Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene

(homologous recombination/gene targeting/fertility)

DENNIS B. LUBAHN^{*†‡§}, JEFFREY S. MOYER^{*}, THOMAS S. GOLDING^{¶||}, JOHN F. COUSE[¶],
KENNETH S. KORACH^{¶**}, AND OLIVER SMITHIES^{*}

Departments of ^{*}Pathology and [†]Pediatrics and [‡]Laboratories for Reproductive Biology, University of North Carolina, Chapel Hill, NC 27599; and [§]Receptor Biology Section and [¶]Gamete Biology Section, Laboratory of Reproduction and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, P.O. Box 12233, Research Triangle Park, NC 27709

Contributed by O. Smithies, September 1, 1993

ABSTRACT Estrogen receptor and its ligand, estradiol, have long been thought to be essential for survival, fertility, and female sexual differentiation and development. Consistent with this proposed crucial role, no human estrogen receptor gene mutations are known, unlike the androgen receptor, where many loss of function mutations have been found. We have generated mutant mice lacking responsiveness to estradiol by disrupting the estrogen receptor gene by gene targeting. Both male and female animals survive to adulthood with normal gross external phenotypes. Females are infertile; males have a decreased fertility. Females have hypoplastic uteri and hyperemic ovaries with no detectable corpora lutea. In adult wild-type and heterozygous females, 3-day estradiol treatment at 40 $\mu\text{g}/\text{kg}$ stimulates a 3- to 4-fold increase in uterine wet weight and alters vaginal cornification, but the uteri and vagina do not respond in the animals with the estrogen receptor gene disruption. Prenatal male and female reproductive tract development can therefore occur in the absence of estradiol receptor-mediated responsiveness.

The estrogen receptor (ER) is a member of the steroid receptor superfamily of ligand-activated transcription factors (1). ER and its hormone ligand, the female sex steroid 17 β -estradiol, have long been known to play critical roles in the development of feminine secondary sexual characteristics as well as in the female reproductive cycle, infertility, and maintenance of pregnancy. Estradiol is also thought to be essential for embryonic and fetal development (2).

Supporting evidence for the crucial role of estrogen is based on pharmacological and genetic data that estrogen antagonists and inhibitors of estrogen synthesis interfere with placental function and cause abortions (2). Overexpression mutations in the mammalian estrogen biosynthetic enzyme, aromatase, have been reported (3) to lead to gynecomastia in males because of excess conversion of androgens to estrogens. Lack of function mutations in human placental aromatase have been reported to cause maternal virilization and fetal pseudohermaphroditism in female offspring (4). This observation suggested the need for estrogens in normal prenatal sexual development. This interpretation may be confounded, however, by the effects of secondarily increased androgen concentrations. In addition, the absence of reported human ER mutations supports the suggestion that if natural ER functional mutations do occur, they may be lethal in eutherian animals (2).

Lack of known ER mutations is contrasted with the situation in regards to the male sex steroids, the androgens.

Mutations in genes for the androgen receptor protein or for androgen synthetic enzymes result in abnormalities in male sexual differentiation and development (5–7) with little effect in females except for a decrease in fertility (8).

The role of estrogen action in prenatal sexual development, however, is controversial. Endocrinological evidence for the importance of estrogens in sexual development is found in rat and rabbit embryogenesis studies (9, 10), where estrogen synthesis is activated in male and female embryos at the time of blastocyst implantation in the uterus. Recently, ER mRNA has been detected by the very sensitive reverse transcriptase/PCR technique in blastocysts and two-cell-stage embryos (11). In contrast, Jost (12), in a series of classical organ ablation experiments, demonstrated that fetal gonadectomy of mammalian males and females resulted in both sexes developing as phenotypic females, suggesting that estrogen is not needed for female sexual development. However, the presence of maternal and placental estrogens left the question of estrogen's importance in prenatal sexual development unresolved.

Nevertheless, there is no doubt that estrogen plays a central role in normal postnatal female physiology and in female pathology, where its importance in breast and uterine cancer, osteoporosis, and cardiovascular disease is well known although poorly understood. Besides the normal physiology and endocrinology of estrogen action, the mechanisms for hormonal stimulation have also been postulated to involve other cellular signaling mechanisms (13–15).

Because of the various uncertainties regarding the roles of estrogen, we decided to disrupt the ER gene in mouse embryonic stem cells by homologous recombination, so as to create an animal lacking a functional estrogen receptor. Experimental development of this animal model was expected to provide a clear role for ER action in a variety of systems under physiological conditions.

MATERIALS AND METHODS

Gene-Targeting Plasmid. The mouse ER gene has been cloned and found to have nine exons (16). Utilizing the published sequence and PCR (17) with oligonucleotide primer pairs 1–2 and 3–4 (see below), we amplified two DNA fragments from the most N-terminal exon of the mouse ER gene. The two amplified fragments were used as probes to allow the cloning of a 10-kb *Bam*HI fragment containing the

Abbreviations: ER, estrogen receptor; TK, thymidine kinase; PGK, phosphoglycerate kinase; ES, embryonic stem; Neo, neomycin resistance.

[§]Present address: Department of Cellular Biochemistry, Glaxo Inc. Research Institute, Durham, NC 27709.

^{**}To whom reprint requests should be addressed.

second exon of the mouse ER gene from a lambda Dash (Stratagene) library constructed from *Bam*HI-digested DNA obtained from E14TG2a cells (18). To assemble the targeting construct (Fig. 1*B*), a Neo gene driven by the phosphoglycerate kinase (PGK) promoter and having a PGK poly(A) addition signal (19) was cloned in a 5'-to-3' orientation in a *Not* I site within exon 2 contained in a 7.5-kb *Bam*HI/*Spe* I fragment, made from the 10-kb *Bam*HI fragment. This insertion disrupts the reading frame. A flanking herpes simplex TK gene driven by the same promoter and enhancer was added to the 3' end of the *Bam*HI/*Spe* I fragment (20). Because of difficulty in cloning of the 5' 7-kb *Bam*HI/*Not* I fragment in plasmids, the targeting construct was assembled in a lambda Dash bacteriophage vector. The Neo gene/*Not* I/*Spe* I fragment and TK gene were first cloned in pBlue-script (Stratagene) and then subcloned in a lambda Dash vector containing the ER 7-kb *Bam*HI/*Not* I fragment. A *Bam*HI site in the Neo gene was destroyed prior to cloning in lambda Dash vector so that *Bam*HI could be used to remove the λ arms.

Embryonic Stem (ES) Cells. The ES cell line E14TG2a, derived originally from a 129/J strain mouse (18), was cultured on primary embryonic fibroblast feeder layers previously irradiated with 3000 rads (30 Gy); the fibroblasts were isolated from neomycin-resistant embryos (21) and were resistant to G418 (Sigma).

Electroporation. Electroporation of about 1×10^7 ES cells was in 0.5 ml of Dulbecco's modified Eagle's medium (DMEM)/15% fetal bovine serum/0.1 mM 2-mercaptoethanol/2 mM glutamine with 5 nM targeting construct with 1-sec discharge from a 150- to 250- μ F capacitor charged to 250–400 V. In early electroporations the λ arms were removed from the targeting construct, but in later ones they were not removed.

Selection. After electroporation, the surviving cells were plated in 100-mm Petri dishes and exposed for 10–14 days to G418 at 200 μ g/ml and 1 μ M ganciclovir (Syntex, Palo Alto, CA). Colonies were picked into 24-well plates (15-mm diameter wells) and grown for 10 days, when about one-tenth of a colony was replated into a single well of the 24-well plates, with the remainder being used to isolate DNA. DNA pre-

pared from these plates was pooled into groups of four and was screened by PCR amplification to detect targeting (22).

PCR Primers. Oligo 1, used for making the 5' probe during cloning of the ER gene: 5'-CGCTGCTGAGCCCTCTGCGTG-3'. Oligo 2, used for making the 5' probe during cloning of ER gene: 5'-GTTGAACCTCGTAGGCGGCGCCCTC-3'. Oligo 3, used for making the 3' probe during cloning of the ER gene and for determination of the presence of the wild-type ER gene: 5'-CGGTCTACGGCCAGTCGGGCACC-3'. Oligo 4, used for making the 3' probe during cloning of the ER gene and for determination of the presence of the wild-type ER gene: 5'-GTAGAAGGCGGAGGGCCGGTGTC-3'. Oligo 5, from sequence in the 3' end of the PGK gene, used for determination of Neo disruption of the ER gene after recombination: 5'-TTCCACATACATTTCATTCTCA-3'. Oligo 6, from sequence external to the targeting construct in intron 2 of the ER gene, used for determination of Neo disruption of the ER gene after recombination: 5'-CTCCACTGGCCTCAAACACCTG-3'.

PCR Amplification. The 3' end of the PGK gene, containing the poly(A) addition signal, was sequenced. From this sequence, oligo 5 was designed for use in conjunction with oligo 6 to amplify a 649-bp fragment diagnostic of a successfully targeted ER gene (see Fig. 1). For PCR screening the DNA was from pools of four colonies of ES cells. PCR was also used to distinguish normal, heterozygous, and homozygous mutant animals (see Fig. 2).

Mouse Breeding. The targeted ES cells were injected into blastocysts from C57BL/6J mothers and were returned to pseudopregnant C57BL/6J hosts to complete their development. Chimeras were identified by coat color and males were bred to C57BL/6J females. Tail DNA from agouti coat color F₁ offspring was screened by PCR with primers 5 and 6 for presence of the targeted ER gene (a 649-bp fragment). F₂ offspring and subsequent generations were screened by PCR for presence of the targeted ER gene, and with primers 3 and 4 for absence of the wild-type gene (a 239-bp fragment).

RESULTS

Gene Targeting. To make the targeting construct for interrupting the reading frame of the mouse ER gene on chromosome 10 (23), exon 2 of the ER gene was isolated in a 10-kb *Bam*HI genomic DNA fragment (Fig. 1*A* and *B*). Exon 2 contains the start codon and the N-terminal domain of the ER gene, which is important in transcriptional control. The Neo gene was inserted into a *Not* I site in this domain to disrupt the ER reading frame. The TK gene was added to increase the efficiency of finding targeted mouse ES cells.

Electroporation was used to introduce the targeting construct into ES cells, which were then exposed to ganciclovir and G418. About 1800 surviving colonies were screened with primers 5 and 6 to find two in which homologous recombination had occurred as judged by the amplification of the 649-bp fragment diagnostic of targeting (Fig. 1*C*). Southern blot analysis with a Neo probe confirmed that the targeted construct was integrated at a single site (data not shown).

Generating Animals. Cells from one of these targeted ES clones were injected into blastocysts, and four male chimeras and four female chimeras were obtained. After several matings with different males the chimeric females showed no germ-line transmission. Two chimeric males transmitted the targeted gene through their germ line to produce heterozygous male and female animals. Heterozygous animals were then mated to produce homozygous animals. Fig. 2 shows the screening profile for a representative litter from one of the heterozygous matings. Primers 3 and 4, which bracket the Neo integration site in the ER gene, allow detection of the presence of the wild-type ER. PCR amplification was used to distinguish homozygous mutants (a 649-bp band only) from

FIG. 1. Diagram of the portion of the mouse ER gene targeted for disruption. (A) Exon 2 and surrounding sequences. (B) Targeting construct containing the neomycin resistance (Neo) and thymidine kinase (TK) sequences. (C) Disrupted mouse ER gene after targeting, which inserted the Neo gene (hatched) into mouse ER exon 2 (solid black). Restriction enzyme sites: B, *Bam*HI; H, *Hind*III; N, *Not* I; S, *Spe* I. Position of the ATG start site in exon 2 is shown by a right-angle arrow. Identity of PCR primers and their sequence positions are shown by the numbered arrowheads corresponding to the primers listed in *Materials and Methods*.

FIG. 2. PCR analysis of a representative litter from a mating of heterozygotes. Even-numbered lanes represent PCRs using primer sets 5 and 6, and odd-numbered lanes used primer sets 3 and 4. Heterozygote parents are shown in the upper gel, and male ($\square$) and female ($\circ$) offspring are depicted in the lower gel. Two PCR assays have been employed in the genotyping of the mice. Primers 3 and 4 amplify a 239-bp product specific for the wild-type ER gene, and primers 5 and 6 amplify a 649-bp product specific for the disrupted gene.

heterozygotes (a 649-bp and a 239-bp band) and from normal animals (only the 239-bp band). PCR products were further characterized by restriction enzymes: the nontargeted band (239 bp) was cut by *Not* I, producing two bands of expected size 43 and 186 bp. As expected, the targeted 649-bp PCR ER product was not cut with *Not* I; subsequent nucleotide sequencing showed that the targeted fragment contained the predicted sequence.

Breeding Data. A total of 48 heterozygous matings have been performed, generating 361 offspring with a normal average litter size of 8 pups. The genotype frequencies are shown in Table 1. There was no apparent gender preference, since almost equal numbers of male and female progeny were produced throughout all three genotype groups. Relative birth frequency of homozygous mutant animals (ER-disrupted) is slightly lower than expected for a Mendelian distribution and is less than that found in the wild-type group. However, statistical analysis using a χ^2 goodness-of-fit test indicated that the deviation from expectation was not significant ($P = 0.07$).

Phenotype of the Animals. The external phenotypes of animals of both sexes that are homozygous for the disrupted

Table 1. Frequency of pup genotypes from heterozygous matings

	W	WM	M	Total
Males	56 (15)	95 (26)	31 (9)	182 (50)
Females	54 (15)	86 (24)	39 (11)	179 (50)
Total	99 (30)	181 (50)	70 (20)	361 (100)

Numbers of offspring from a total of 48 heterozygous matings are reported for each group: wild-type ER (W), heterozygote (WM), and homozygous mutant ER (M). Numbers in parentheses are the percentage of offspring in each group. χ^2 goodness-of-fit test indicated that the deviation from Mendelian ratio was not significant ($P = 0.07$).

gene were normal. Internally only the females showed noticeable gross differences from normal, with hypoplastic uteri as well as ovaries that lacked corpora lutea (see Fig. 4).

Histologically, the uteri and hyperemic cystic ovaries were abnormal. Fig. 3A shows a histological section of an ovary from a wild-type animal. Primary follicles can be seen, with examples of preantral tertiary follicles and corpora lutea present in the section. In contrast, ovaries from the homozygous mutant animals show classic cystic and hemorrhagic follicles containing few if any granulosa cells (Fig. 3B). A few primary follicles can be seen, but no corpora lutea were observed in these ovaries. The histology of the uterus showed the presence of all major uterine cell types, but the stromal, epithelial, and myometrial tissue compartments were present in diminished size. Uterine glands were present as well as ciliated epithelial cells in the oviduct (data not presented).

Males appear overtly normal, but testis weight was low compared with wild-type or heterozygote animals. On the other hand, seminal vesicle and coagulating gland weights were not significantly different. Sperm were present in the testis and epididymus, but the sperm count was low, only 10% of the control level. Functionality and motility of the sperm from the recessive males are not yet known.

Responses to Estrogen. To test for estrogen responsiveness, adult females were treated for 3 days with estradiol at levels known to increase uterine wet weight, induce hyperemia, and alter vaginal epithelial cytology in normal animals (24). The wild-type and heterozygous animals responded normally compared with untreated controls (Fig. 4A) by an increase in uterine wet weight of 3- to 4-fold (Fig. 4B). The uteri from wild type (Fig. 4B) and heterozygotes (data not presented) became hyperemic after a 3-day bioassay. The uteri of the homozygous ER-disrupted animals did not respond with either an increase in uterine weight wet or hyperemia (Fig. 4C and D). Unlike wild-type and heterozygous animals, ER-disrupted animals treated with estradiol for 4-6 hr showed no uterine water imbibition or uterine hyperemia (data not presented). Vaginal cytology after estradiol treatment showed an increased number of cornified epithelial cells in the wild-type and heterozygous animals but was unaltered in ER-disrupted animals (data not shown).

Estrogen Binding. Ligand-binding analyses of uterine tissue from the ER-disrupted animals showed detectable estradiol binding (25) at about 5% of the wild-type level (60-70 fmol/100 μ g of DNA), with a dissociation constant similar to that of the wild type (0.7 nM). Low concentrations of ER protein were confirmed by an ELISA (Abbott) using two antibodies to the C terminus of the ER protein. "Splicing over" of insertional mutations (26) or nonsense codons (27) has been observed previously. *In vivo* reinitiation of translation after a nonsense mutation, resulting in a ligand-nonresponsive receptor protein, has also been seen previously in the steroid receptor superfamily (28).

Fertility. All homozygous mutant females are infertile. None showed any lordosis posture or receptiveness to wild-type males even when treated with estrogen. This behavior suggests absence of estrogen responsiveness in the central nervous system. Male fertility was tested with harem pairings to known fertile wild-type females. Fertility was reduced, but not abolished, since only 3 of 15 paired males produced any offspring. However, the males that initially demonstrated fertility have not to date sired any more litters after several pairings. No vaginal plugs were found in any of the matings of both sexes of homozygous mutant animals, with the exception of the 3 fertile males.

DISCUSSION

The homozygous mutant mice with ER-disrupted genes appear healthy, and with the exception of fertility problems in

FIG. 3. Histology of mouse ovaries from untreated animals stained with hematoxylin and eosin. (A) Control wild type. ($\times 22$.) (B) ER-disrupted homozygous mutant mice. ($\times 45$.)

both male and female animals, there are no obvious problems in prenatal sexual development. This is surprising because of the known importance of estrogens in breast and uterine growth and because of their effects on cardiovascular disease and in preventing bone loss after menopause and after ovariectomy in mice. Possibly there is a parallel regulatory pathway that can make up for the lack of estrogen responsiveness during prenatal sexual differentiation and development. It is also possible (see below) that some residual functions are retained by the disrupted gene, but in either case, these alternate pathways do not enable the uterus or vagina to respond to estradiol. Growth factors, such as insulin-like growth factor 1, epidermal growth factor, and transforming growth factor α , may be involved in possible alternative regulatory pathways, since they have been linked to estrogen action in uterine tissue (14, 15, 29).

Other members of the steroid receptor superfamily have been targeted by homologous recombination. In some isoforms of the retinoic acid receptor (RAR) family the gene disruptions have resulted in alterations in phenotype while in others they have not (30–32). Disruption of one N-terminal exon of the RAR α -1 and RAR γ -2 isoforms resulted in a

normal phenotype, while disruption of a different N-terminal exon of the RAR α and RAR γ genes produced early postnatal lethality.

Traditional pronuclear injection transgenic techniques which produce overexpression of the ER result in aberrant reproductive phenotypes with alterations in the length of parturition.^{††} However, as in our ER-disrupted animals, no abnormalities in prenatal sexual development are seen, although at the neuroendocrine, end organ, and gonadal level, reproductive function appears to be severely compromised. The observation of apparently normal development of prenatal sexual phenotypes, associated with fertility problems, suggests that ER defects may be responsible for some human infertility problems. It will now be interesting to see if any human male or female infertilities might be caused by ER mutations or in genes regulated by ER. Past reports have suggested silent ER codon polymorphisms may be linked

^{††}Davis, V. L., Couse, J. F., Goulding, E. H., Eddy, E. M. & Korach, K. S., Proceedings of the American Association for Cancer Research, Mechanism of Action of Retinoids, Vitamin D, and Steroid Hormones, C10, 1993, Banff, Canada.

FIG. 4. Uteri from wild-type (A and B) and ER-disrupted homozygous mutant (C and D) mice after treating daily for 3 days with a 100- μ l s.c. injection of propylene glycol vehicle (A and C) or a 40- μ g/kg dose of estradiol in the same vehicle (B and D). ($\times 1$.) Uteri were removed, and the following data are expressed as the ratio of uterine wet weight (mg) to body weight (g). (A) Control wild type given propylene glycol (1.0). (B) Wild type given estradiol (3.8). (C) Homozygous mutant given propylene glycol (0.8). (D) Homozygous mutant given estradiol (0.7). Results are representative of four separate experiments.

with hypertension (34) and spontaneous abortions as well as increased susceptibility to breast cancer (35), but to date there have been no reports of ER changes in relation to fertility. Further studies into the susceptibility of the ER-disrupted mice to hypertension and mammary gland cancer will be valuable in assessing nonestrogenic risk factors. Assessment of bone strength, cardiovascular disease susceptibility, and atherosclerosis during aging will also help us learn more about the roles played by the ER in these pathophysiologicals.

Our observation that we can still detect about 5% of normal estradiol binding in uterine preparations from animals homozygous for the gene disruption and demonstrably unresponsive to estradiol is especially interesting. The binding has the same affinity as determined for normal estrogen receptor. While the gene disruption that we have constructed abolished estrogen responsiveness, it is possible that a protein product able to bind estrogen may still be generated; for example a "splicing over" event may have occurred that eliminated exon 2. Examples are known of exons being "spliced over" when a nonsense codon is found in an exon (27) or when Neo has been inserted into the exon by targeting (26). Examples of mutant ER isoforms lacking different exons have been identified by mRNA analysis from primary tumors or cancer cell lines (36). A recent report has shown in normal rat brain tissue an ER isoform lacking exon 4 (37); however this protein would not be capable of binding ligand. An altered form of the normal receptor may be synthesized after the ER gene disruption. If so, it is incapable of mediating estrogen responsiveness, either because of its structure or because it is synthesized at too low a level. We have shown earlier that uterine responsiveness to estrogen does not occur in mice if receptor levels are decreased to below 20% of control (38).

Interestingly, the testicular feminization mouse (39) does not respond to androgen because of a frameshift mutation in the androgen receptor N-terminal domain. Low levels of androgen receptor binding are detectable. Reinitiation of translation downstream of the nonsense codon is believed to occur, such that the C-terminal ligand-binding domain is present in very low amounts without the N-terminal domain (28). It is tempting to speculate that something similar may be happening with our ER-disrupted mice.

Alternatively, the ER form detected by binding in the homozygous mutant animals may represent another, previously undetected, estrogen-binding protein. Further studies will be needed to characterize the molecular nature and possible function of the residual estrogen binding activity in our homozygous mutant mice.

In any case, the homozygous mutants with the ER-disrupted genes are not physiologically responsive to estrogens by several classical bioassays of estrogenic activity— increase in uterine water imbibition, stimulation of uterine hyperemia, and change in vaginal cornification. Detection of ER early in development (11) and the apparent role of estrogen in blastocyst activation (9, 10, 33) make it remarkable that prenatal sexual development is not altered and that lethality did not occur in the animals with their ER genes disrupted.

We thank Vicky Valancius for ES cell DNA used to construct the bacteriophage library, Nobuyo Maeda for helpful discussions in trouble-shooting the screening of the ES cells, Denise Lee for providing embryonic fibroblast feeder plates, John Hagaman and Paula Oliver for injecting and implanting the blastocysts, Edward Eddy and David Schomberg for discussions regarding reproductive tract morphology, Beth Gladen for the statistical analyses, and Sarah Bronson, Judson Van Wyk, and Richard Paules for critical reading of the manuscript. D.B.L. was a Pew Scholar supported by the Pew Scholars Program in the Biomedical Sciences. The work was sup-

ported in part by a National Institute of Environmental Health Sciences contract to D.B.L. and O.S., and by a National Institute of General Medical Sciences grant (GM20069) to O.S. The scientific contributions of D.B.L., K.S.K., and O.S. should be considered equal.

- Evans, R. M. (1988) *Science* **240**, 889–899.
- George, F. W. & Wilson, J. D. (1988) in *The Physiology of Reproduction*, eds. Knobil, E., Neil, J. D., Ewing, L. L., Greenwald, G. S., Markert, C. L. & Pfaff, D. W. (Raven, New York), pp. 3–26.
- Hemsell, D. L., Edman, C. D., Marks, J. F., Siiteri, P. K. & MacDonald, P. C. (1977) *J. Clin. Invest.* **60**, 455–464.
- Shozu, M., Kazutomo, A., Harada, T. & Kubota, Y. (1991) *J. Clin. Endocrinol. Metab.* **72**, 560–566.
- Lubahn, D. B., Brown, T. R., Simental, J. A., Higgs, H. N., Migeon, C. J., Wilson, E. M. & French, F. S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9534–9538.
- Brown, T. R. & Migeon, C. J. (1987) in *Hormone Resistance and Other Endocrine Paradoxes*, eds. Cohen, M. P. & Foa, P. P. (Springer, New York), pp. 157–203.
- Wilson, J. D., Griffin, J. E., Leshin, M. & MacDonald, P. C. (1983) in *The Metabolic Basis of Inherited Disease*, eds. Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L. & Brown, M. S. (McGraw-Hill, New York), pp. 1001–1026.
- Lyon, M. F. & Glenister, P. H. (1980) *Proc. R. Soc. London B* **208**, 1–12.
- George, F. W. & Wilson, J. D. (1978) *Science* **199**, 200–202.
- Dickman, Z., Gupta, J. S. & Dey, S. (1977) *Science* **195**, 687–688.
- Hou, Q. & Gorski, J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 9460–9464.
- Jost, A. (1971) in *Hermaphroditism: Genital Anomalies and Related Endocrine Disorders*, eds. Jones, H. W., Jr., & Scott, W. W. (Williams & Wilkins, Baltimore), pp. 16–64.
- McEwen, B. S. (1991) *Trends Pharmacol. Sci.* **12**, 141–147.
- Ignar-Trowbridge, D. M., Nelson, K. G., Bidwell, M. C., Curtis, S. W., Washburn, T. F., McLachlan, J. A. & Korach, K. S. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4658–4662.
- Ignar-Trowbridge, D. M., Teng, C. T., Ross, K. A., Parker, M. G., Korach, K. S. & McLachlan, J. A. (1993) *Mol. Endocrinol.* **7**, 992–998.
- White, R., Lees, J. A., Needham, M. & Parker, M. (1987) *Mol. Endocrinol.* **1**, 735–744.
- Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. & Erlich, H. (1986) *Cold Spring Harbor Symp. Quant. Biol.* **51**, 263–273.
- Hooper, M., Hardy, K., Handyside, A., Hunter, S. & Monk, M. (1987) *Nature (London)* **326**, 292–295.
- Boer, P. H., Potten, H., Adra, C. N., Jardine, K., Mullhofer, G. & McBurney, M. W. (1990) *Biochem. Genet.* **28**, 299–307.
- Mansour, L. S., Thomas, K. R. & Capecchi, M. R. (1988) *Nature (London)* **336**, 348–352.
- Koller, B. H., Hyung-Suk, K., Latour, A. M., Brigman, K., Boucher, R. C., Jr., Scambler, P., Wainwright, B. & Smithies, O. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10730–10734.
- Kim, H.-S. & Smithies, O. (1987) *Nucleic Acids Res.* **16**, 8887–8903.
- Sluyter, M., Rijkers, A. W. M., de Goeij, C. C. J., Parker, M. & Hilkens, J. (1988) *J. Steroid Biochem.* **31**, 757–761.
- Turner, C. D. (1965) *General Endocrinology* (Saunders, New York).
- Korach, K. S. (1979) *Endocrinology* **104**, 1324–1332.
- Luetteke, N. C., Qiu, T. H., Peiffer, R. L., Oliver, P., Smithies, O. & Lee, D. C. (1993) *Cell* **73**, 263–278.
- Dietz, H. C., Valle, D., Francomano, C. A., Kendzior, R. J., Jr., Pyritz, R. E. & Cutting, G. R. (1993) *Science* **259**, 680–683.
- Gaspar, M.-L., Meo, T., Bourgaire, P., Guenet, J.-L. & Tosi, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8606–8610.
- Murphy, L. J. & Ghadhap, A. (1990) *Endocr. Rev.* **11**, 443–453.
- Li, E., Sucov, H. M., Lee, K.-F., Evans, R. M. & Jaenisch, R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1590–1594.
- Lohnes, D., Kastner, P., Dierich, A., Mark, M., LeMeur, M. & Chambon, P. (1993) *Cell* **73**, 643–658.
- Lufkin, T., Lohnes, D., Mark, M., Dierich, A., Gorry, P., Gaub, M.-P., LeMeur, M. & Chambon, P. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 7225–7229.
- Nieder, G. L., Weitlauf, H. M. & Suda, H. M. (1987) *Biol. Reprod.* **36**, 687–699.
- Lehrer, S., Rabin, J., Kalir, T. & Schachter, B. S. (1993) *Hypertension* **21**, 439–441.
- Lehrer, S., Sanchez, M., Song, H. K., Dalton, J., Levine, E., Savoretti, P., Thung, S. N. & Schachter, B. (1990) *Lancet* **335**, 622–624.
- McGuire, W. L., Chamness, G. C. & Fuqua, S. A. W. (1991) *Mol. Endocrinol.* **5**, 1571–1577.
- Skipper, J. K., Young, L. J., Bergeron, J. M., Tetzlaff, M. T., Osborn, C. T. & Crews, D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 7172–7175.
- Gibson, M. K., Nemmers, L. A., Beckman, W. C., Jr., Davis, V., Curtis, S. A. & Korach, K. S. (1991) *Endocrinology* **129**, 2000–2010.
- Charest, N. J., Zhou, Z., Lubahn, D. B., Olsen, K. L., Wilson, E. M. & French, F. S. (1991) *Mol. Endocrinol.* **5**, 573–581.