The Hypogonadal Mouse: Reproductive Functions Restored by Gene Therapy

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The hypogonadal (*hpg*) mouse lacks a complete gonadotropin-releasing hormone (GnRH) gene and consequently cannot reproduce. Introduction of an intact GnRH gene into the genome of these mutant mice resulted in complete reversal of the hypogonadal phenotype. Transgenic *hpg*/*hpg* homozygotes of both sexes were capable of mating and producing offspring. Pituitary and serum concentrations of luteinizing hormone, follicle-stimulating hormone, and prolactin were restored to those of normal animals. Immunocytochemistry and in situ hybridization showed that GnRH expression was restored in the appropriate hypothalamic neurons of the transgenic *hpg* animals, an indication of neural-specific expression of the introduced gene.

MICE HOMOZYGOUS FOR THE HYPOGONADAL (*hpg*) MUTATION are sexually immature and have arrested germ cell development (*1*). Immunocytochemical and radioimmunoassay measurements (*1, 2*) have shown that these mice lack detectable levels of hypothalamic gonadotropin-releasing hormone (GnRH), leading to low levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

As described in the accompanying article (*3*), the genetic basis for this mutation is a deletion of at least 33.5 kilobases encompassing the distal half of the gene for the common biosynthetic precursor of GnRH and GnRH-associated peptide (GAP) (*4, 5*). This deletion does not seem to affect the number and location of the GnRH neurons in the *hpg* hypothalamus since messenger RNA (mRNA) transcripts from the truncated *hpg* gene can be detected by in situ hybridization in the expected locations of the *hpg* brain (*3*). The normal architecture of GnRH neurons and neuronal circuitry necessary to control the pulsatility and correct amplitude of GnRH and GAP (*6*) output may therefore not be directly affected by the *hpg* mutation.

To try and restore a functional hypothalamic-pituitary-gonadal axis, we introduced a DNA fragment containing the mouse GnRH gene with 5 kb of 5′ flanking and 3.5 kb of 3′ flanking sequences into a wild-type mouse. By a series of matings with *hpg*/+ heterozygotes, the transgene was introduced into a *hpg*/*hpg* homozygous background. Transgenic *hpg*/*hpg* homozygotes displayed tissue-specific expression of the GnRH transgene, they reached full sexual maturity, and were capable of mating and raising healthy litters.

Introduction of GnRH-GAP gene into *hpg*/*hpg* homozygotes. Since homozygous *hpg*/*hpg* mice are sterile, we introduced the GnRH-GAP gene into the *hpg*/*hpg* homozygous background as outlined in Fig. 1A. The genotype of each animal from this series of matings was established by Southern genomic DNA hybridization with probe 2 (Fig. 1, B and D). This Bam HI–Eco RI fragment, which encompasses the 3′ breakpoint of the *hpg* deletion and thus does not detect the presence of the transgene, could distinguish between genomic DNA from wild-type, *hpg*/+, and *hpg*/*hpg* mice (Fig. 1D). Sequential hybridization of the same genomic DNA's with probe 1, which is specific for intron B and exon III of mouse GnRH gene, was used to establish which of the mice contained the introduced transgene (Fig. 1, B and C).

Approximately 200 copies of a gel-purified 13.5-kb Sal I–BstE II fragment (Fig. 1B) containing the mouse GnRH gene flanked by 5 kb of upstream and 3.5 kb of downstream sequences were microinjected into 250 fertilized wild-type mouse eggs (C57BL/6J × SJL). A total of 27 pups were born, of which two were shown to be transgenic, each containing more than 20 copies of the transgene. To introduce the GnRH gene into a *hpg*/+ heterozygous background, transgenic mice A and B were backcrossed to *hpg*/+ heterozygotes (Fig. 1A). Of the 18 offspring of mouse B, 14 inherited the transgene. Only 6 of the 30 offspring of transgenic mouse A were transgenic. Although this result suggested that the germ line of mouse A was mosaic for the transgene, an identical copy number of the transgene was maintained in the offspring of this mouse. As was expected, approximately half of the offspring from the above crosses were *hpg*/+ heterozygotes.

Mice identified as transgenic *hpg*/+ heterozygotes from both families were crossed with either another transgenic *hpg*/+ heterozygote from the same family or a nontransgenic *hpg*/+ heterozygote. Approximately one-quarter of the offspring from these crosses were *hpg*/*hpg* homozygotes (see Fig. 1D). Of a total of 12 *hpg*/*hpg* homozygotes identified in family B, seven were also transgenic, two being males (B-41, B-61) and five females (B-26, B-50, B-66, B-68, B-69). A surgical examination of the transgenic *hpg*/*hpg* homozygotes and their five nontransgenic *hpg*/*hpg* homozygous littermates revealed that the transgenic mice had well-developed gonads and accessory sexual glands compared to their *hpg* littermates whose gonadal tissue was developmentally arrested at the prepubertal stage. The same results were observed with the A family, in which each of seven transgenic *hpg*/*hpg* homozygotes was sexually mature.

Mating and pregnancy can occur in transgenic *hpg* animals. To investigate whether the transgenic *hpg* males were capable of mating and fathering offspring, two transgenic *hpg* males (B-41 and B-61) were each paired with a normal female. The females found to have vaginal plugs (a sign of successful copulation) were killed and most of their eggs were fertilized as seen by microscopic examination. The fertilized eggs were transplanted into a pseudopregnant female, and

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approximately 12 days after transplantation the foster female was killed and genomic DNA was extracted from each of the embryos. Results of a Southern analysis of this DNA showed that the nine embryos fathered by mouse B-41, and ten embryos fathered by mouse B-61 were all heterozygous for the *hpg* allele, thus confirming our assignment of B-41 and B-61 as *hpg/hpg* homozygotes (Fig. 1E). This experiment demonstrated that the transgenic *hpg* males were capable of correct mating behavior and of fertilizing eggs.

Transgenic *hpg* females had also regained their full reproductive ability, since three such females from the B family when paired with transgenic *hpg*/+ heterozygote males became pregnant and gave birth to healthy litters of between 6 and 11 mice. Analogous experiments with mice from the A family showed that the males and females were reproductively competent. Offspring from such crosses were genotyped and those identified as transgenic *hpg/hpg* homozygotes were killed for detailed endocrinological and expression studies.

Gonadal development in transgenic *hpg/hpg* homozygotes. To investigate the extent of gonadal development in the transgenic *hpg* mice the following study was performed. From the B family, five transgenic *hpg/hpg* homozygous females and four transgenic *hpg/hpg* homozygous males approximately 12 weeks old were killed along with 10-week-old wild-type male and female mice and 8-week-old *hpg/hpg* homozygotes as controls. The testes, seminal vesicles, preputial glands, and adrenals were surgically removed from the male mice and weighed. Similarly, the ovaries, uteri, and adrenals were removed from the females and their weight was determined (Fig. 2A). In the transgenic *hpg/hpg* homozygous males the testes, seminal vesicles, and preputial glands had developed to normal size. Similar results are seen on comparison of normal ovarian and uterine weights in normal and transgenic *hpg/hpg* homozygous females. The weights of the ovaries and uteri from transgenic *hpg* females were slightly higher, but not significantly, than those of the normal females. The weights of the adrenals of the three groups of male and

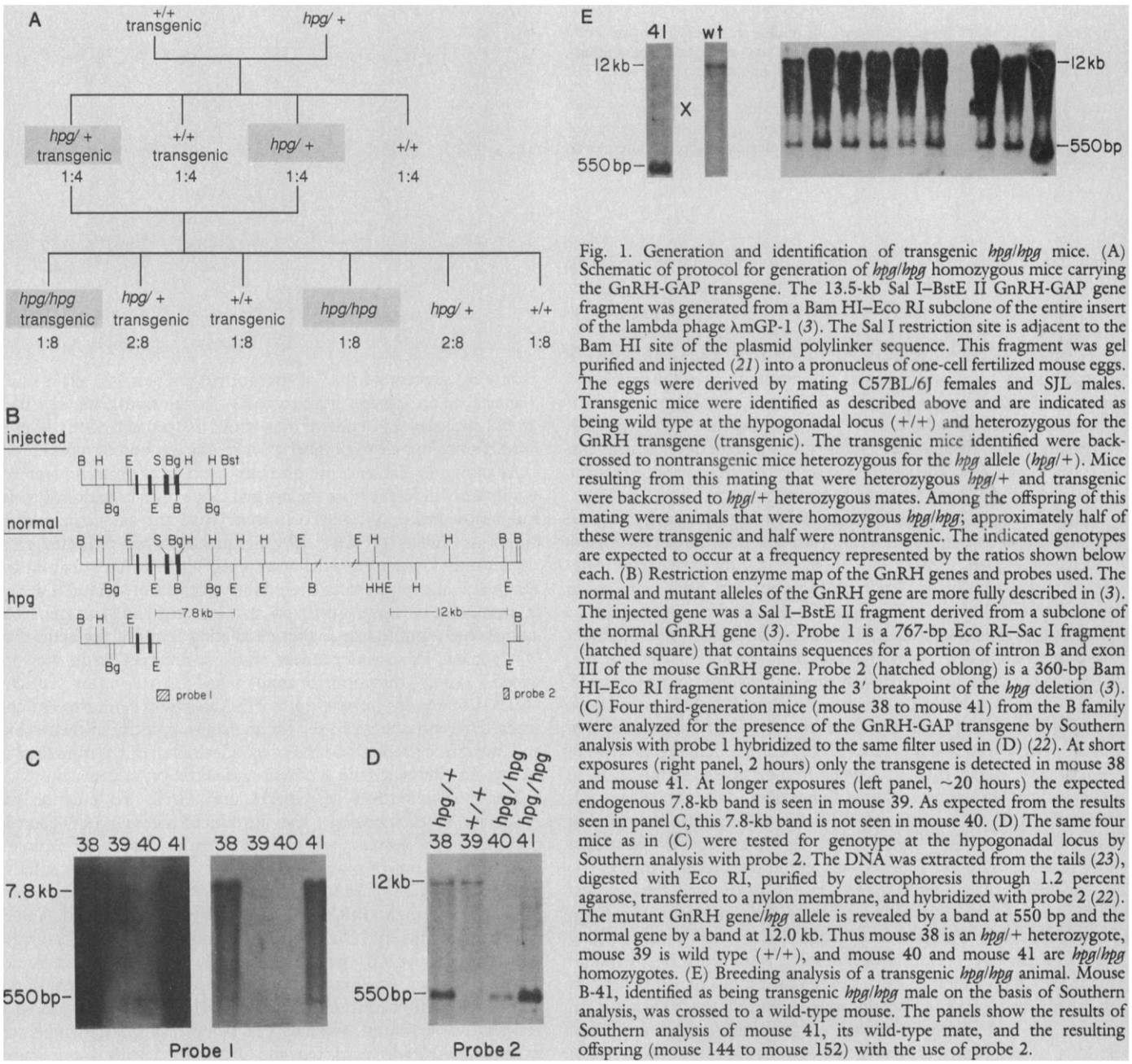
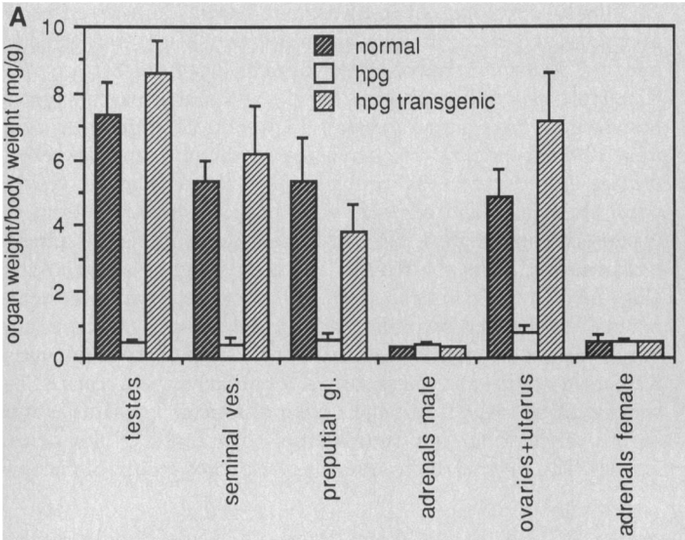
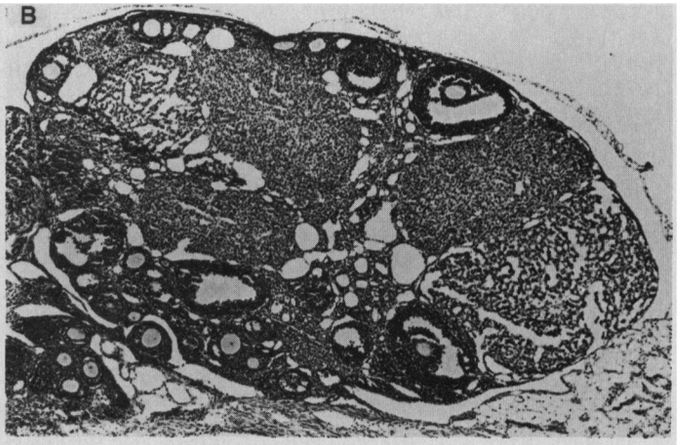


Fig. 1. Generation and identification of transgenic *hpg/hpg* mice. (A) Schematic of protocol for generation of *hpg/hpg* homozygous mice carrying the GnRH-GAP transgene. The 13.5-kb Sal I–BstE II GnRH-GAP gene fragment was generated from a Bam HI–Eco RI subclone of the entire insert of the lambda phage λmGP-1 (*3*). The Sal I restriction site is adjacent to the Bam HI site of the plasmid polylinker sequence. This fragment was gel purified and injected (*21*) into a pronucleus of one-cell fertilized mouse eggs. The eggs were derived by mating C57BL/6J females and SJL males. Transgenic mice were identified as described above and are indicated as being wild type at the hypogonadal locus (+/+) and heterozygous for the GnRH transgene (transgenic). The transgenic mice identified were backcrossed to nontransgenic mice heterozygous for the *hpg* allele (*hpg*/+). Mice resulting from this mating that were heterozygous *hpg*/+ and transgenic were backcrossed to *hpg*/+ heterozygous mates. Among the offspring of this mating were animals that were homozygous *hpg/hpg*; approximately half of these were transgenic and half were nontransgenic. The indicated genotypes are expected to occur at a frequency represented by the ratios shown below each. (B) Restriction enzyme map of the GnRH genes and probes used. The normal and mutant alleles of the GnRH gene are more fully described in (*3*). The injected gene was a Sal I–BstE II fragment derived from a subclone of the normal GnRH gene (*3*). Probe 1 is a 767-bp Eco RI–Sac I fragment (hatched square) that contains sequences for a portion of intron B and exon III of the mouse GnRH gene. Probe 2 (hatched oblong) is a 360-bp Bam HI–Eco RI fragment containing the 3′ breakpoint of the *hpg* deletion (*3*). (C) Four third-generation mice (mouse 38 to mouse 41) from the B family were analyzed for the presence of the GnRH-GAP transgene by Southern analysis with probe 1 hybridized to the same filter used in (D) (*22*). At short exposures (right panel, 2 hours) only the transgene is detected in mouse 38 and mouse 41. At longer exposures (left panel, ~20 hours) the expected endogenous 7.8-kb band is seen in mouse 39. As expected from the results seen in panel C, this 7.8-kb band is not seen in mouse 40. (D) The same four mice as in (C) were tested for genotype at the hypogonadal locus by Southern analysis with probe 2. The DNA was extracted from the tails (*23*), digested with Eco RI, purified by electrophoresis through 1.2 percent agarose, transferred to a nylon membrane, and hybridized with probe 2 (*22*). The mutant GnRH gene/*hpg* allele is revealed by a band at 550 bp and the normal gene by a band at 12.0 kb. Thus mouse 38 is an *hpg*/+ heterozygote, mouse 39 is wild type (+/+), and mouse 40 and mouse 41 are *hpg/hpg* homozygotes. (E) Breeding analysis of a transgenic *hpg/hpg* animal. Mouse B-41, identified as being transgenic *hpg/hpg* male on the basis of Southern analysis, was crossed to a wild-type mouse. The panels show the results of Southern analysis of mouse 41, its wild-type mate, and the resulting offspring (mouse 144 to mouse 152) with the use of probe 2.





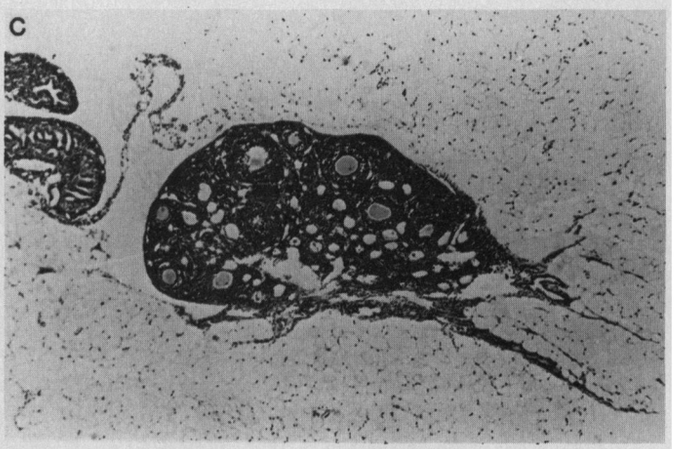


Fig. 2. (A) Organ weights (means in milligrams per gram of body weight ± standard deviation) in normal, *hpg*, and transgenic *hpg* animals. The results were obtained from 10-week-old normal female (n = 6) and male (n = 6) mice; 8-week-old *hpg* females (n = 6), and males (n = 7); 12-week-old transgenic *hpg* females (n = 5) and males (n = 4) of the B family. (B and C) Micrograph of ovaries of a transgenic *hpg* female (B) compared with that of a *hpg* female (C). Well-developed follicles and corpus luteum can be seen in the transgenic *hpg* ovary.

female mice were comparable. These results showed that circulating gonadotropins of sufficient concentration to stimulate steroid-dependent gonadal growth had been restored in the transgenic *hpg* mice.

Histological examination of the testes from transgenic *hpg*/*hpg* homozygotes showed a normal arrangement of fully developed interstitial tubules, Leydig cells, and Sertoli cells. Examination of histological sections of the ovaries from transgenic *hpg*/*hpg* females revealed characteristic follicles at different stages of development and corpora lutea (Fig. 2B), confirming our earlier observations that the females were undergoing normal estrous cycles.

Gonadotropin levels in transgenic *hpg*/*hpg* homozygotes. The fact that the transgenic *hpg* homozygotes had fully developed gonads and accessory glands and produced offspring showed that a normally functioning reproductive endocrine system had been restored in these animals. To confirm this, serum levels and pituitary contents of luteinizing hormone, follicle-stimulating hormone, and prolactin were determined in 12-week-old transgenic *hpg* mice of the B family. These values were compared with those of *hpg*/*hpg* homozygotes (8 weeks of age) and normal controls (10 weeks of age). The results are summarized in Table 1. Serum levels of FSH in transgenic *hpg* females were essentially identical to normal females and were significantly higher than in *hpg*/*hpg* homozygotes. Serum FSH in transgenic *hpg* males was slightly higher than in the control animals but well within the normal range. FSH levels were also measured in a male transgenic *hpg* mouse and a female transgenic *hpg* mouse from the A family and found to be within the normal range.

The pituitary content of FSH in transgenic *hpg* females was slightly lower than normal controls but significantly higher than *hpg* homozygotes. In transgenic *hpg* males and normal mice, pituitary FSH contents did not differ significantly, both being clearly higher than *hpg* homozygotes. The LH levels in serum (Table 1) of transgenic *hpg* females and males were somewhat lower than normal levels, yet both were much higher than *hpg*/*hpg* homozygotes. Pituitary LH content in transgenic *hpg* female mice was approximately 50 percent of that of the normal animals but again much higher than in *hpg*/*hpg* homozygotes. In the transgenic *hpg* male group, pituitary LH content was about 70 percent of the level in normal mice but clearly higher than in *hpg*/*hpg* homozygotes.

As shown in Table 1, the pituitary content of prolactin was not significantly different from the normal range in transgenic *hpg* males and female mice, a result consistent with the prolactin release-inhibiting effects of GAP (*5*). Serum levels of prolactin were significantly lower in normal and transgenic *hpg* males than in the *hpg* males, also supporting the inhibitory effect of GAP. However, in females the opposite was found, that is, normal and transgenic *hpg* animals had significantly higher circulating levels of prolactin than *hpg* females. In an independent study, a different result was obtained, namely, that normal animals had higher serum prolactin values than *hpg*/*hpg* homozygotes (*7*). This could be due to age and strain differences between the three groups, cyclicity in the normal and transgenic groups, and the effect of estradiol or other, unknown factors that influence the synthesis and secretion of prolactin (*8*).

Tissue distribution of GnRH and GAP. To examine the specificity of expression of the introduced GnRH-GAP gene in transgenic *hpg* animals, we analyzed various tissues by radioimmunoassay. Brains of nine transgenic *hpg* mice (four males and five females from family B) at approximately 12 weeks of age were extracted, and their GnRH and GAP contents determined. Values were compared with those of *hpg*/*hpg* homozygotes (8 weeks of age) and normal mice (10 weeks of age). In addition to brain, the following tissues were extracted and assayed for their content of the two peptides from one individual of each group: testes, seminal vesicles, ovaries, uterus, adrenal, liver, kidney, pancreas, thymus, and spleen. GnRH was extracted and determined with a previously

described assay based on a "conformational" antibody, EL-14 (*9*). This antibody detects only the mature processed form of the decapeptide with blocked terminals. GAP was extracted and assayed (*10*) with an antibody (56A) produced against residues 20 to 43 of rat GAP. Rat GAP and mouse GAP are essentially identical in the region recognized by this antibody, with only one amino acid change (*3, 4*). The results of these experiments are summarized in Table 2.

The overall tissue distribution of GnRH immunoreactivity and GAP immunoreactivity in the transgenic *hpg*/*hpg* homozygotes resembled that of normal animals. In all tissues found to contain GnRH and GAP, GAP levels on a molar basis were always twofold higher than that of GnRH. This reflects the ratio of precursor to processed hormone, since the GAP antibody can also detect the unprocessed GnRH-GAP precursor (*11*). Brain and testicular contents of both GnRH and GAP or prohormone in the transgenic *hpg*/*hpg* homozygous males were within the range of normal levels as shown in Table 2. The only major difference between normal and transgenic *hpg* males was the presence of very low levels of the two gene products in the livers of the latter. In females, contents of

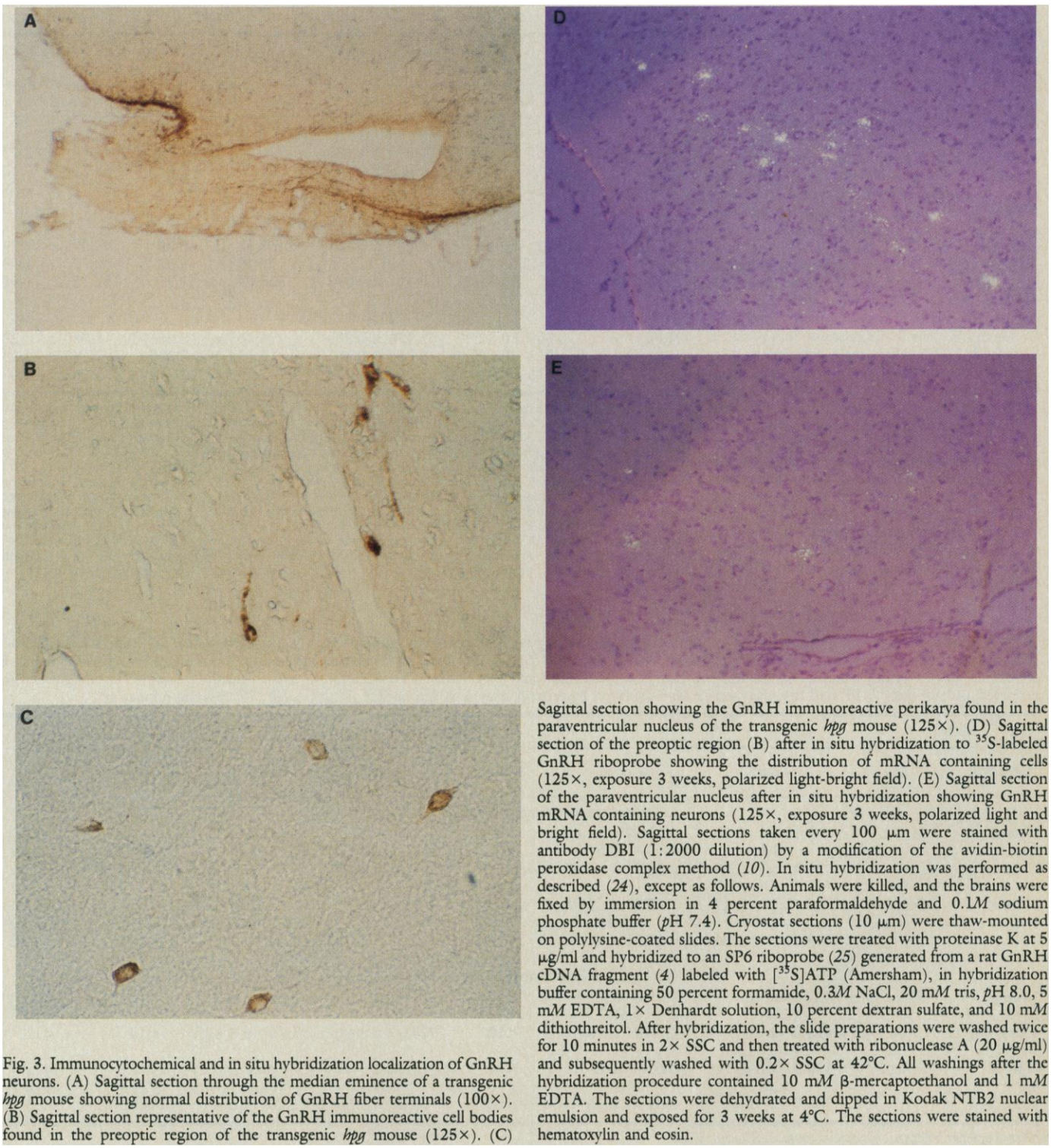


Fig. 3. Immunocytochemical and in situ hybridization localization of GnRH neurons. (A) Sagittal section through the median eminence of a transgenic *hpg* mouse showing normal distribution of GnRH fiber terminals (100×). (B) Sagittal section representative of the GnRH immunoreactive cell bodies found in the preoptic region of the transgenic *hpg* mouse (125×). (C) Sagittal section showing the GnRH immunoreactive perikarya found in the paraventricular nucleus of the transgenic *hpg* mouse (125×). (D) Sagittal section of the preoptic region (B) after in situ hybridization to ^{35}S-labeled GnRH riboprobe showing the distribution of mRNA containing cells (125×, exposure 3 weeks, polarized light-bright field). (E) Sagittal section of the paraventricular nucleus after in situ hybridization showing GnRH mRNA containing neurons (125×, exposure 3 weeks, polarized light and bright field). Sagittal sections taken every 100 μm were stained with antibody DBI (1:2000 dilution) by a modification of the avidin-biotin peroxidase complex method (*10*). In situ hybridization was performed as described (*24*), except as follows. Animals were killed, and the brains were fixed by immersion in 4 percent paraformaldehyde and 0.1*M* sodium phosphate buffer (*p*H 7.4). Cryostat sections (10 μm) were thaw-mounted on polylysine-coated slides. The sections were treated with proteinase K at 5 μg/ml and hybridized to an SP6 riboprobe (*25*) generated from a rat GnRH cDNA fragment (*4*) labeled with [^{35}S]ATP (Amersham), in hybridization buffer containing 50 percent formamide, 0.3*M* NaCl, 20 m*M* tris, *p*H 8.0, 5 m*M* EDTA, 1× Denhardt solution, 10 percent dextran sulfate, and 10 m*M* dithiothreitol. After hybridization, the slide preparations were washed twice for 10 minutes in 2× SSC and then treated with ribonuclease A (20 μg/ml) and subsequently washed with 0.2× SSC at 42°C. All washings after the hybridization procedure contained 10 m*M* β-mercaptoethanol and 1 m*M* EDTA. The sections were dehydrated and dipped in Kodak NTB2 nuclear emulsion and exposed for 3 weeks at 4°C. The sections were stained with hematoxylin and eosin.

GnRH and GAP or prohormone in the central nervous system (CNS) were close to those of normal animals. Gonadal content of the two peptides was slightly less than normal. In the transgenic *hpg* female a low amount of GnRH and GAP was again detected in the liver. Liver-specific expression of GnRH and GAP was also demonstrated in transgenic *hpg* animals of the other transgenic line. Because of the sensitivity of the radioimmunoassay, we cannot discount that an equally low level of expression is also occurring in other tissues. Extremely low levels of GnRH and GAP were detected in the kidneys of normal and transgenic *hpg* males, but only GAP was detected in the kidney of transgenic *hpg* females. The finding of GnRH and GAP in the kidney may be attributable to the fact that the kidney is the major degradation site for GnRH (*12*) and presumably for GAP.

Localization of GnRH-GAP neurons in *hpg* transgenic animals. The distribution and density of cells containing GnRH were compared between normal and transgenic *hpg* animals of the A and B family by immunocytochemistry and in situ hybridization (Fig.

Table 1. Serum levels and pituitary content of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (PRL) in transgenic *hpg*/*hpg* homozygotes (Trans. *hpg*) compared with normal and *hpg* control groups. Hormone concentrations were determined by double-antibody radioimmunoassays (RIA) as described (*26*) using materials provided by the National Hormone and Pituitary Program, NIADDK, for the corresponding rat hormones. These included FSH-RP-2, FSH-I-6, FSH-S-11, LH-RP-2, LH-I-6, LH-S-9, PRL-RP-3, PRL-I-5, and PRL-S-9. The assay sensitivities allowed individual determinations of FSH and PRL concentrations in both serum and pituitary and LH in pituitary but sera from two animals per group had to be pooled to obtain a large enough sample for the LH RIA. There were obvious substantial differences between males and females for pituitary FSH, serum FSH, pituitary LH, and serum prolactin. Hence, these data were analyzed separately for males and females. However, analysis of variance with sex and treatment group indicated no notable differences between males and females for serum LH ($P = 0.1032$) or pituitary prolactin ($P = 0.1862$). Therefore, males and females were combined for the analysis of these data. Two-sample *t* tests were used to compare each pair of treatment groups. The *P* values (unadjusted for multiple comparisons) are listed below. These were adjusted for the fact that multiple comparisons were being made. Conservatively, Bonferroni's inequality was used for this adjustment (*27*). Of all the *P* values listed, only one changed from being significant ($P = 0.0289$) to nonsignificant as a result of this adjustment. This was in the case of serum FSH between normal males compared to transgenic males, and therefore the difference between these groups is questionable.

Mouse genotype	Serum level (ng/ml ± SD)		Pituitary content (ng/pituitary ± SD)	
	Male	Female	Male	Female
	*Follicle-stimulating hormone (FSH)**			
Normal	27.957 ±4.809	12.390 ± 4.430	320.87 ± 66.16	44.79 ± 3.97
hpg	3.679 ±1.490	4.906 ± 1.248	32.59 ± 2.67	18.75 ± 0.17
Trans. *hpg*	38.650 ±6.553	12.332 ± 5.358	332.89 ± 28.59	33.72 ± 1.55
	Luteinizing hormone (LH)†			
Normal	0.42 ±0.12	0.48 ± 0.18	1168.36 ± 116.04	299.68 ± 19.89
hpg	<0.05	<0.05	79.19 ± 4.53	48.52 ± 1.58
Trans. *hpg*	0.27 ±0.11	0.31 ± 0.09	833.04 ± 41.93	152.15 ± 11.44
	Prolactin (PRL)‡			
Normal	0.940 ±0.327	2.970 ± 0.800	3.165 ± 0.665	3.080 ± 0.230
hpg	1.753 ±0.720	1.264 ± 0.461	2.055 ± 0.200	2.195 ± 0.086
Trans. *hpg*	0.676 ±0.203	2.747 ± 0.267	2.700 ± 0.210	2.765 ± 0.405

**FSH pituitary levels*: transgenic *hpg* females versus normal females ($P < 0.00005$), both versus *hpg*/*hpg* homozygotes ($P < 0.00005$). Transgenic *hpg* males versus normal males ($P = 0.4066$), both versus *hpg*/*hpg* homozygotes, significance ($P < 0.00005$). FSH serum levels: transgenic *hpg* females versus normal females ($P = 0.9898$), normal versus *hpg*/*hpg* homozygotes ($P < 0.0089$), transgenic versus *hpg*/*hpg* homozygotes ($P = 0.0141$). Transgenic *hpg* males versus normal males ($P = 0.0289$), both versus *hpg*/*hpg* homozygotes, significance ($P < 0.00005$). †*LH pituitary levels*: transgenic *hpg* females versus normal females ($P = 0.0002$), both versus *hpg*/*hpg* homozygotes ($P < 0.00005$). Transgenic *hpg* males versus normal males ($P < 0.00005$), both versus *hpg*/*hpg* homozygotes, significance ($P < 0.00005$). LH serum levels: transgenic *hpg* versus normal ($P = 0.00002$), both versus *hpg*/*hpg* homozygotes ($P = 0.00005$). ‡*PRL pituitary levels*: transgenic *hpg* versus normal ($P = 0.0040$), normal versus *hpg*/*hpg* homozygotes ($P < 0.00005$), transgenic versus *hpg*/*hpg* homozygotes ($P < 0.0055$). *PRL serum levels*: transgenic *hpg* females versus normal females ($P = 0.0002$), both versus *hpg*/*hpg* homozygotes ($P = 0.00005$). Transgenic *hpg* males versus normal males ($P = 0.0084$), both versus *hpg*/*hpg* homozygotes, significance ($P < 0.00005$).

Table 2. Tissue contents of GnRH and GAP in normal, *hpg*/*hpg* homozygous, and transgenic *hpg*/*hpg* homozygous (transgenic *hpg*) male and female mice. Brains from 10-week-old normal mice ($n = 6$), 8-week-old *hpg* mice ($n = 6$), and 12-week-old transgenic *hpg* mice ($n = 5$ females, $n = 4$ males, family B) were removed and quickly frozen in liquid nitrogen. The other tissues indicated were obtained from one mouse in each group. Tissues were homogenized and extracted as described (*9, 10*) and the supernatants were analyzed by protein assay (*28*) and GnRH (*9*) and GAP (*10*) radioimmunoassays.

Mouse		Tissue content (pg/mg)*						
Genotype	Sex	Brain	Gonads	Adrenals	Liver	Kidneys	Pancreas	Spleen plus thymus
		Gonadotropin-releasing hormone (GnRH)						
Normal	Male	41.15 ± 3.86	28.39 ± 4.68	<0.40	<0.40	0.59 ± 0.11	<0.40	<0.40
hpg		<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40
Trans. *hpg*		35.82 ± 2.89	26.91 ± 4.85	<0.40	1.45 ± 0.13	0.68 ± 0.18	<0.40	<0.40
Normal	Female	26.75 ± 2.17	17.45 ± 1.98	<0.40	<0.40	<0.40	<0.40	<0.40
hpg		<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40
Trans. *hpg*		19.89 ± 1.86	12.21 ± 0.95	<0.40	0.52 ± 0.11	<0.40	<0.40	<0.40
		GnRH-associated peptide (GAP)						
Normal	Male	432.0 ± 88	295.0 ± 16	<10	<10	11.2 ± 3.3	<10	<10
hpg		<10	<10	<10	<10	<10	<10	<10
Trans. *hpg*		495.0 ± 83	366.0 ± 93	<10	24.0 ± 3.4	15.2 ± 5.4	<10	<10
Normal	Female	516.0 ± 181	368.0 ± 88	<10	<10	<10	<10	<10
hpg		<10	<10	<10	<10	<10	<10	<10
Trans. *hpg*		385.0 ± 107	184.0 ± 35	<10	15.6 ± 5.5	27.0 ± 6.9	<10	<10

*Picograms per milligram of protein, means ±SD.

3). Transgenic *hpg/hpg* homozygous mice of both sexes showed immunoreactive cells in the medial preoptic and septal nuclei, superchiasmatic region of the hypothalamus, the bed nucleus of the stria terminalis, the olfactory bulbs, and paraolfactory regions. The same distribution and approximately the same number of cells was seen after in situ hybridization of serial sections to a ^{35}S-labeled GnRH-GAP probe. An additional group of cells was noted in the paraventricular nucleus (PVN) of the thalamus in transgenic animals that was not seen in normal controls (Fig. 3, C and E). Unlike the typical GnRH neurons observed in the hypothalamus, the GnRH-containing PVN neurons did not display any immunoreactive processes (Fig. 3C). Immunoreactive cells outnumbered those found in the preoptic-septal region and were found concentrated along the midline following the curve of the third ventricle from beneath the habenular nucleus and proceeding ventrally through the PVN and the paratenial nucleus. These cells extended ventrally past the third ventricle into the bed nucleus of the stria terminalis, contributing to the increased cell counts in this region. The GnRH content of these cells is approximately five times lower than that found in other areas of the brain as judged by the intensity of immunocytochemical staining (Fig. 3, B compared to C) and the grain densities after in situ hybridization (Fig. 3, D compared to E). These results demonstrate that sequence elements present on the transgene can direct GnRH synthesis in all the normal neuronal sites of GnRH expression as well as the thalamic PVN with high specificity.

Phenotype of rescued mice demonstrates regulated GnRH gene expression. Our results demonstrate that the phenotypic effects of the *hpg* mutation can be reversed by the germ-line integration of an intact mouse GnRH-GAP gene contained on a 13.5-kb DNA fragment. Transgenic animals homozygous for the *hpg* allele developed fully their reproductive functions. Females had estrous cycles and could conceive and carry healthy litters to term. Males displayed correct mating behavior. This phenotypic reversal is due to the neural-specific as well as developmentally regulated expression of the GnRH-GAP precursor gene and the subsequent restoration of the hypothalamic-pituitary-gonadal axis. It is important to note that the hypothalamic neurons expressing the transgene must be correctly controlled in pulsatility and amplitude of GnRH release. This control is believed to occur through a complex set of neural connections with the GnRH-secreting cells (*6*). These connections are probably not affected by the gene deletion and therefore are intact in the *hpg* mouse or, conversely, are established as a result of embryonal GnRH expression. The importance of this control is illustrated by the failure of preoptic area (POA) brain grafts in the adult *hpg* brains to restore normal reproductive functions in the mutant mouse (*13, 14*). Thus grafted *hpg* females are in a constant estrous state and exhibit reflux ovulation (*13*), and grafted males develop near normal-sized testes but display no mating behavior (*14*). The inability of grafted *hpg* females to maintain estrous cycles indicates that GnRH expression is not being correctly regulated. The failure of these males to mate can be traced to a lack of testosterone in the neonatal *hpg* males, the presence of which is required for adult mating behavior to ensue (*15*). The development of normal-sized testes was never achieved in grafted males (*14*) but occurred in transgenic males, indicating either a requirement for pre- or neonatal GnRH expression for the presence of testicular GnRH or GAP (or both).

As expected from the phenotype of the rescued mice, immunocytochemical and in situ hybridization studies on the brains of transgenic animals revealed a normal number of GnRH-containing neurons in every brain area previously reported to be a site of GnRH expression (*16*). We have shown (*3*) that *hpg* brains contain no detectable GnRH by immunocytochemistry but that distinct neurons in the hypothalamus express an aberrant GnRH mRNA at a low level. Our results showing a good overlap of cells positive for GnRH expression by in situ hybridization and immunocytochemistry indicate that the transgene is being expressed in the same neurons that also express the aberrant *hpg* GnRH mRNA. In addition, a discrete population of neurons in the PVN of the thalamus, which have never been observed to express GnRH, exhibited a low level of GnRH expression. Such uncharacteristic expression was also observed at a low level in the livers of rescued animals. The finding of GnRH expression in cells in PVN of the thalamus and in the liver could be explained by the absence of a negative regulatory element in the transgene. Alternatively, the high transgene copy number could be titrating out a negative repressor present in these cell types. Neither of these sites of expression should influence GnRH concentrations in pituitary portal blood. Cells from the PVN of thalamus do not extend to the median eminence, and any GnRH released from the liver is expected to be rapidly degraded by GnRH-degrading enzymes present in peripheral plasma (*17*).

The major finding of our study is that the sequences present on the 13.5-kb GnRH genomic fragment are sufficient to direct quantitative GnRH and GAP expression in specific neural areas as well as in the testes and ovaries. Such tissue-specific expression has been reported for several other genes (*18*), but our results provide, in addition, an example of neural-specific expression in a transgenic animal.

The use of gene replacement to rescue the *hpg* mutation illustrates well the potential uses of gene therapy. Particularly striking is the ability to obtain qualitative and quantitative neural-specific expression and the consequent restoration of the normal circulating levels of the gonadotropins and prolactin. The ability to cure genetic defects by gene replacement has been reported in other systems. A murine β-thalassemia was reversed by the introduction of the natural human β-globin gene (*19*). Attempts to correct a growth deficiency in the dwarf little (*lit*) mouse, by the introduction of the human and rat growth hormone genes failed to reverse this defect. The use of a heterologous gene fusion (metallothionein–rat growth gene) did restore growth to the dwarf mice, but these mice had vastly elevated growth hormone levels, and the females were sterile (*20*).

The approach that we have described above can now be extended and used to define the individual roles of GnRH and GAP in regulating gonadotropin release and the role of GAP in modulating prolactin secretion. This neuroendocrinological issue can be further pursued by studying transgenic *hpg* animals containing a mutated GnRH and intact GAP coding sequence and vice versa.

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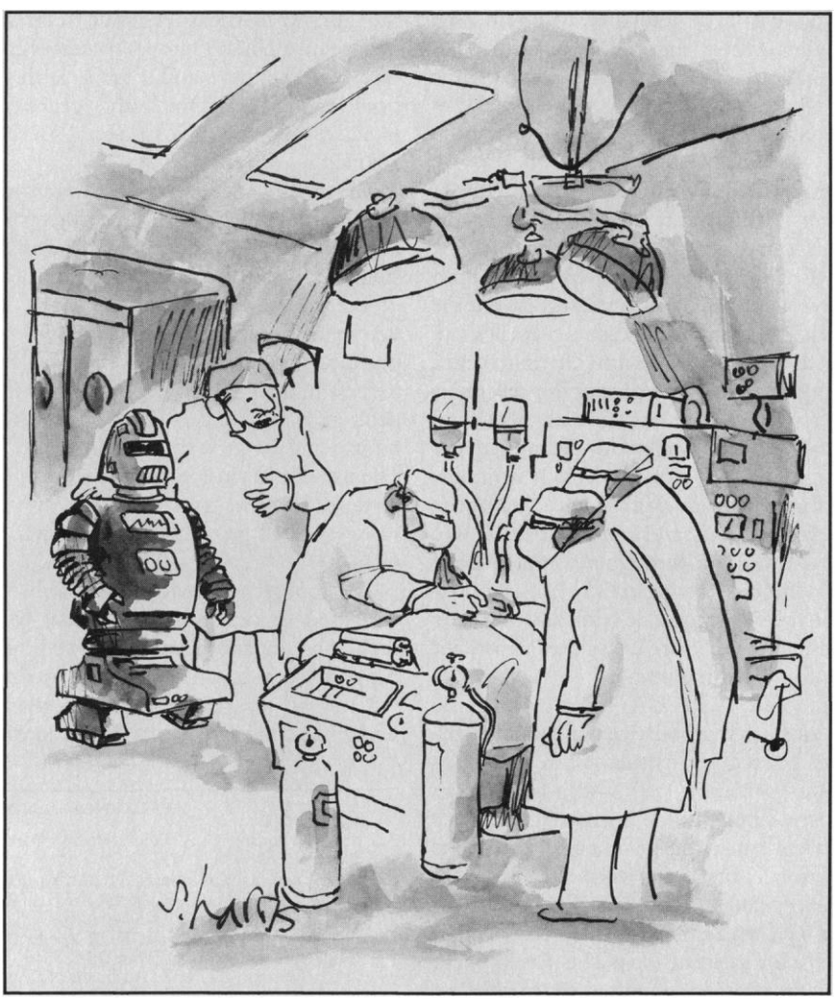

"Dr. KT-25 will now install the artificial heart."