Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 515–520. Pergamon Press. Printed in Great Britain

GENETIC REGULATION OF THE ANDROGEN RECEPTOR—A STUDY OF TESTICULAR FEMINIZATION IN THE MOUSE*

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SUMMARY

Testicular feminization (TFM) is an inherited disorder which is transmitted by females to half their male offspring. Affected individuals are genetic males with end organ insensitivity to androgens. As a consequence, they lack androgen dependent differentiation and present as phenotypic females. Studies on rats and mice indicate that a major tissue abnormality in testicular feminization is decreased cytosol androgen receptor activity. Deficiency of this cytosol binder could account for the lowered nuclear binding of testosterone and dihydrotestosterone in these animals. If androgen uptake and binding in the nucleus is required to initiate RNA and DNA synthesis, then the inability to concentrate androgens at the active site in the nucleus could account for the androgen insensitivity in testicular feminization. The findings in the present study support a direct correlation between the TFM gene, androgen receptor activity and androgen response.

INTRODUCTION

Testicular feminization was first described in man [1]. Patients with this disorder are genotypic males with an inherited end organ insensitivity to exogenous and endogenous androgens. As a consequence, they lack androgen dependent development and present phenotypically as women with primary amenorrhea. In the complete form of this syndrome, patients have no or very sparse pubic and axillary hair and well developed breasts. The vagina, when present, is short and ends in a blind pouch. The derivatives of the gonaducts do not develop and are represented only by fibrous rudiments of the wolffian and mullerian systems. The testis are located interabdominally or in the inguinal canal [1–3].

In 1964, Stanley and Gumbreck[4] reported their observations on hereditary male pseudohermaphroditism in a rat (tfm) which had resulted from a mutation isolated in their colony. Affected males were phenotypically and karyotypically similar to patients with testicular feminization. In the tfm rat as in man, the genetic defect was transmitted by the female to half her male offspring in a pattern consistent with an x-linked recessive or a sex-limited autosomal dominant gene mutation [5].

Since TFM rats do not have a prostate or seminal vesicles, androgen action on other tissues was examined. Studies from our own and other laboratories indicated that the physiologic doses of testosterone or dihydrotestosterone did not produce the expected response on a variety of end organs such as liver, preputial gland, kidney and pituitary [6]. It was significant, however, that on several of the above end organs, pharmacologic doses of testosterone produced a low, but detectable biological effect in the TFM animal. To investigate these observations in detail, normal and TFM rats were treated with large doses of testosterone enanthate and the effect on the preputial gland measured. In the normal animal, all growth indices (RNA, DNA, total protein and enzyme synthesis) showed a maximal response to doses of testosterone enanthate below 6 mg per day. In the tfm rat, although no changes were seen below this dose, a definite effect on preputial gland growth was observed with 30 and 60 mg doses. These observations indicated that the androgen insensitivity in the TFM rat is relative rather than absolute. Even though the tfm animals required a large dose of testosterone, the response was qualitatively similar to that of the normal rat. The fact that simultaneous stimulation of all cellular elements was produced once androgen action was initiated suggested that tfm rats have a pretranscriptional regulatory gene defect [7]. To investigate this possibility, *in vivo* and *in vitro* studies of androgen metabolism were performed in the TFM rat which indicated that these animals could not concentrate dihydrotestosterone in the nucleus of the cell [8]. This latter defect correlated with the lack of demonstrable 7·9S cytosol androgen receptor protein [6]. These observations are consistent with the hypothesis that androgen insensitivity in the TFM rat is a consequence

* This work was supported in part by Grant No. HD-05276 and Contract No. N01-HD-2-2730 from the National Institutes of Health.

The abbreviations and trivial names used are: Testosterone : 17β-hydroxy-4-androsten-3-one; dihydrotestosterone: 17β-hydroxy-5α-androstan-3-one; androstanediol(s): A mixture of 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol; androstenedione: androstene-3,17-dione; medroxyprogesterone acetate (MPA): 6α-methyl-17α-hydroxy-4-pregnene-3,20-dione acetate; 5α-reductase-NADPH: 4ene-3-ketosteroid 5α-oxidoreductase; 3-ketoreductase: 3α-hydroxysteroid NAD(P) oxidoreductase (EC 1.1.1.50).

of the inability of cells to effect receptor mediated transfer of androgens into the nucleus.

While the above studies were in progress, Lyon and Hawkes[9] described a mouse with testicular feminization. Male animals with this defect (TFM/y) are similar to the TFM rat in that they lack androgen dependent differentiation and have absence of a reproductive tract other than abdominal testes. This disorder, like its prototype in the rat, is transmitted by the female to half her male offspring. This pattern of inheritance coupled with linkage studies, indicates that the TFM mutation in the mouse is on the X-chromosome [9]. A variety of studies suggested that the TFM/y mouse like the TFM rat had an abnormality of the androgen receptor [10–12]. Animals which carried the TFM gene thus provided an excellent opportunity to study the genetics of the androgen receptor in the mouse. In the present report, we review studies which correlated the androgen responsiveness and androgen receptor activity with the TFM gene in normal female (+/+), male (+/y), androgen insensitive (TFM/y) and carrier female (TFM/+) mice. It should be emphasized that the results present were derived from our own laboratory. Reference to all the important papers in this area must await a more encyclopedic review.

EXPERIMENTAL PROCEDURE

Animals

The androgen insensitive TFM rats and their normal litter-mates were obtained from the University of Oklahoma colony established by Stanley and Gumbreck. Mice were from a colony maintained at the Milton S. Hershey Medical Center which originated from the colony of Dr. Susumu Ohno. These included male (+/y), female (+/+), androgen insensitive male (TFM/y) and heterozygous female (TFM/+) animals.

Methods

The procedures used in the majority of the studies have been described in previous publications as follows: isolation of androgen metabolites [8]; assay of androgen receptor with sucrose density gradients and Dextran-coated charcoal [10, 13]; Sephadex G-200 gel filtration, isoelectric focusing and DNA-cellulose binding [14], enzyme assays [8, 15].

Radioactive steroids

The details for synthesis of [^{3}H]-androgens which were not available from commercial suppliers have been reported [8, 10]. [^{3}H]-medroxyprogesterone acetate ([^{3}H]-MPA) was prepared as follows: MPA was refluxed in a benzene solution with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone to yield 6α-methyl-17-hydroxy-1,4-pregnadiene-3,20-dione acetate. This latter compound was selectively reduced with tritium and chlorotris rhodium to yield [1,2-^{3}H] 6α-methyl-17-hydroxy-4-pregnene-3,20-dione acetate.

Isolation of kidney nuclei and chromatin

Mouse kidney nuclei were prepared according to Bush *et al.*[16] with the exception that in the initial homogenization the sucrose concentration was 2·0 M. After washing the isolated nuclei once with 0·32 M sucrose, 2 mM $MgCl_2$ and 0·2% Triton X-100, the kidney chromatin was prepared using modifications of the methods by Shaw and Huang[17] and Cox *et al.*[18] as follows: repeated washings with 0·075 M NaCl, 0·024 M EDTA (pH 8·0) and 10 mM Tris–HCl (pH 8·0); centrifugation of the sample through 1·7 M sucrose, 10 mM Tris–HCl (pH 8·0); suspension of the chromatin pellet in 0·4 mM Tris–HCl (pH 8·0).

Solubilization and purification of kidney RNA polymerases

Mouse and rat kidney nuclear RNA polymerases were solubilized and separated by DEAE-Sephadex chromatography essentially as described by Sajdel and Jacob[19] for rat liver RNA polymerases.

Chromatin template activity was measured using unlimiting amounts of kidney RNA polymerase II. The amount of chromatin DNA in an incubation was approximately 10 μg. The template activities of various preparations were expressed as nanomoles UMP incorporated into acid insoluble material/mg DNA [20].

RESULTS AND DISCUSSION

Response of normal, TFM/y and TFM/+ mice to androgens

Since the TFM/y animal has no prostate and the preputial glands are small, androgen action and metabolism were studied in kidney. β-glucuronidase is a renal protein which increases 50-fold in kidneys of normal male and female mice after treatment with steroids including dihydrotestosterone, testosterone, and the 3α- and 3β-androstanediols [6]. Females, heterozygous for TFM (TFM/+) exhibited androgen responsiveness which was 50–70% that of the normal mice [21–23]. By contrast, tfm/y mice had no detectable response to any androgen dose tested [6, 21–23]. From these observations, we conclude that the TFM/y mouse is more androgen resistant than the TFM/y rat.

In the above studies, the effect of androgen or enzyme activity was measured several days following the initial steroid treatment. To determine whether androgens would influence an earlier step in steroid activation of the cell, the effect of testosterone on chromatin template activity was investigated. The results of these studies are summarized in Fig. 1. Testosterone produced a progressive increase in chromatin activity which peaked at 1 h in normal animals. No response was seen in TFM/y animals. These observations are consistent with the hypothesis that the TFM/y mouse like the TFM rat has a defect of a regulatory gene which influences transcriptional events.

In vivo studies from our laboratory [24] demonstrated that mouse kidney has a very limited capacity to reduce testosterone to dihydrotestosterone. More importantly, however, these experiments showed that testosterone rather than dihydrotestosterone was bound in kidney nuclei after the administration of

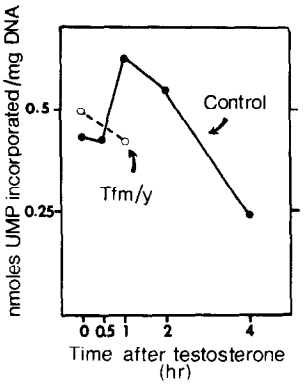

Fig. 1. Chromatin template activity in normal female (control) and TFM/y mouse kidneys following a single intraperitoneal dose of testosterone (0·5 mg). Chromatin was isolated and assayed for the template activity using partially purified kidney RNA polymerase II, as described in the text.

[^{3}H]-testosterone. These observations suggested that testosterone actions on kidney and perhaps in other organs in the mouse are not mediated by dihydrotestosterone as had been proposed for other species [25]. To clarify this possibility, *in vitro* androgen metabolism was extensively studied in mouse kidney.

In vitro *androgen metabolism in mouse kidney*

Kidney slices were incubated with either [^{3}H]-testosterone or [^{3}H]-dihydrotestosterone (10^{-7} M) at 37°. After 1 h only 3% of the substrate [^{3}H]-testosterone had been metabolized and no dihydrotesterone or 5α-androstanediols could be isolated from the incubation mixture [15]. These observations are consistent with the conclusion that mouse kidney has very little 5α-reductase activity. By contrast, when [^{3}H]-dihydrotestosterone was incubated with kidney slices, 70% was reduced to the 5α-androstanediols in 30 min; thus, this organ has potent 3-ketoreductase activity. When cell fractions were isolated, this enzyme was primarily located in cytosol. Its pH optimum was between 6·5 and 7·5 and it utilized NADPH as cofactor with a maximum activity at 37°C. Of particular interest was the observation that the 3-ketoreductase retained 10% of its activity at 0°C. As a consequence, physiologic concentrations of dihydrotestosterone (10^{-9} M) were rapidly metabolized to the 5α-androstanediols at 0–4°C even without addition of NADPH. These observations are particularly pertinent since a large fraction of dihydrotestosterone added to kidney homogenates *in vitro* may be converted to 5α-androstanediols and not be available for interaction with the cytosol androgen receptor.

In vivo *androgen metabolism in mouse kidney*

In vivo metabolism of testosterone, dihydrotestosterone and other androgens was next examined. Castrated male mice were functionally hepatectomized by removal of the intestinal tract [24]. Fifty μCi of [^{3}H]-testosterone, [^{3}H]-dihydrotestosterone, [^{3}H]-androstenedione or one of the [^{3}H]-5α-androstanediols were administered intravenously and the animals were killed 1 h later. Kidneys from 2–3 animals were pooled and the nuclei and cytoplasm were isolated. [^{3}H]-androgens in these cell fractions were isolated by thin layer chromatography and quantified by double isotope dilution. The results are summarized in Figs. 2 and 3. When [^{3}H]-testosterone was administered to castrated male mice, testosterone was the dominant steroid found in both cytoplasm and nuclei (Fig. 2). As noted above, these observations indicated that there was very little *in vivo* testosterone reduction to dihydrotestosterone and that in the absence of 5α-reduction, testosterone *per se* was transformed to the nucleus of the cell (Figs. 2, 3). When [^{3}H]-androstenedione was given, testosterone was again the major metabolite and little or no dihydrotestosterone was found (Fig. 3). By contrast, after the intravenous administration of [^{3}H]-dihydrotestosterone, 5α-androstanediols and dihydrotestosterone were the major cytoplasmic androgens. In these studies, almost all the nuclear radioactivity was dihydrotestosterone. A similar pattern was observed when the [^{3}H]-5α-androstane-3α,17β-diol or its 3β-isomer was given. These latter studies indicated that dihydrotestosterone as well as testosterone may be transferred in the nucleus of the mouse kidney (Fig. 3). The uptake of testosterone and dihydrotestosterone in kidney nuclei was shown to be specific in that 98% of the [^{3}H]-androgens could be displaced by an excess of unlabeled testosterone, dihydrotestosterone, or cyproterone acetate (Fig. 3). By contrast, there was no specific uptake of testosterone or dihydrotestosterone in nuclei of TFM/y androgen insensitive mice (not shown). Since the transfer of steroids to the nucleus

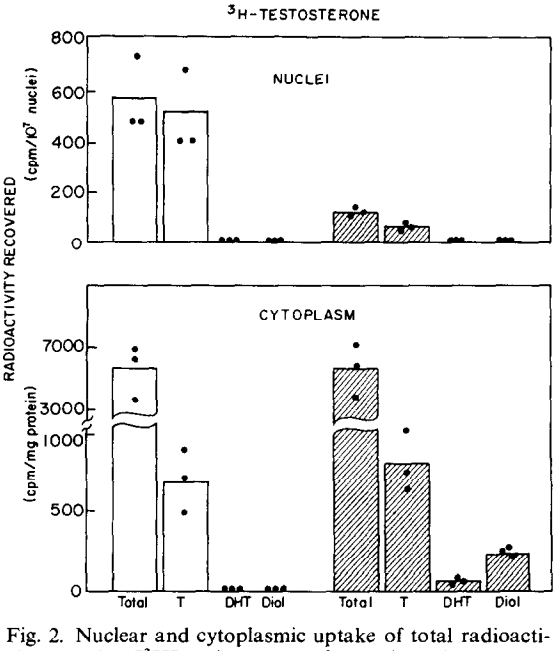

Fig. 2. Nuclear and cytoplasmic uptake of total radioactivity and [^{3}H]-androgens after the intravenous administration of [^{3}H]-testosterone. The [^{3}H]-androgens were isolated from cell fractions by multiple thin layer chromatographies as described in the text.

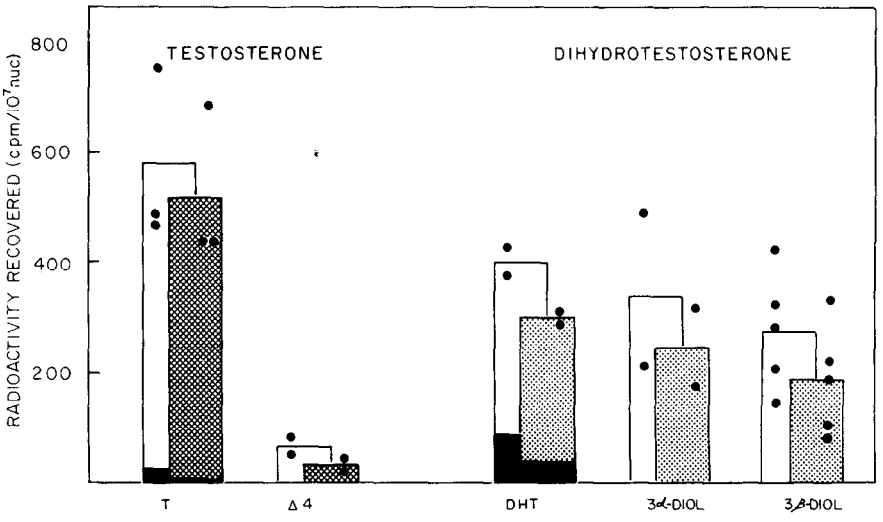

Fig. 3. Nuclear uptake of total radioactivity (open bars) and [^{3}H]-androgens (shaded bars) after the intravenous administration of [^{3}H]-testosterone (T), [^{3}H]-androstenedione (Δ_4), [^{3}H]-5α-dihydrotestosterone (DHT), [^{3}H]-5α-androstane-3α,17β-diol (3α-diol) and [^{3}H]-5α-androstane-3β,17β-diol (3β-diol). When either testosterone or androstenedione was administered, testosterone was the predominant androgen isolated from kidney nuclei (left). When one of the 5α-reduced steroids was administered, dihydrotestosterone was the predominant steroid isolated from kidney nuclei (right). The solid portion of the testosterone and dihydrotestosterone bars indicates the nuclear androgen uptake when cyproterone acetate was administered before the [^{3}H]-androgen.

of the cell is believed to be mediated by cytosol receptor, these studies suggest that testosterone and dihydrotestosterone share a common intracellular binding protein in mouse kidney. They further suggest that this receptor protein is defective in the TFM/y mouse.

Androgen receptors in mouse kidney

Kidneys from normal mice were homogenized and centrifuged at 150,000 ***g***. The supernatant fraction was incubated for 2 h with either [^{3}H]-testosterone or [^{3}H]-dihydrotestosterone (10^{-9} M) and was analyzed by sucrose gradients [10]. Testosterone and dihydrotestosterone labeled macromolecules sedimented in the 7·9S portion of the gradient. Both [^{3}H]-androgens were displaced from their cytosol binders by unlabeled cyproterone acetate, testosterone, or dihydrotestosterone (Fig. 4). These *in vitro* observations are consistent with the above *in vivo* studies and together indicate that testosterone and dihydrotestosterone share a common cytosol receptor protein in kidney. With these procedures, the physico-chemical properties of the androgen receptor complex were determined. These are summarized in Table 1. These observations indicate that the androgen receptor in mouse kidney is an asymmetric acidic protein which has a high affinity for its steroid ligand. Studies with protein specific reagents suggest that both cysteine and tryptophan residues may be necessary for maintaining the functional configuration associated with androgen binding. The kidney receptor can promote the association of testosterone with purified DNA. We conclude that these properties of the receptor in mouse kidney are remarkably similar to those for the androgen receptor in male accessory sexual tissues [14].

We next attempted to measure androgen receptor activity in the TFM/y mouse. In these animals, no demonstrable high affinity androgen binding activity was detected with any of the assays used to characterize the normal kidney receptor: sucrose gradients, Dextran-coated charcoal, Sephadex gel filtration, isoelectric focusing or DNA-cellulose. These studies

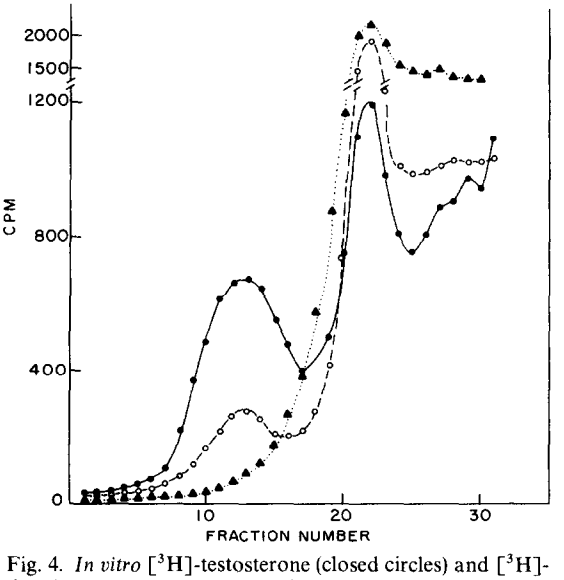

Fig. 4. *In vitro* [^{3}H]-testosterone (closed circles) and [^{3}H]-dihydrotestosterone (open circles) binding in kidney cytosol from normal female mice. The [^{3}H]-steroids were incubated with kidney cytosol and analyzed on sucrose gradients. [^{3}H]-androgens in the 7·9S peak are shown. Displacement studies indicate that testosterone and dihydrotestosterone are binding to the same macromolecule (not shown). When either [^{3}H]-androgen was incubated with cytosol from TFM/y mice, no androgen binding was demonstrated (closed triangles).

Table 1. The physicochemical properties of the cytoplasmic androgen receptor in mouse kidneys

Sedimentation coefficient (S)	7·9
Stokes radius (A)	82
Axial ratio (f/f_0)	1·98
Isoelectric point	4·8
K_d (nM)	1·3

have led us to conclude that the TFM/y mouse like the TFM rat has a defective androgen receptor.

Wilson and Goldstein[26] have detected a stable 3S dihydrotestosterone binding protein in cytosol from submaxillary glands of mice. This androgen binder has a low affinity and a higher capacity than most other androgen receptors. Interestingly, these investigators found less dihydrotestosterone binding activity in cytosol from normal males than in females and in TFM/y mice. These studies are interpreted as evidence against the possibility that reduced receptor activity was responsible for androgen insensitivity in TFM/y animals. At present, it is not possible to resolve the differences in the experiments of these investigators and those reported from our laboratory.

[^{3}H]-MPA binding to the androgen receptor in kidney

The fact that androgen insensitive mice do not respond to MPA suggested that androgens and progestins may share a common receptor [27]. To test this possibility, kidney cytosol was incubated with [^{3}H]-testosterone and [^{3}H]-MPA in the presence and absence of cold steroids and sedimented on sucrose gradients. The results of these studies are summarized in Fig. 5. [^{3}H]-testosterone and [^{3}H]-MPA labeled macromolecules sedimented in the 7·9S region of the gradient. Cold testosterone and MPA displaced both [^{3}H]-ligands. Furthermore, neither [^{3}H]-testosterone nor [^{3}H]-MPA was bound by kidney cytosol from TFM/y mice (not shown). These observations suggest that androgens and progestins may share a common receptor protein in mouse kidney.

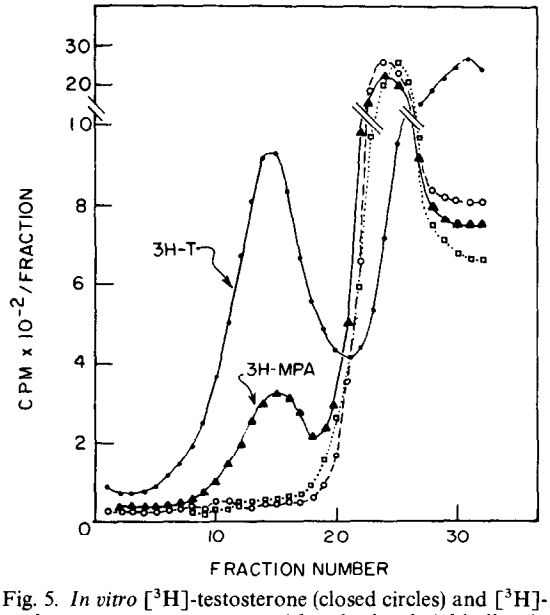

Fig. 5. *In vitro* [^{3}H]-testosterone (closed circles) and [^{3}H]-medroxyprogesterone acetate (closed triangles) binding in kidney cytosol. [^{3}H]-steroids were incubated with kidney cytosol from castrate mice and analyzed on sucrose gradients. Cold MPA could displace [^{3}H]-testosterone (open squares) and cold testosterone could displace [^{3}H]-MPA (open circles) from their respective binding sites. Neither [^{3}H]-testosterone nor [^{3}H]-MPA demonstrated specific binding in the cytoplasm of TFM/y mice (not shown).

Receptor activity in carrier females (TFM/+) mice

The kidney androgen receptor from carrier females had a similar sedimentation coefficient, Stokes radius and pI compared to normal litter-mates. The average dissociation constant (K_d) for testosterone binding in kidney cytosol from carrier females was also similar to that of normal mice (1·3 ± 0·1 nM (mean ± SE) vs. 1·2 ± 0·1 nM for carrier and normal mice, respectively). These findings suggested that the physical properties of the receptor in TFM/+ mice were not grossly altered. By contrast, the number of binding sites in cytosol from carrier females was only 69% of normal as measured with the Dextran-coated charcoal assay (4·4 ± 0·2 vs 6·4 ± 0·4 × 10^{-14} mol/mg cytosol protein). It was possible to confirm this decrease in sites using sucrose gradient analysis. Kidney cytosol from normal and TFM/y mice was incubated with 3 concentrations of [^{3}H]-testosterone on 7 different days. Since the amount of specifically bound [^{3}H]-testosterone varied between experiments, binding in carrier cytosol was expressed as a per cent of binding in the corresponding samples of normal cytosol. The average binding by cytosol from carrier females was again found to be 69% of normal. Analysis of variance indicated that this difference in binding between normal and carrier mice (TFM/+) was highly significant ($P < 0·01$).

According to the Lyon hypothesis, one X-chromosome is inactivated in each female cell resulting in hemizygous expression of genes on the active X [28]. Since the tfm gene is on the X-chromosome, the intermediate androgen responsiveness of the carrier female (TFM/+) is consistent with the variable percentage of normal vs TFM carrying X-chromosomes that are active in the target organ. In the present study, a correlation between the androgen receptor, androgen responsiveness and the TFM gene is demonstrated.

REFERENCES

1. Naftolin F. and Judd H. L.: In *Obstetrics and Gynecology Annual* (Edited by R. W. Wynn), Appleton-Century-Crofts, New York (1973), pp. 25–53.
2. Morris J. M.: *Am. J. Obstet. Gynecol.* **65** (1953) 1192–1211.
3. Simmer H. H., Pion R. J. and Dignam W. J.: In *Testicular Feminization* (Edited by W. A. Selle). Springfield, Ill., Charles C. Thomas (1965).
4. Stanley A. J. and Gumbreck L. G.: 46th Meeting of the Endocrine Society (1964), p. 40.
5. Stanley A. J., Gumbreck L. G., Allison J. E. and Easley R. B.: *Recent Prog. Horm. Res.* **29** (1973) 43–64.
6. Bardin C. W., Bullock L. P., Sherins R. J., Mowszowicz I. and Blackburn W. R.: *Recent Prog. Horm. Res.* **29** (1973) 65–109.

7. Sherins R. J. and Bardin C. W.: *Endocrinology* **89** (1971) 835–841.
8. Bullock L. P. and Bardin C. W.: *J. steroid Biochem.* **4** (1973) 139–151.
9. Lyon M. F. and Hawkes S. G.: *Nature., Lond.* **227** (1970) 1217–1219.
10. Bullock L. P. and Bardin C. W.: *Endocrinology* **94** (1974) 746–756.
11. Goldstein J. L. and Wilson J. D.: *J. clin. Invest.* **51** (1972) 1647–1658.
12. Gehring U., Tomkins G. M. and Ohno S.: *Nature New Biol.* **232** (1971) 106–107.
13. Bullock L. P. and Bardin C. W.: *J. clin. Endocr. Metab.* **35** (1972) 935–937.
14. Bullock L. P., Mainwaring W. I. P. and Bardin C. W.: *Endocr. Res. Commun.* (1974) (in press).
15. Mowszowicz I. and Bardin C. W.: *Steroids* **23** (1974) 793–807.
16. Busch H., Narayan K. S. and Hamilton J.: *Exp. Cell Res.* **47** (1967) 329–336.
17. Shaw L. M. J. and Huang R. C. C.: *Biochemistry* **9** (1970) 4530–4542.
18. Cox R. F., Haines M. E. and Carey N. H.: *Eur. J. Biochem.* **32** (1973) 513–524.
19. Sajdel E. M. and Jacob S. T.: *Biochem. biophys. Res. Commun.* **45** (1971) 707–715.
20. Jacob S. T.: *Progr. Nuc. Acid. Res. Mol. Biol.* **13** (1973) 93–126.
21. Ohno S. and Lyon M. F.: *Clin. Genet.* **1** (1970) 121–127.
22. Tettenborn U., Dofuku R. and Ohno S.: *Nature, New Biol.* **234** (1971) 37–40.
23. Lyon M. F., Hendry I. and Short R. V.: *J. Endocr.* **58** (1973) 357–362.
24. Bullock L. P., Bardin C. W. and Ohno S.: *Biochem. biophys. Res. Commun.* **44** (1971) 1537–1543.
25. Wilson J. D. and Gloyna R. E.: *Recent Prog. Horm. Res.* **26** (1970) 309–336.
26. Wilson J. D. and Goldstein J. L.: *J. biol. Chem.* **247** (1972) 7342–7348.
27. Mowszowicz I., Bieber D. E., Chung K. W., Bullock L. P. and Bardin C. W.: *Endocrinology* **95** (1974) 1589–1599.
28. Lyon M. F.: *Biol. Rev.* **48** (1972) 1–35.