Fund, and NSF grant BNS 76-22943. D.C. and R.S. have also been supported by the H. F. Guggenheim Foundation, NSF grant BNS 83-15039, and NIH grant NH 19826. B.S. received support from the L. S. B. Leakey Foundation, the Wenner-Gren Foundation, the W. T. Grant Foundation, and NSF grant BNS 83-03677.

Research Articles

A Deletion Truncating the Gonadotropin-Releasing Hormone Gene Is Responsible for Hypogonadism in the *hpg* Mouse

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Hereditary hypogonadism in the hypogonadal (*hpg*) mouse is caused by a deletional mutation of at least 33.5 kilobases encompassing the distal half of the gene for the common biosynthetic precursor of gonadotropin-releasing hormone (GnRH) and GnRH-associated peptide (GAP). The partially deleted gene is transcriptionally active as revealed by in situ hybridization histochemistry of *hpg* hypothalamic tissue sections, but immunocytochemical analysis failed to show the presence of antigen corresponding to any part of the precursor protein.

THE CENTRAL REGULATION OF REPRODUCTIVE COMPETENCE in mammals is mediated by the activity of hypothalamic neurons and relies on the correct expression of a single distinguishing gene that encodes the precursor protein for the decapeptide GnRH (gonadotropin-releasing hormone) and the 56–amino acid peptide GAP (GnRH-associated peptide) (*1, 2*). These biosynthetically linked hypophysiotropic peptides are released at intervals into the portal circulation where they stimulate the pulsatile release of gonadotropins and suppress prolactin secretion from the anterior pituitary (*1–3*). Failure in this system may lead to the common reproductive disorder of hypogonadism, which is often associated with hyperprolactinemia (*4*).

The hypogonadal (*hpg*) mouse, first described in 1977 (*5*), is an appropriate animal model to study this disorder. In this mouse, an isolated deficiency in pituitary gonadotropin secretion leading to a failure of testes and ovaries to develop postnatally is genetically linked to an autosomal recessive mutation. The primary genetic lesion seems to lie within the structural gene for GnRH or in a gene or genes required for its correct expression since GnRH cannot be detected in *hpg* mouse brain, although *hpg* mouse pituitary gonadotrophs are responsive to the decapeptide (*6*). Phenotypic reversal of the *hpg* disorder was achieved by grafts from preoptic parts of the normal fetal brain, which restored functional GnRH- (and presumably GAP-) secreting hypothalamic neurons (*7*).

To determine whether and how the GnRH gene is altered in the *hpg* mouse, we isolated this gene locus from normal and *hpg* mice. Detailed analysis revealed a deletion in the *hpg* genome of at least 33.5 kilobases (kb); this deletion removed the two GnRH gene exons that encode most of the GAP peptide, resulting in a transcriptionally active, but translationally incompetent, truncated gene.

The GnRH-GAP gene from mouse. To provide a basis for comparison of the GnRH gene in normal and *hpg* animals, we isolated the cloned normal gene and delineated its structure. The map of this gene locus and the complete nucleotide sequence of the gene are shown in Figs. 1 and 2. Exon-intron boundaries were assigned on the basis of homologous rat hypothalamic complementary DNA (cDNA) sequences (*8*). The distribution of exons shows close homology of the mouse gene to the corresponding human and rat genes (*8*). The three coding exons (II, III, and IV) encode amino acids 1 to 45, 46 to 77, and 78 to 90, respectively, of the GnRH-GAP precursor. The sequence of the mouse precursor, 90 amino

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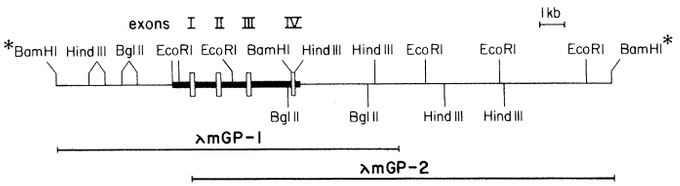


Fig. 1. Map of mouse GnRH-GAP gene locus. Exons I to IV are shown as stippled boxes, and the positions of restriction sites are as indicated. The accentuated black region represents the sequenced region shown in Fig. 2. The origins of the two overlapping lambda clones (λMGP-1 and -2) are shown below the map. The Bam HI sites delineated with an asterisk may have been created by cloning into the lambda vector and are not necessarily present in the genome.

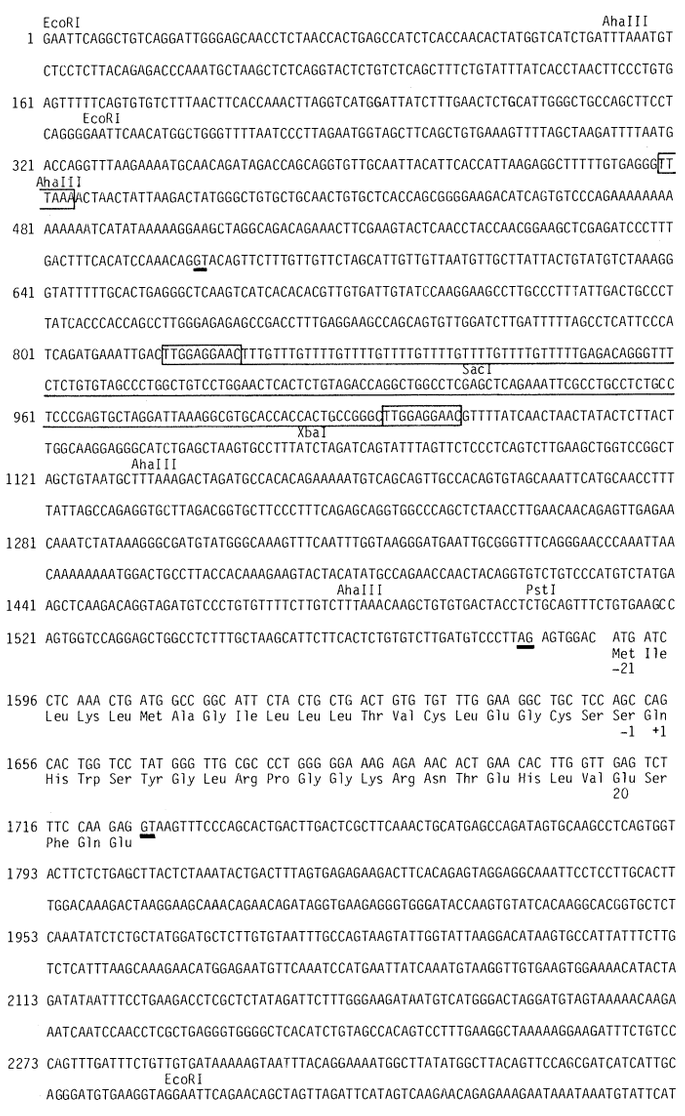
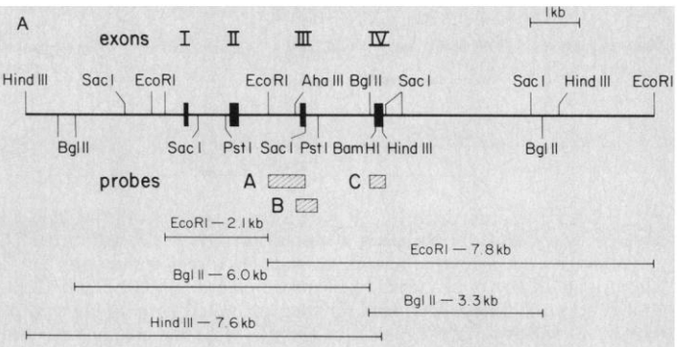


Fig. 2. The nucleotide sequence comprising 5054 bp, extending from an Eco RI site to a Sac I site contains the coding strand for the mRNA. The following features of the DNA sequence are indicated: the boxed TATA sequence (*21*) is the same one used for transcription of the highly homologous rat gene in rat brain (*22*); the GT and AG boundaries of intron 1 are assigned by homology with the rat 5′-untranslated region and are underlined; a repetitive sequence element of 178 bp (underlined) between two direct repeats of 10 bp (boxed) is situated in the first intron; the polyadenylation signal AATAAA is boxed and the 3′ end of the mRNA as predicted from the homologous rat mRNA (*8*) is indicated by an asterisk. Deduced amino sequences of the signal peptide, GnRH and GAP peptides are shown below the DNA sequence. All restriction sites relevant to Fig. 3A are shown above the DNA sequence. The clones λmGP-1 and -2 were isolated by screening 1×10^6 clones from a murine partial Mbo I genomic library with a 462-bp Bam HI–Sal I fragment from cloned rat GnRH-GAP cDNA (*8*). Restriction enzyme sites were deduced by restriction mapping and Southern analysis with the cDNA probe, or from DNA sequence data. Various restriction fragments over the 5-kb sequenced region were isolated and subcloned into the M13 vectors mp18 or mp19 (*23*). Single-stranded template was isolated and the sequence determined using the chain termination method (*24*). Exon-intron boundaries were assigned by homology with the rat GnRH cDNA sequence (*8*).

acid residues long as deduced from the gene sequence, is two amino acids smaller than the rat and human precursors (*8, 9*) because of a shorter signal peptide. There is strong homology between the mouse and rat GnRH-GAP precursor proteins with only nine amino acid differences, of which five are within the signal peptide and the other four within the GAP peptide. Homology with the human precursor is approximately 75 percent.

The analyzed gene was derived from a genetic background different from that of the *hpg* mouse (BALB/c compared to C3H/HeH×101/H). Southern analysis of DNA from mice congenic with *hpg* showed identical GnRH gene restriction fragments. These results confirm the presence of a single GnRH-GAP gene in mouse as found for human and rat (*8, 9*).



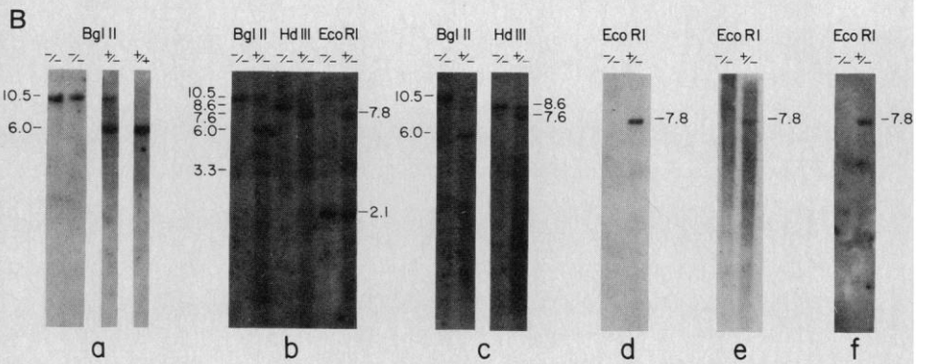


Fig. 3. Southern analysis of the GnRH-GAP gene in the *hpg* mouse. (A) Restriction map of the normal GnRH-GAP gene. The sizes of the Eco RI, Bgl II and Hind III restriction fragments from this region are shown below the map. The positions of probe A (767-bp Eco RI–Sac I, nucleotides 2367 to 3134, Fig. 2), probe B (462-bp Aha III–Pst I, nucleotides 2885 to 3347) and probe C (310-bp Bam HI–Sac I, nucleotides 4413 to 4723) are shown by stippled boxes below the restriction map. (B) Genomic blots of DNA from homozygous wild type (+/+), heterozygous *hpg* (+/−), and homozygous *hpg* (−/−) mice. DNA's digested with the enzymes shown were separated by electrophoresis on a 1 percent agarose gel, transferred to nitrocellulose, and hybridized with the following labeled probes [panels a and b: rat cDNA 462-bp Sal I–Bam HI (*6*); panel c: probe A (Fig. 3A); panel d: probe B (Fig. 3A); panel e: probe C (Fig. 3A); panel f; rat gene fragment encompassing same region as probe C]. DNA fragments to be used as hybridization probes were isolated from plasmid subclones by cleavage with restriction enzymes and electrophoresis in 6 percent polyacrylamide gels. Labeled probes were prepared to a specific activity of 10^8 cpm/μg by random priming in the presence of [α-^{32}P]dCTP and [α-^{32}P]dATP (deoxycytidine and deoxyadenosine triphosphates) and *Escherichia coli* DNA polymerase I, large fragment (*25*). Genomic DNA's were prepared from mouse livers (Jackson Laboratory mice) as described (*26*). The DNA's were digested to completion with the appropriate enzyme and subjected to electrophoresis in a 1 percent horizontal agarose gel in TAE buffer (40 m*M* tris, 20 m*M* sodium acetate, and 2 m*M* EDTA, *p*H 7.8) at 40 V for 18 hours. The DNA was transferred to a nitrocellulose filter (*27*) and hybridized with the appropriate probe at 42°C in 5× SSC (standard saline citrate), 50 m*M* sodium phosphate buffer (*p*H 6.8), 0.1 percent sodium pyrophosphate, 5× Denhardt's solution, sonicated salmon sperm DNA (at 0.04 g/liter), 50 percent formamide, and 10 percent dextran sulfate and washed at 65°C in 0.1× SSC, 0.1 percent SDS. Filters were exposed for autoradiography at −70°C with an intensifying screen for 1 to 2 days. Numbers alongside the autoradiograms indicate the size in kilobases (kb) of the hybridizing DNA fragments. These sizes were determined from co-electrophoresis with a Hind III digest of λc1857 (Bethesda Research Labs).

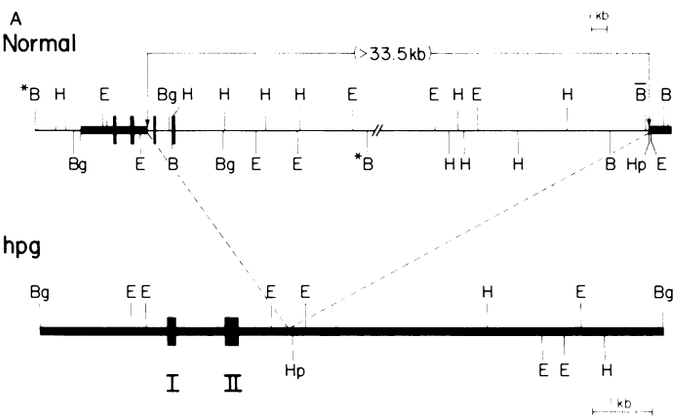


Fig. 4. The *hpg* deletion as characterized by restriction map (A) and DNA sequence (B). The extent of the deletion is shown by the thin line between the two arrows. The slashes in this line indicate an unknown length in excess of 33.5 kb. The map 5′ to the slashed lines is derived from λmGP-1 and -2 (Fig. 1) and 3′ from λmGP-3. Broken lines extend from the 5′ and 3′ breakpoints and meet on the *hpg* locus depicted underneath (note difference in scale) contained on a Bgl II fragment from a *hpg* gene library. GnRH gene exons are indicated by filled-in boxes in both maps. Restriction enzyme cleavage sites are abbreviated by B (Bam HI), Bg (Bgl II), H (Hind III), Hp (Hpa I), and E (Eco RI). The two Bam HI sites at the 3′ end of the normal map are not shown on the *hpg* map. The starred Bam HI sites in the normal map represent the ends of the cloned mouse DNA in λmGP-1 and -2 and therefore may not be present in the mouse genome. The *hpg* DNA sequence (middle sequence) shows the region around the convergence of the broken lines and is compared to DNA sequences around the 5′ (top sequence) and 3′ (bottom sequence) breakpoints in wild-type DNA. The nucleotide numbering of the top and middle sequences is that of the nucleotide sequence shown in Fig. 2. Bottom sequence numbering starts at the overlined Bam HI site close to the 3′ breakpoint. Sequence identities are indicated by continuous lines. λmGP-3 was isolated by screening a mouse genomic library with a 185-bp Hpa I–Eco RI fragment (see text). To isolate clones containing the *hpg* GnRH-GAP gene, Bgl II–digested homozygous *hpg* genomic DNA was fractionated on a 10 to 40 percent sucrose gradient in 1*M* NaCl, 10 m*M* tris-HCl, *p*H 8.0, 1 m*M* EDTA, and fractions containing 8- to 15-kb fragments were pooled and precipitated with ethanol. Lambda EMBL-3 DNA (Promega Biotec) was digested with Bam HI and Eco RI and isopropanol precipitated to obtain vector arms. Genomic DNA was ligated to 1 μg of vector DNA and packaged in vitro (Gigapack). Independent recombinants (~10^6) were obtained and screened with a cloned labeled 462-bp Sal I–Bam HI rat cDNA fragment (*8*) under hybridization conditions described in Fig. 3. Six independent positive phage were grown and purified. Lambda DNA's were analyzed by standard methods (*28*). The 10.5-kb Bgl II fragment containing the *hpg* GnRH locus was isolated from phage DNA via Sal I cleavage and subcloned into the Sal I site of plasmid pSP65 (*29*). Plasmid DNA was prepared (*28*) and used for restriction analysis. A 767-bp Eco RI–Sac I fragment from the normal gene containing exon III sequences was subcloned into M13mp19 RF-DNA (*23*), as was the 550-bp Eco RI fragment from the *hpg* gene, which covers the deletion and the 360-bp Eco RI–Bam HI, which contains the normal 3′ breakpoint sequences. Single-stranded templates were prepared, and all sequence reactions performed by the chain termination method (*24*).

B

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Normal   2531  TCTAGACAGAGTCTCTTCCCAGGTGATTCTACATTGGGTCAAGTTGACTATTAAAAGCAACCATCACAACTCCACCCCCTGTCAATTTGAGATGCAAACA
hpg      2531  TCTAGACAGAGTCTCTTCCCAGGTGATTCTACATTGGGTCAAGTTGACTATTAAAAGCAACCATCACAACTCCACCCCCTGTCAATTTGAGATGCAAACA
Normal   Δ+1   GGATCCTCCTTTAAAATAAGCACCTCTAGTTACTTACCCACTAGGGAAATTTACAAAGCAGGCCATGCATTGTATCTTCTAAAACAGCCTCTAAATCTTA

Normal   2631  CACCACTCTAAGCTAGTTCAAGGTTAGACCAGGATACATAATGAGTCTCAGAGCAATCTGGGGTACAGAGTGAAATCCCAATTGAGAGAAAAATGAAAGA
hpg      2631  CACCACTCTAAGCTAGTTCAAGGTTAGACCAGGATACATAATGAGTCTCAGAGCAATCTGGGGTACAGAGTGGCTGAGGAATGTTAACTAATTTGCCTGT
Normal   Δ+100 ATCGTAACTCTTAGCCACTCTTAGTCTTGGTTTTTGTTTATCTGTTTGTTTGTTTTTAGGGTGGAACCATAGGCTGAGGAATGTTAACTAATTTGCCTGT

Normal   2731  ACAGCAGCAACAAAACCAATGTAGCAAAGAGAAACCCAGCCCTATCAGGACTGGAAAGCCTTGTATCATTTGTACAGTCTGTTCATGAGGCTCAGAGCAC
hpg      2731  TTCAAGAGAGTAGAAACAACTAAAATTTCAAAGTCTTCAATAAATTCCAACCGCTTTCCAGTGACAGCCTCGGAGCTCCCAGGCTCTCAATGAACTCTGA
Normal   Δ+200 TTCAAGAGAGTAGAAACAACTAAAATTTCAAAGTCTTCAATAAATTCCAACCGCTTTCCAGTGACAGCCTCGGAGCTCCCAGGCTCTCAATGAACTCTGA

Normal   2831  TTCACTCCTGTCTGGGAGAACACTGTCCCATTACCTCCTAAACTCCAGCTCATTCTTTAAA
hpg      2831  ACCAGACCTGAGAGGATGCACTGAAACCTGGAACCAAACTTGTCATTCCCGTATAGAATTC
Normal   Δ+300 ACCAGACCTGAGAGGATGCACTGAAACCTGGAACCAAACTTGTCATTCCCGTATAGAATTC
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GnRH-GAP gene altered in *hpg* mouse. Genomic DNA's from normal mice (+/+), *hpg* heterozygotes (+/−), and homozygotes (−/−) were digested with endonuclease Bgl II, Hind III, or Eco RI and analyzed by Southern blotting for the presence of restriction fragments hybridizing to rat GnRH-GAP cDNA sequences (Fig. 3B, panels a and b). As would be expected from the map shown in Fig. 3A, two Bgl II fragments (6.0 and 3.3 kb), one Hind III fragment (7.6 kb), and two Eco RI fragments (2.1 and 7.8 kb) should be, and were, present in DNA from normal mice and also from *hpg* heterozygotes. The 3.3-kb Bgl II fragment contains exon IV and is seen only after long exposures since as few as 59 bp of the hybridization probe are present on this exon.

A change in the *hpg* GnRH gene was demonstrated by the presence of differently sized hybridizing Bgl II and Hind III DNA restriction fragments in the *hpg* genome. Thus, the only hybridizing Bgl II fragment from *hpg* homozygotes is 10.5 kb in size. This fragment coexists with the normal Bgl II fragments (6.0 and 3.3 kb) in DNA samples from heterozygotes (Fig. 3B, panels a and b). Similarly, an abnormal 8.6-kb Hind III fragment is present in the *hpg* genome and is observed along with the normal 7.6-kb fragment in heterozygous DNA (Fig. 3B, panel b). These abnormally sized restriction fragments hybridize with reduced intensities when compared to the normal fragments in heterozygote DNA (Fig. 3B, panel b), suggesting that some exon sequences are missing from the *hpg* GnRH gene. Such deleted gene regions were localized to exons III and IV since the 7.8-kb Eco RI fragment containing these exons is not detected in homozygous *hpg* DNA (Fig. 3B, panel b). The 2.1-kb Eco RI fragment containing exons I and II is present in DNA from all three genotypes.

On the basis of this finding, we expected the breakpoint of the *hpg* deletion to lie within the second intron. Its approximate position was localized with the use of several defined hybridization probes for Southern analysis of *hpg* DNA (Fig. 3A). Probe A, consisting of part of the second intron and exon III, hybridized with the 10.5-kb Bgl II and 8.6-kb Hind III fragments characteristic of *hpg* DNA (Fig. 3B, panel c), indicating that the breakpoint lies within this probe. Probe B, which contained less of the second intron but also included exon III sequences, did not hybridize to *hpg* homozygous DNA (Fig. 3B, panel d) and neither did probe C, which contained exon IV and flanking intron sequence. These data show that the 5′ breakpoint of the deletion in the GnRH gene lies between the Eco RI site and the Aha III site in the second intron (Fig. 3A) within a 518-bp region (Fig. 2B). Analysis of DNA obtained from several male and female *hpg* homozygotes and heterozygotes confirmed these results (Fig. 3B, panel a) obtained from the DNA of a single mouse.

Truncation of the *hpg* GnRH gene is part of large deletion. To characterize the *hpg* GnRH gene locus in detail, we isolated a cloned 10.5-kb Bgl II fragment containing the gene from a *hpg* gene library constructed in bacteriophage λ (Fig. 4A). The *hpg* gene is identical to the normal gene up to nucleotide 2700 (Fig. 2B); after that it diverges into an unrelated sequence that should border the 3′ end of the *hpg* deletion in normal mouse DNA.

The size of the deletion was estimated by labeling a fragment from the *hpg* gene (185-bp Hpa I–Eco RI fragment), which originates 10 bp downstream from the point of sequence divergence from the normal gene and hybridizing it to λmGP-2 that contained the complete normal gene. No hybridization was obtained, suggesting that the deletion was larger than 15.5 kb (see Fig. 1). In a further attempt to find the 3′ end of the *hpg* deletion, we screened the

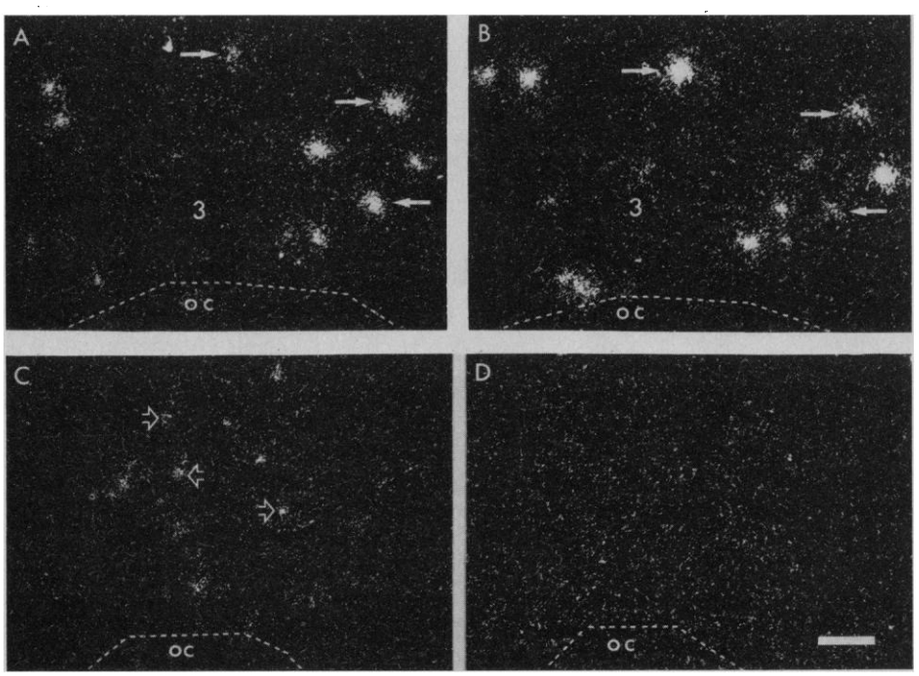

Fig. 5. In situ hybridization histochemistry in the normal and hypogonadal mouse at the level of the organum vasculosum of the lamina terminalis. Silver grains are white in these darkfield photomicrographs of autoradiograms. (A and B) These adjacent sections show normal mouse cells labeled by probes 1 and 2, respectively. Solid arrows indicate three cells that are labeled. (C) Cells in the hypogonadal mouse are labeled less intensely with probe 1 than those in the normal mouse. The open arrows indicate three labeled cells. In (D), which shows a section adjacent to the one in (C), no cells are labeled by probe 2. Abbreviations: oc, optic chiasm (indicated by a broken line); 3, third ventricle. Bar indicates 100 μm. Two synthetic oligodeoxynucleotides, 48 bases in length, and complementary to mouse GnRH gene nucleotides 1677 to 1724 (Fig. 2, exon II, amino acids 9 to 24 of mature precursor) and 3095 to 3143 (exon III, last 16 amino acids), respectively (probes 1 and 2), were labeled with terminal deoxynucleotidyl transferase (BRL) and [α-^{35}S]dATP (>1000 Ci/mmol; New England Nuclear) to a specific activity of 9000 Ci/mmol. The average tail length was 12 bases. For in situ hybridization histochemistry, the tissues were obtained and processed as described (*30*). Briefly, six hypogonadal (*hpg*/*hpg*; confirmed by the presence of gonadal hypoplasia) and seven normal littermates (+/+ or *hpg*/+) were perfused with 4 percent formaldehyde, 0.12*M* phosphate buffer (*p*H 7.4). Frozen sections (12 μm) were thaw-mounted onto gelatin-coated slides and prepared for hybridization by placing them in 0.25 percent acetic anhydride in 0.1*M* triethanolamine and 0.9 percent NaCl for 10 minutes at room temperature. They were then dehydrated and delipidated through a graded series of ethanol and chloroform, and rehydrated through ethanol. The sections were next hybridized with the probe—1×10^6 to 2×10^6 dpm for two sections per slide. The sections were held overnight at room temperature and washed in four 15-minute changes of 2× SSC and 50 percent formamide at 40°C (about 20°C below the theoretical melting temperature) (*31*). The sections were dried and placed against cover slips coated with NTB3 nuclear emulsion (1:1 with water, Kodak) (*32*). Autoradiograms were developed after 3-week exposures, and the tissues were stained with cresyl violet. Neurons were considered labeled if the grain density was five times background over the optic chiasm. Specificity of probes was demonstrated by use of one additional oligodeoxynucleotide complementary to the first 48 bases downstream to the *hpg* deletion. Although its base composition was similar to probes 1 and 2, this probe failed to label any cells in normal mouse or *hpg* tissue.

genomic library derived from normal mouse DNA with the same 185-bp DNA. The only isolate found (λmGP-3) extended, by 18 kb, 5′ to the point of divergence. Since the structure of cloned DNA contained in this isolate did not overlap with that of λmGP-2, the size of the deletion exceeded 33.5 kb (Fig. 4A).

The DNA sequence across the 3′ end of the deletion breakpoint was determined and compared, along with the sequence across the 5′ breakpoint, to that present in the *hpg* GnRH gene (Fig. 4B). No special sequence features, such as inverted repeats or homologous flanking regions, were found at the ends of the large deletion that might account for the generation of the *hpg* genotype.

The *hpg* GnRH gene is transcriptionally active. The intact part of the truncated *hpg* GnRH gene includes the presumptive promoter region responsible for the regulation of transcriptional activity. Such activity was visualized with the use of in situ hybridization histochemistry (*10*) on thin sections of normal and mutant mouse brain. Two labeled synthetic oligodeoxynucleotides complementary to GnRH gene sequences encoding prohormone amino acids 9 to 24 (probe 1, exon II) and 41 to 56 (probe 2, exon III, present in normal gene only) served as probes. Brain sections were from the level of the organum vasculosum of the lamina terminalis and rostral-most third ventricle (OVLT region), which contains a relatively high density of GnRH immunoreactive neurons (*11*).

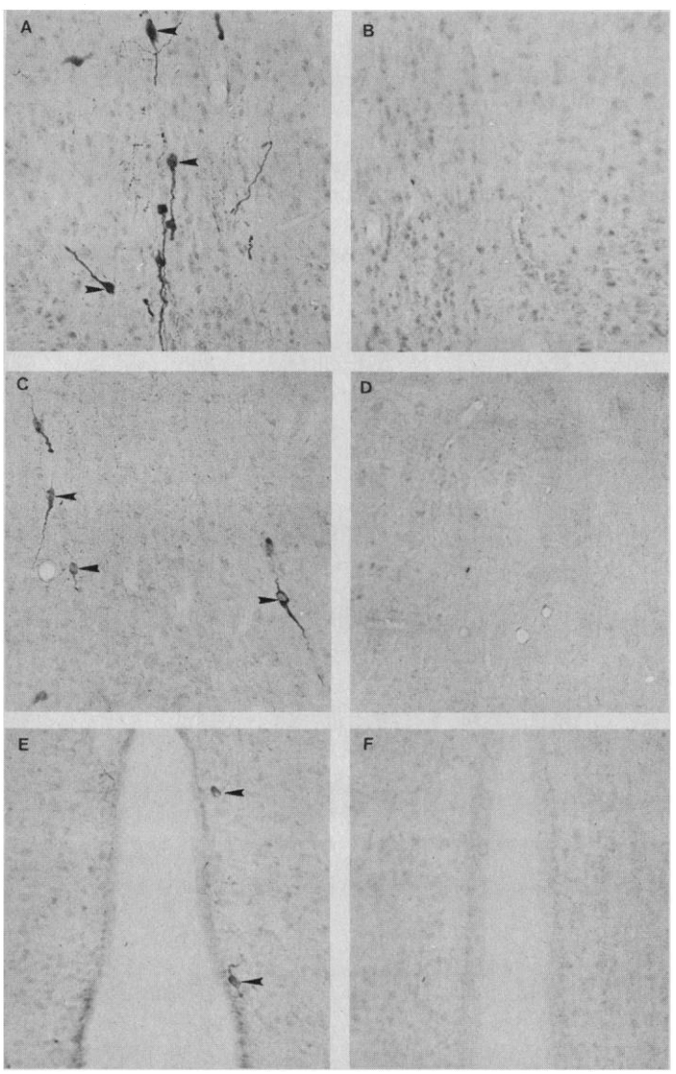

Fig. 6. Immunocytochemical localization of pro-GnRH sequences in normal and *hpg* mouse brain. A, C, and E are sections of normal mouse brain; B, D, and F are sections through comparable regions of *hpg* mouse brain. Arrowheads indicate examples of immunoreactive perikarya. Approximate magnification is 830× for A to F. (A and B) Sections through diagonal band of Broca reacted with DB1 antiserum (to GnRH). Numerous intensely stained perikarya are apparent (A) in the normal mouse. No immunoreactivity is detectable in (B) the *hpg* mouse. (C and D) Sections through diagonal band of Broca reacted with serum 56A, an antiserum to proGnRH(33–56). Immunoreactive perikarya are abundant (C) in the normal mouse, but absent (D) in the *hpg* mouse. (E and F) Sections through the medial preoptic area reacted with serum 24A, an antiserum to proGnRH(14–24). Few weakly stained perikarya are apparent in the normal mouse (E). No immunoreactivity is detectable in the *hpg* mouse (F).

In the normal (or heterozygous) littermates, both probes I and II labeled 5 to 25 cells of the thousands of cells in each processed section in a pattern similar to that observed when rodent hypothalamic sections are immunohistochemically labeled for GnRH and GAP (*11–14*). Most labeled cells were partly contained in each of two consecutive sections, indicating the expected presence of exons II and III in the transcriptional product of the same cell (Fig. 5, A and B). In the hypogonadal mice, only probe I was reactive; it labeled up to 15 cells in a distribution similar to that in normal mice, although the signal intensity was significantly less (Fig. 5C), indicating a lowered rate of transcription (or stability) of the aberrant transcript. Probe II, complementary to a deleted portion of the gene, revealed no signal above background (Fig. 5D), as would be expected with the truncated structure of *hpg* gene.

GnRH and GAP are absent from *hpg* brain. Since the *hpg* GnRH gene shows transcriptional activity, translation of the ensuing messenger RNA (mRNA) should result in an altered precursor protein with only the signal sequences, the GnRH decapeptide structure, and the amino-terminal 11 residues of GAP identical to the normal precursor. To determine whether the *hpg* mouse synthesizes such a protein containing regions of the GnRH-GAP prohormone, we performed immunocytochemical analysis of normal and *hpg* mouse brain with three antibodies to synthetic peptides representing three regions of GnRH-GAP prohormone. Antiserum DB1 was generated against an analog of GnRH (residues 1 to 10 of the prohormone), antiserum 24A was derived by immunization against residues 14 to 24 of the prohormone (amino acids 1 to 11 of GAP), and antiserum 56A was raised against prohormonal residues 33 to 56 (amino acids 20 to 43 of GAP). Antisera 24 and 56 detect antigens, the approximate coding regions of which were visualized in the cytoplasm of hypothalamic neurons by in situ hybridization probes 1 and 2. All sera showed excellent staining of neuronal perikarya and axons in regions of rat brain previously demonstrated to contain GnRH and GAP immunoreactivity (*14*). Immunocytochemical reaction of normal mouse brain with antisera DB1 and 56A produced a staining pattern qualitatively identical to that observed in rat brain. Numerous immunoreactive perikarya were observed in the septopreoptic region (Fig. 6, A and C), and axonal terminals were particularly abundant in the median eminence and the organum vasculosum of the lamina terminalis. Reaction of normal mouse brain with antiserum 24A paralleled the pattern observed with sera DB1 and 56A (Fig. 6E), but produced weaker signals, reflecting perhaps the difference in rat and mouse GAP residue 7 (Asp compared to Glu). In the *hpg* mouse brain, no immunoreactivity was detectable with antibodies to any of the three prohormone sequences (Fig. 6, B, D, and F). Septal, preoptic, and hypothalamic regions were completely devoid of reactive neuronal elements. These results extend earlier reports indicating a lack of GnRH-like immunoreactivity in the *hpg* mouse brain (*7*). As suggested by our data on the transcriptional activity of the mutant locus, this apparent lack of immunoreactivity in *hpg* mouse neurons could be due to the low sensitivity of our immunodetection method for the hypothetical mutant protein.

The genetic basis for hypogonadism in the *hpg* mouse. The comparison of the GnRH gene from normal and mutant mice has revealed a deletion in the chromosomal *hpg* locus that removes a region in excess of 33 kb, consisting of two exons of the GnRH gene that encode most of the GAP portion of the GnRH precursor protein. We propose that this deletion is the cause of infertility in the *hpg* mouse, a defect of autosomal recessive inheritance (*5*). The lack of GAP, which possesses potent prolactin release inhibitory activity (*2*), may explain the steep decrease in pituitary prolactin content in *hpg* females (*15*).

Interestingly, although it is well established that the *hpg* mouse is genetically deficient in GnRH production, the part of the gene coding for the decapeptide is left intact. Also left intact is the promoter region of the gene and the coding sequences for the signal peptide, the Gly-Lys-Arg processing and amidation site and the amino-terminal 11 amino acid residues of GAP. The lack of GnRH still requires explanation since in situ hybridization histochemistry has revealed the presence of cytoplasmic *hpg* GnRH mRNA sequences, and translation of this mutant mRNA may generate the decapeptide structure.

It can be argued that, possibly through splicing the primary *hpg* transcript, a sequence unrelated to the normal coding sequence for most of GAP serves to substitute for the carboxyl terminal half of the normal GnRH precursor and that this mutant protein cannot be processed to yield GnRH. We have tried to find such a mutant protein by using immunocytochemistry and antisera to different sections of the normal GnRH precursor. Our failure to detect any cross-reacting antigens in *hpg* brain extends previous results that the hypothalamic content of GnRH is undetectable, as estimated by radioimmunoassay (*5*) and immunocytochemistry (*16*). These results might argue against any translation of the mutant mRNA, but low sensitivity of the antibody to the amino-terminal 11 residues of rat GAP and poor cross-reactivity of the GnRH antiserum to unprocessed precursor sequences may have precluded detection of low amounts of the hypothetical mutant protein.

Several factors could bring about such low amounts. Thus, mutant RNA levels were found to be much reduced; whether this is due to impaired stability or lowered transcriptional activity remains undetermined. Reduced transcription from the *hpg* GnRH gene might derive from the fact that the *hpg* deletion, in addition to truncating this gene, spans a region that may be considerably in excess of 33 kb. It is conceivable that DNA sequences may be affected and, either directly or through their transcriptional or translational products, are involved in allowing the correct expression of the GnRH gene. Moreover, such sequences could exert an effect on the number of neuroendocrine cells that express this gene. This suggestion is supported by the finding that heterozygous *hpg* animals contain only 20 percent of the hypothalamic GnRH content of their normal counterparts (*17*). Indeed, this reduction in GnRH content seems to exceed the possible contribution from a gene dosage effect resulting from the mutant GnRH allele. Furthermore, altered translational efficiency of the mutant mRNA and reduced stability of the protein product are likely to play a major role in the failure of detection by immunocytochemistry. A similar situation was encountered in the case of the vasopressin gene in the Brattleboro rat, where a one-nucleotide deletion proved deleterious to the expression of the affected mRNA (*18*).

It should be stressed that the detection of transcriptional activity from the *hpg* GnRH gene has given us important information about the existence of a population of hypothalamic neuroendocrine cells which seem to develop in the correct locations in *hpg* brain despite the mutant phenotype. We have therefore found and described a convenient neuronal marker for the *hpg* equivalent of normal GnRH-secreting neurons which elude detection by immunocytochemistry.

The *hpg* mouse is an important animal model for the common reproductive disorder of hypogonadism. Kallmann's syndrome and familial gonadotropin deficiency are two forms of secondary hypogonadism in man, which are treated by GnRH therapy (*19, 20*). It remains to be seen whether these deficiencies are a result of a GnRH mutation.

REFERENCES AND NOTES

1. E. Knobil, *Recent Prog. Horm. Res.* **36**, 53 (1980); A. V. Schally *et al.*, *Science* **173**, 1036 (1971).
2. K. Nikolics, A. J. Mason, E. Szonyi, J. Ramachandran, P. H. Seeburg, *Nature (London)* **316**, 511 (1985).
3. I. J. Clarke *et al.*, in preparation.
4. D. A. Leong, L. S. Frawley, J. D. Neill, *Annu. Rev. Physiol.* **45**, 109 (1983).
5. B. M. Cattanach, C. A. Iddon, H. M. Charlton, S. A. Chiappa, G. Fink, *Nature (London)* **269**, 338 (1977).
6. G. Fink, W. J. Sheward, H. M. Charlton, *J. Endocrinol.* **94**, 283 (1982).
7. D. T. Krieger *et al.*, *Nature (London)* **298**, 468 (1982).
8. J. P. Adelman, A. J. Mason, J. S. Hayflick, P. H. Seeburg, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 179 (1986).
9. P. H. Seeburg and J. P. Adelman, *Nature (London)* **311**, 666 (1984).
10. P. Hudson *et al.*, *Endocrinology* **108**, 353 (1981).
11. G. E. Hoffman, *Peptides* **6**, 439 (1985).
12. C. Bennett-Clarke and S. A. Joseph, *Cell Tissue Res.* **221**, 493 (1982).
13. Y. Ibata *et al.*, *Biomed. Res.* **4**, 161 (1983).
14. H. S. Phillips, K. Nikolics, D. Branton, P. H. Seeburg, *Nature (London)* **316**, 542 (1985).
15. H. M. Charlton *et al.*, *Endocrinology* **113**, 545 (1983).
16. M. F. Lyon, B. M. Cattanach, H. M. Charlton, in *Mechanisms of Sex Differentiation in Animals and Man*, C. R. Austin and R. G. Edwards, Eds. (Academic Press, New York, 1981), pp. 329–386.
17. D. T. Krieger *et al.*, in *Fernstrom Foundation Series*, in press.
18. H. Schmale and D. Richter, *Nature (London)* **308**, 705 (1984); H. Schmale, R. Ivell, M. Breindl, D. Darmer, D. Richter, *EMBO J.* **3**, 3289 (1984).
19. P. Troen and H. Oshima, in *Endocrinology and Metabolism*, P. Felig, J. D. Baxter, A. E. Broadus, L. A. Frohman, Eds. (McGraw-Hill, New York, 1981), pp. 627–668.
20. R. W. Ewer, *J. Clin. Endocrinol. Metab.* **28**, 783 (1968).
21. J. Corden *et al.*, *Science* **209**, 1406 (1980).
22. J. S. Hayflick *et al.*, in preparation.
23. J. Norrander, T. Kempe, J. Messing, *Gene* **26**, 101 (1983).
24. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).
25. J. M. Taylor, R. Illmensee, J. Summers, *Biochim. Biophys. Acta* **442**, 324 (1976).
26. M. Gross-Bellard, P. Oudet, P. Chambon, *Eur. J. Biochem.* **36**, 32 (1973).
27. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
28. T. Maniatis, E. F. Fritsch, J. Sambrook, Eds., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
29. D. A. Melton *et al.*, *Nucleic Acids Res.* **12**, 7035 (1984).
30. W. S. Young III, E. Mezey, R. E. Siegel, *Neurosci. Lett.*, in press.
31. R. Lathe, *J. Mol. Biol.* **183**, 1 (1985).
32. W. S. Young III and M. J. Kuhar, *Brain Res.* **179**, 255 (1979).

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