Interaction of Testosterone and Testosterone Receptor Complexes with Nuclei of Skeletal Muscle from Intact Male Mice and from Mice Bearing the Testicular Feminization (Tfm) Mutant Gene

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With the nuclear exchange assay and the nuclear retention assay it is shown that the androgen insensitivity of Tfm mice is probably due to a defect of the nuclear acceptor sites for the testosterone receptor complex. Furtheron the results obtained point strongly to the possibility that hormone free androgen receptor is localized in the nuclei and in the cytoplasma according to the "equilibrium model". A practicable method for separation of unbound steroids from nuclei is described.

Introduction

In mice bearing the testicular feminization (Tfm) gene Dahlberg *et al.* [1] found that at least in skeletal muscle the androgen receptor content was the same as in intact male mice *i.e.* 200 fmol per g tissue. Still the Tfm mice do not react on testosterone application. There might be four reasons conceivable for this:

1) The androgen receptor is defective and therefore not able to translocate into the nucleus (this is relevant if the "two step model" of steroid hormone action [2] is right).
2) The receptor is *a priori* in the nucleus and binds hormone but is unable to transform into a DNA binding form (this is relevant for the "nuclear model" [3, 4]).
3) Androgen insensitivity of Tfm mice is due to a defective nuclear acceptor which is unable to bind the testosterone receptor complex (this is relevant for the "two step model", "nuclear model" and the "equilibrium model" [5, 6]).
4) There is a defect in the membrane of the nucleus which does not allow testosterone and the testosterone receptor complex respectively to permeate the nuclear membrane (this is also relevant for all three models).

To find out what was the reason for the androgen insensitivity of the Tfm mice, we made *in vitro* experiments with isolated nuclei of male mice (castrated and uncastrated) and of Tfm mice respectively. We compared the two different sorts of nuclei with regard to their binding capability for free testosterone (test for nuclear acceptor bound receptors – "nuclear model") and for testosterone receptor complexes obtained from skeletal muscle of male mice (test for nuclear acceptors). The latter test was principally done by Krieg *et al.* [7] with nuclei from heart muscle and androgen receptors from prostate, bulbo cavernosus/levator ani and heart muscle respectively.

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Materials and Methods

(1,2,6,7,16,17)-^{3}H-testosterone (5.78 TBq/mmol) from New England Nuclear (Dreieich). Radioinert testosterone, crude collagenase type IA, dithiothreitol (DTT), phenylmethylsulfonylflouride (PMSF) from Sigma (München). Charcoal Norit A, dextran 60, bovine serum albumine cryst. and Triton-X-100 were from Serva (Heidelberg). All other reagents used were of analytical grade and obtained from E. Merck (Darmstadt).

The purity and identity of radioactive and radioinert testosterone were ascertained by thin layer chromatography.

Buffers:

Buffer A: 10 mM Tris containing 0.33 M sucrose, 0.15 M KCl, 3 mM $MgCl_2$ and 0.1 mM PMSF, adjusted to pH 7.4 at room temperature with HCl.

Buffer B: Buffer A containing 0.88 M sucrose instead of 0.33 M sucrose.

Buffer C: Buffer A containing 0.2% Triton-X-100.

Buffer D: 50 mM Tris containing 0.15 M KCl, 3 mM $MgCl_2$, 2 mM EDTA adjusted to pH 7.4 at room temperature with HCl.

Buffer E: 50 mM Tris containing 0.25 M sucrose, 0.15 M KCl, 3 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT and 0.1 mM PMSF, adjusted to pH 7.4 at room temperature with HCl.

Collagenase-buffer: 100 mM Tris containing 66.74 mM NaCl, 6.7 mM KCl, 4.8 mM $CaCl_2$ adjusted to pH 7.6 at room temperature with HCl.

Cytosol-buffer: 50 mM Tris containing 2 mM EDTA, 3 mM $MgCl_2$, 1.5 mM DTT and 0.1 mM PMSF, adjusted to pH 7.4 at room temperature with HCl.

Nuclei-buffer: Buffer E containing 0.14 mM PMSF instead of 0.1 mM PMSF but without DTT.

Dextran coated charcoal (DCC)

4 g charcoal Norit A and 0.1 g dextran 60 per 100 ml of cytosol-buffer were stirred over night at 0–4 °C.

Scintillation cocktail

3.5 g PPO (2,5-diphenyloxazole), 0.14 g POPOP (2,2-*p*-phenylene-bis-5-phenyloxazole), 300 ml Triton-X-100 and 700 ml toluene were stirred under exclusion of light.

Preparation of nuclei

Six adult male C57BL6J mice (when necessary castrated 24 h before sacrifice) were killed (in case of Tfm mice 11 mice were needed to obtain the same amount of muscle (20 g) as with intact males). The hindlimbs were isolated from bones and fat and placed immediately in ice-cold 0.9% NaCl solution. All subsequent procedures were performed at 0–4 °C. The muscles were transferred into 50 ml collagenase-buffer containing 0.02% crude collagenase type IA and continuously stirred over night at 2 °C. Thereafter the redbrown gelatinous suspension was diluted by adding 120 ml of collagenase-buffer and was then centrifuged for 15 min at $800 \times g$. The pellet was suspended in 170 ml of buffer A and centrifuged for 15 min at $800 \times g$. The pellet was divided into 2 g portions and homogenized in a 60 ml Dounce homogenizer using three strokes with the loose fitting pestle. It is important that the pudding like consistency of the collagenase digest is achieved, because poor digestion needs more violent homogenization which leads to a serious loss of nuclei. After homogenization the homogenate was filtered on four layers of surgical gauze. The filtrate (about 600 ml) was centrifuged at $800 \times g$ for 10 min. The resulting pellet was cautiously suspended in a small volume of buffer A and then diluted with 300 ml of buffer B. This suspension was centrifuged again at $800 \times g$ for 10 min. The resulting pellet was carefully resuspended in a small volume of buffer A and diluted with 170 ml of buffer C. After 5 min at 2 °C this suspension was centrifuged at $800 \times g$ for 10 min. The resulting pellet was washed with 300 ml of buffer A. After centrifugation at $800 \times g$ for 10 min the pellet was carefully suspended in 50 ml of buffer D containing 1.6 M sucrose. In centrifugation tubes of a Beckman SW 25/1 rotor 3 ml of buffer D containing 1.8 M sucrose was layered onto the top of 6 ml of buffer D containing 2 M sucrose. Then the nuclei suspension was layered onto the top of this gradient and centrifuged for 2 h at 20000 rpm at 2 °C. The resulting pellet was suspended carefully in nuclei-buffer and centrifuged at $800 \times g$ for 5 min. The pellet was cautiously resuspended in 7.5 ml of nuclei-buffer and 3 ml of buffer D containing 2 M sucrose. This suspension was used as nuclei suspension. The nuclei examined by phase contrast and Nomarski interference microscopy appeared free of cytoplasmatic tags. For chemical examination a certain amount of nuclei were processed according to [8]. DNA was assayed by the diphenylamine method [9] using calf thymus DNA as a standard. RNA was directly measured at 260 nm using yeast RNA as a standard. Protein was determined according to Lowry *et al.* [10]. The protein/DNA relation was 7.7 and the RNA/DNA relation was 0.59.

Preparation of cytosol

The cytosol was prepared according to [11] with the modification that the buffer contained 1.5 mM DTT instead of 2 mM 2-mercaptoethanol. Specific binding was determined according to [12].

Nuclear exchange assay

0.9 ml of cytosol-buffer were pipetted into 10 ml tubes and increasing concentrations of [^{3}H]testosterone were added so, that the concentration of this compound in the tubes was 0.1 nM, 0.2 nM, 0.3 nM, 0.4 nM, 0.5 nM, 0.75 nM, 1 nM, 1.5 nM, 2 nM, 2.5 nM and 3 nM, respectively. Cytosol-buffer was added to

each tube to get a final volume of 1 ml. Then 0.5 ml of nuclei suspension (10^6 nuclei/ml) were added to each tube. In parallel to this an equal charge was prepared but here a 100-fold excess of radioinert testosterone was added. Both charges were prepared in duplicate. After incubation for 2 h at 0–4 °C, 2 ml of buffer E were added and the nuclei suspended. To separate unbound testosterone from the nuclei this suspension was layered onto the top of 0.3 ml of buffer D containing 1 M sucrose which itself was underlayered with 0.2 ml of buffer D containing 2 M sucrose. The tubes were centrifuged for 10 min at $5500 \times g$. The pellet on the top of the 2 M sucrose layer was collected and submitted two more times to this gradient centrifugation process. The nuclei obtained were resuspended in 2 ml buffer E and centrifuged (without gradient) for 10 min at $800 \times g$. The resulting pellets were transferred into scintillation vials containing 10 ml of scintillation cocktail. Radioactivity was measured in a liquid scintillation counter.

Nuclear retention assay

Cytosol was incubated with 5 nM [^{3}H]testosterone and in parallel with 5 nM [^{3}H]testosterone plus a 100-fold excess of radioinert testosterone for 2 h at 0–4 °C. Both charges were prepared in duplicate. Unbound steroid was removed by DCC treatment. Specifically bound steroid was determined according to [12], the amount was 4500 dpm/ml. From both charges of cytosol concentration series were pipetted into 10 ml vials: 50 µl, 100 µl, 150 µl, 200 µl, 250 µl, 300 µl, 400 µl, 600 µl, 800 µl, and 1000 µl, respectively. To the different vials an amount of cytosol-buffer and KCl solution was added such that the final volume was 1 ml and the final concentration of KCl 150 mM. To all vials 500 µl of nuclei suspension were added and then incubated for 2 h at 0–4 °C by occasional shaking. Thereafter the vials were centrifuged at $800 \times g$ for 5 min. The resulting pellets were washed for three times with 3 ml of nuclei-buffer. These pellets were then suspended in 0.3 ml of nuclei-buffer and transferred into scintillation vials containing 10 ml of scintillation cocktail. Radioactivity was measured with a liquid scintillation counter.

Results and Discussion

With the nuclear exchange assay and the nuclear retention assay different aspects of the binding behaviour of the nuclei can be determined. As to the nuclear exchange assay, the mice which were used for nuclei preparation had not been castrated *i.e.* endogenous testosterone was not removed from these nuclei. Furtheron the assay is done without addition of hormone labelled cytosol. In this assay the ^{3}H-labelled testosterone exchanges against the endogenous testosterone. That means, free acceptor sites are not detectable. Only those acceptor sites are determinable by this assay to which hormone receptor complexes and hormone-free receptors are bound, respectively.

As to the nuclear retention assay, the mice used for nuclei preparation have to be castrated (at least 24 h before sacrifice, if they are not Tfm mice). In this assay [^{3}H]testosterone-labelled cytosol is used instead of free [^{3}H]testosterone. By this assay the unoccupied acceptor sites in the nuclei are determined. Therefore both assays are yielding results complementary to each other. By the nuclear exchange assay the K_D of the receptor bound to the nuclear acceptor sites is determinable whereas by the nuclear retention assay the K_D of the nuclear acceptor sites is determinable.

From the nuclear retention assay we obtained a sigmoidal binding curve when nuclei of castrated male mice were used (Fig. 2). The reason for the sigmoidal shape of this curve is not yet known. From this binding curve a nonlinear Satchard plot emerged (Fig. 2). The apparent K_D for the nuclear acceptor sites was 2.3×10^{-11} M and 15.8 fmol binding sites/mg DNA were determined. Nuclei of Tfm mice showed nonspecific binding and yielded a linear binding curve (Fig. 2).

From the nuclear exchange assay we obtained a hyperbolic binding curve when nuclei from intact male mice were used (Fig. 1). From the linear Scatchard plot a K_D of 4.9×10^{-10} M for the acceptor bound receptor and 23.5 fmol binding sites/mg DNA were determined (Fig. 1). Nuclei of Tfm mice showed no specific binding and yielded a linear binding curve (Fig. 1).

When we modified the nuclear exchange assay in as far as we used nuclei of castrated male mice and added a constant amount (900 µl) of cytosol (of castrated male mice) to each vial, then the results ($K_D = 4.6 \times 10^{-10}$ M and 27.5 fmol binding sites/mg DNA) were nearly equal to those obtained from the unmodified nuclear exchange assay. That means, no additional binding (as was expected) could be achieved

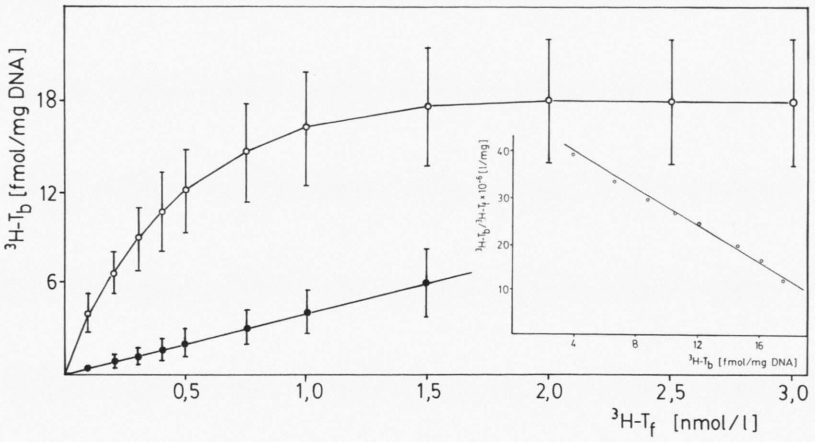

Fig. 1. Nuclear exchange assay with nuclei of intact male mice. ^{3}H-T$_b$: specifically bound [^{3}H]testosterone; ^{3}H-T$_f$: unbound [^{3}H]testosterone ○——○. Inset: Scatchard plot of the binding curve (^{3}H-T$_b$ is expressed in fmol/mg protein and ^{3}H-T$_f$ is expressed in mol/l. Therefore ^{3}H-T$_b$/^{3}H-T$_f$ has the dimension l/mg). Nuclear exchange assay with nuclei of Tfm mice. ^{3}HT$_b$: specifically bound [^{3}H]testosterone; ^{3}H-T$_f$: unbound [^{3}H]testosterone ●——●.

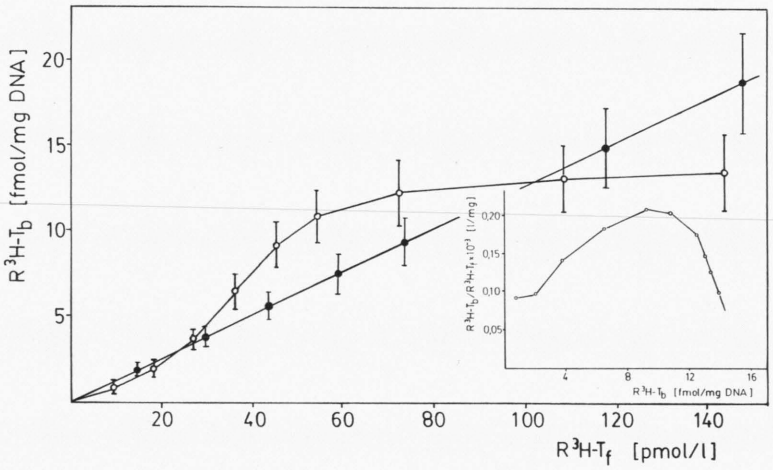

Fig. 2. Nuclear retention assay with nuclei of castrated male mice. R^{3}H-T$_b$: acceptor bound receptor-[^{3}H]testosterone-complex; R^{3}H-T$_f$: unbound receptor-[^{3}H]testosterone-complex ○——○. Inset: Scatchard plot of the binding curve (dimension of ^{3}H-T$_b$/^{3}H-T$_f$ see legend to the inset of Fig. 1). Nuclear retention assay with nuclei of Tfm mice. R^{3}H-T$_b$: acceptor bound receptor-[^{3}H]testosterone-complex; R^{3}H-T$_f$: unbound receptor-[^{3}H]testosterone-complex ●——●.

by this method (Fig. 3). An explanation for this result is: In this modified nuclear exchange assay we have nuclei of castrated mice and labelled cytosol plus unbound [^{3}H]testosterone. If there are only free acceptor sites in the nuclei of castrated mice we should obtain the same results as by the nuclear retention assay. As we got more specific binding by this modified nuclear exchange assay (*i.e.* about twice as much), it is only possible that in castrated male mice there is still hormone-free receptor in the nuclei which binds the free [^{3}H]testosterone that was not removed in this assay.

When the nuclear retention assay was performed with nuclei of uncastrated mice the apparent K_D was 1.8×10^{-11} M and the specific binding of these nuclei was 10.3 fmol binding sites/mg DNA which is about ⅔ of that obtained by the unmodified nuclear retention assay done with nuclei of castrated mice, *i.e.* castration leads not to a dramatic increase of free acceptor sites in these nuclei (Fig. 4). It results from these experiments that nuclei of castrated male mice must contain a relatively high amount of hormone-free androgen receptor. On the other hand, by the unmodified nuclear retention assay it is possible to translocate hormone receptor complexes into the nuclei which is an indication for the possibility that cytoplasmic hormone receptor complexes are able to permeate into the nucleus. All this points to the pos-

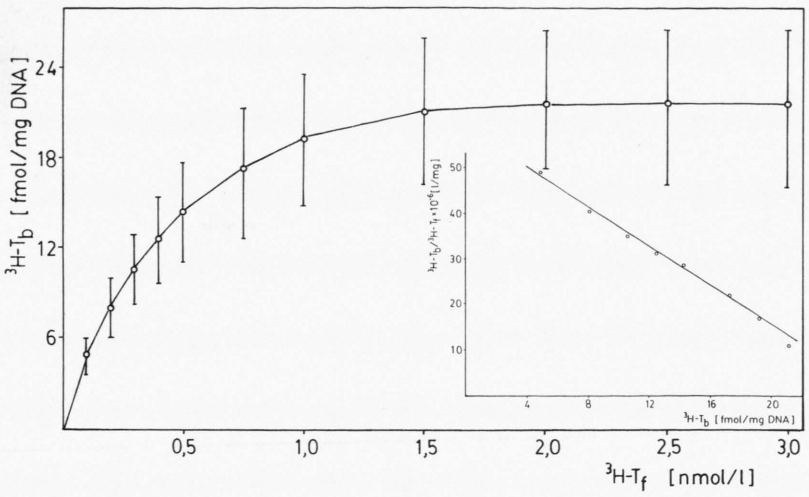

Fig. 3. Modified nuclear exchange assay with nuclei of castrated male mice and a constant amount of cytosol (0.9 ml). ^{3}H-T$_b$: specifically bound [^{3}H]testosterone (and eventually acceptor bound receptor-[^{3}H]-testosterone-complexes); ^{3}H-T$_f$: unbound [^{3}H]testosterone (and eventually unbound receptor-[^{3}H]testosterone-complexes). Inset: Scatchard plot of the binding curve (dimension of ^{3}H-T$_b$/^{3}H-T$_f$ see legend to the inset of Fig. 1).

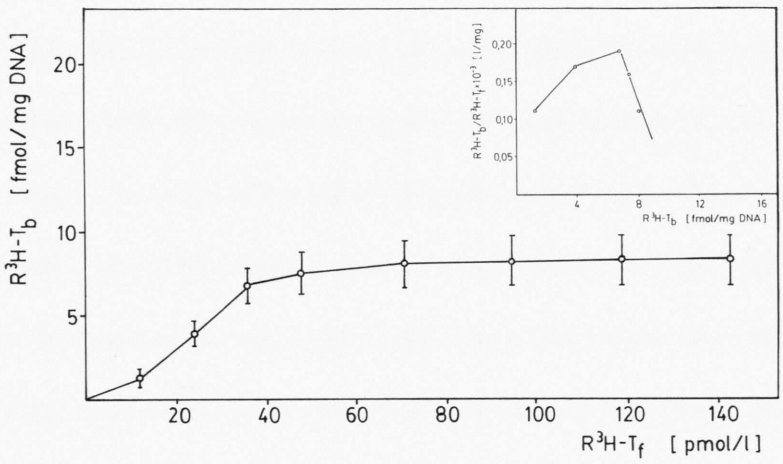

Fig. 4. Nuclear retention assay of nuclei of uncastrated male mice. R^{3}H-T$_b$: acceptor bound receptor-[^{3}H]testosterone-complex; R^{3}H-T$_f$: unbound receptor-[^{3}H]testosterone-complex. Inset: Scatchard plot of the binding curve (dimension of ^{3}H-T$_b$/^{3}H-T$_f$ see legend to the inset of Fig. 1).

sibility that hormone-free androgen receptors are localized in the nucleus and in the cytoplasm which is in accordance with the "equilibrium model" [5, 6].

Although skeletal muscle of Tfm mice contain a normal amount of androgen receptors which are still able to bind androgen [1] no specific binding could be obtained by the nuclear exchange assay (Fig. 1). There are two possible explanations for this:

1) The androgen receptor of Tfm mice is not able to bind to the acceptor sites in the nuclei because it is defective in this respect. In this case all acceptor sites in the nucleus would be free and therefore not detectable with the nuclear exchange assay.
2) The acceptor sites in nuclei of Tfm mice are defective and therefore unable to bind the hormone receptor complex which should be formed by the hormone offered and that part of receptor which is *a priori* in the nucleus.

To examine the first possibility we performed the nuclear retention assay. As by this assay it is possible to determine those nuclear acceptor sites which are free of hormone receptor complexes and hormone-free receptors, we should have got a positive result if free acceptor sites would have been in the Tfm nuclei because we used androgen receptor of normal male mice. But as shown in Fig. 2 no specific binding could

be detected. A possible explanation for the results obtained could have been that the nuclear membrane was not permeable for the hormone (nuclear exchange assay) and the hormone receptor complex (retention assay), respectively. But the nuclei prepared by the method described were stripped from their outer membrane by Triton X-100. That leaves the conclusion that the nuclear acceptor sites are defective in skeletal muscles of Tfm mice. This would also be an obvious interpretation of the results of Thiedemann *et al.* [13] who observed that in striated urethral muscle Tfm nuclei are not stimulated by the intact testosterone receptor complex.

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