Androgen-dependent messenger RNA(s) related to secretory proteins in the mouse epididymis

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Summary. Total RNA from mouse epididymides was translated in a cell-free system derived from rabbit reticulocyte lysate. The androgen dependence of a highly represented mRNA(s) was detected. This mRNA(s) encoded for a band of M_r 26 000 visualized by denaturing gel electrophoresis (SDS-PAGE). No other mouse sexual tissues (testis, vas deferens and seminal vesicle), liver, kidney or striated muscle presented such a band. Furthermore, this mRNA(s) was restricted to the caput epididymidis. Two-dimensional gel electrophoresis (2D-PAGE) showed that this band of M_r 26 000 was composed of 6 basic polypeptides. This translated protein may correspond to a newly synthesized secretory protein of M_r 24 000.

The concentration of translatable mRNA(s) encoding for the band of M_r 26 000 dropped to 31% of the normal level at 3 days after castration and to 7%, 20 days later. Administration of testosterone to 30-day-castrated mice partly reversed these changes: 32% of the control value was reached after 10 days of treatment. Accumulation of this mRNA(s) was also just detectable (7% of the mature level) at 10 days of age. The high levels attained by 20 days of age (60% of the control value) suggest that the greatest increase of accumulation for this mRNA(s) occurs during the prepubertal period.

Keywords: mouse; epididymis; castration; ontogeny; mRNA(s)

Introduction

The mammalian epididymis is an androgen-dependent organ through which spermatozoa undergo a progressive series of morphological and physiological changes (Bedford, 1975) resulting in the ability to fertilize the oocyte (Orgebin-Crist *et al.*, 1975; Orgebin-Crist & Jahad, 1982). These sperm changes which are referred to as the 'maturation process' are under androgenic control of the epithelium and require RNA and protein synthesis (Orgebin-Crist & Jahad, 1978). In rodents, specific epididymal proteins have been identified (Cameo & Blaquier, 1976; Garberi *et al.*, 1979; Brooks & Higgins, 1980; Jones *et al.*, 1980). Some are associated with the sperm surface (Lea *et al.*, 1978; Voglmayr *et al.*, 1980; Moore, 1981; Olson & Danzo, 1981).

Modulation of epididymal gene expression by hormones has been investigated (Brooks, 1981). Most evidence to date suggests that androgens control protein synthesis by regulating gene expression at transcriptional and/or post-transcriptional levels (D'Agostino *et al.*, 1980; Brooks *et al.*, 1986a, b; Morel *et al.*, 1986; Brooks, 1987a; Courty *et al.*, 1987). The mechanism for the mouse epididymis is not known although several androgen-dependent proteins have been identified (Flickinger *et al.*, 1986; Holland & Orgebin-Crist, 1988), amongst which some bind specifically to the surface of spermatozoa (Devine & Carroll, 1984; Murphy & Carroll, 1984, 1987; Lakoski *et al.*, 1988).

The caput epididymidis in the mouse is an active site of synthesis and secretion for numerous proteins (Flickinger, 1979, 1981; Fain-Maurel *et al.*, 1981, 1983; Flickinger, 1983; Devine &

Carroll, 1985). Furthermore, it is generally admitted that most of the physiological changes of the gametes are initiated in this epididymal region (Courrot, 1981; Orgebin-Crist & Fournier-Delpech, 1982; Voglmayr & Dacheux, 1983). Hence, as a preliminary to studies on the regulation of gene expression, it was decided to investigate the effects of testosterone on mRNA activity in the mouse caput epididymidis.

Materials and Methods

Animals. Tissues were isolated from male mice of the Swiss strain (CD1: Charles River, Clon, France) killed under different physiological conditions. Adult mice (60 days) were castrated by the abdominal route. Androgenic replacement was by subcutaneous injection twice daily (75 µg/injection) for 10 days with testosterone heptylate (Theramex Laboratories, Monaco) dissolved in sesame oil. For total RNA isolation, at each age and in each physiological state, up to 40 pooled organs were excised free from adhering adipose tissue, immediately frozen in liquid nitrogen and then stored at -80°C . Just before freezing, the epididymides were cut into the 3 regions of the caput, corpus and cauda (see Fig. 1b).

Isolation of total RNA. Total cellular RNA was isolated by the guanidium extraction procedure of Cathala *et al.* (1983) in which up to 500 mg tissue were homogenized in 8 ml 3 M-guanidium monothiocyanate, 50 mM-Tris-HCl pH 7.5, 10 mM-EDTA, 10% (v/v) β -mercaptoethanol. Total RNA was precipitated using 3.5 M-LiCl at 4°C for 36 h. After centrifugation at 11 000 g for 90 min the pellet was redissolved in 10 mM-Tris-HCl pH 7.5, 1 mM-EDTA, 0.1% (v/v) sodium dodecyl sulphate (SDS) by vortexing. Proteins were then recovered by phenolic extraction and finally total RNA was precipitated in 250 mM-sodium acetate with 2 volumes of ethanol at -20°C .

In-vitro translation of RNA. Total RNA (10 µg) was translated in a cell-free system derived from rabbit reticulocyte lysate treated with micrococcal nuclease (Promega Biotec, Madison, WI, USA) and with added yeast tRNA (5 ng/l: GIBCO BRL, Cergy Pontoise, France). Incubation was carried out with human placental ribonuclease inhibitor (HPRI or RNasine, 1 U/l: Promega Biotec) for 1 h at 30°C according to the manufacturer's instructions, in the presence of 60 Ci [^{35}S]methionine (sp. act. 1000 Ci/mmol: Amersham, Bucks, UK).

Whole cell protein synthesis. To obtain newly synthesized proteins, 4 epididymides were freshly excised and minced finely in ice-cold sterile phosphate-buffered saline to remove spermatozoa and luminal secretions. Tissue fragments of 50 mg were incubated for 5 h at 37°C in a CO_2 -enriched atmosphere in 0.5 ml Eagle's minimum essential medium (MEM) without methionine (GIBCO BRL) containing 100 Ci [^{35}S]methionine (sp. act. 1000 Ci/mmol: Amersham).

The tissues were then rinsed with ice-cold 120 mM-Tris-HCl buffer pH 7.2 and homogenized in the same buffer in the presence of 0.1% SDS (v/v). After centrifugation at 12 000 g for 10 min, supernatants were collected and stored at -80°C . Total soluble proteins were estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Protein electrophoresis and autoradiography. Incorporated radioactivity in newly synthesized proteins and in translated products was determined by protein precipitation with 10% trichloroacetic acid and measurement with scintillation liquid (Beckman EP40) in a scintillation spectrophotometer (Beckman LS9800, Beckman Instruments, Gagny, France). Protein synthesis or translation was investigated by analysis of labelled proteins or translated products on 15% polyacrylamide slab gels in the presence of 0.1% (v/v) SDS (SDS-PAGE) according to the method of Laemmli (1970). Denatured samples containing 150 000 d.p.m. were loaded and one-dimensional electrophoresis was performed for 6 h at 25 mA in Tris-glycine buffer pH 8.3. Two-dimensional electrophoresis (2D-PAGE) consisting of a non-equilibrium pH gradient electrophoresis (NEpHGE) followed by denaturing polyacrylamide gel electrophoresis were also performed according to O'Farrell (1975). In the first dimension, samples containing 500 000 d.p.m. were loaded in the presence of pH 3–10 ampholines (Servalyt: Tebu, Le Perray-en-Yvelines, France) and of a non-denaturing zwitterionic detergent according to Hjelmeland (1980).

Gels were then stained with Coomassie brilliant blue R250 (0.25% in 50% methanol, 10% acetic acid) for 30 min and destained for 12 h in 20% methanol, 5% acetic acid. For 2D-PAGE, fluorography of gels was performed using 20% PPO in DMSO (w/v) as an enhancer (Laskey & Mills, 1975). After drying, gels were exposed for 1 month at -80°C on an X-Omat film (Kodak, Rochester, NY, USA) in the presence of intensifying screens (Dupont Cronex Li+, Wilmington, DE, USA). The absorbance of one-dimensional gels electrophoresis autoradiography was also measured spectrophotometrically at 580 nm (LKB Instruments, Orsay, France).

Results

Evidence for a major mRNA(s) species restricted to the caput epididymidis

Individual mRNA species can be studied by translating them in a cell-free system and analysing the pattern of radiolabelled translated products on SDS-PAGE. Total RNA was isolated from

whole epididymis, testis, vas deferens, seminal vesicle, liver, kidney and striated muscle of normal adult mice and was translated in the rabbit reticulocyte lysate. Autoradiography of the gel electrophoresis indicated that a major band of M_r 26 000 seemed to be present in the epididymis only (Fig. 1a, lane 1). Total RNA was isolated from the caput, corpus and cauda epididymidis (Fig. 1b). SDS-PAGE analysis of translated peptides from 10 μ g total RNA of each part showed that the band of M_r 26 000 was more abundant in the caput (Fig. 1c, lane 1) than in other parts of the epididymis (Fig. 1c, lanes 2 and 3). Three bands of M_r 22 000, 25 000 and 27 000 were translated in the corpus (Fig. 1c, lane 2), while another major band of M_r 28 000 was apparent in the cauda (Fig. 1c, lane 3).

Fig. 1. Tissue specificity and epididymal distribution for the mRNA(s) encoding for the protein at M_r 26 000. **(a)** Autoradiography analysis of radiolabelled peptides translated, in rabbit reticulocyte lysate, from 10 μ g total RNA prepared from adult male mice whole epididymis (lane 1), testis (lane 2), vas deferens (lane 3), seminal vesicle (lane 4), liver (lane 5), kidney (lane 6) and striated muscle (lane 7). Arrow indicates the position of the major epididymal band of M_r 26 000. **(b)** Subdivisions of the epididymis into the caput, the corpus and the cauda. **(c)** Autoradiography analysis of radiolabelled peptides translated, in rabbit reticulocyte lysate, from 10 μ g total RNA isolated from the caput (lane 1), corpus (lane 2) and cauda (lane 3) epididymidis. Lane 4 was the lysate control without added RNA. Arrow indicates the position of the band of M_r 26 000.

Androgenic regulation of the mRNA(s) encoding for the protein at M_r 26 000

To investigate whether testosterone regulated the accumulation of this translatable mRNA(s) species, 10 μ g total RNA extracted from the caput epididymidis of normal adult mice, and mice castrated for 30 days or castrated for 30 days and then treated for 10 days with testosterone, were translated in the cell-free system. Analysis of labelled translated peptides on SDS-PAGE followed by autoradiography revealed that testosterone regulated the concentration of this mRNA(s). Autoradiographs were scanned on a spectrophotometer. The band at M_r 26 000 accounted for 34% of the total [35 S]methionine incorporated for the normal adult mice (Fig. 2a, lane 1). After castration, the proportion declined to 2.5% (Fig. 2a, lane 2) but was partly restored to 13% (Fig. 2a, lane 3) by testosterone administration.

Total RNA was prepared from the caput epididymidis of adult mice (60 days), adult mice castrated for 3, 10, 20 or 30 days and from mice castrated for 30 days then treated with testosterone for 10 days. After translation of 10 μ g total RNA in the cell-free system containing [35 S]methionine, the radioactivity incorporated in the band of M_r 26 000 was investigated by analysing the electrophoresis autoradiographs on a spectrophotometer. Results were expressed as a percentage of pre-castration values (Fig. 2b). Specific translatable mRNA(s) encoding for the band of M_r 26 000 fell by about 70% during the first 3 days after castration and represented about 5% of normal by 30 days castration. Administration of testosterone to these long-term castrated mice resulted in a partial restoration in translatable mRNA(s) concentration (32% of normal value).

Accumulation of the mRNA(s) encoding for the protein at M_r 26 000 during post-natal development

Total RNA (10 μ g) from the caput epididymidis of young mice aged 10, 20 and 30 days and from adult mice (60 days) was translated. The concentration of specific translatable mRNA(s) encoding for the band of M_r 26 000 was assessed as described previously. Results are expressed as a percentage of normal adult values (Fig. 2c). Translatable mRNA(s) for the band of M_r 26 000 was already detectable at 10 days of age, representing about 5% of the normal values. Thereafter, the level of mRNA(s) rose sharply to reach about 60% of the control at 20 days of age and attaining values characteristic of adulthood between 20 and 60 days of age.

Comparison of the protein at M_r 26 000 with proteins synthesized in the caput epididymidis

To correlate the translated band of M_r 26 000 with epididymal proteins, protein synthesis was investigated. Fragments of caput epididymidis from adult mice, adult mice castrated for 30 days and adults castrated for 30 days before treatment with testosterone for 10 days were incubated *in vitro* in the presence of [35 S]methionine. Proteins were separated on SDS-PAGE and gels were stained with Coomassie blue. No accumulated protein of M_r 26 000 was stained. Gels were then autoradiographed to indicate labelled proteins.

Unexpectedly, no band of M_r 26 000 was detected amongst the newly synthesized proteins but a major androgen-dependent band of M_r 24 000 was identified. Direct SDS-PAGE of the media from the incubation sets revealed the presence of a particularly prominent protein of M_r 24 000 for the normal epididymis, suggesting a secreted protein (data not shown). To attempt correspondence between the bands of M_r 26 000 and M_r 24 000, two-dimensional gel electrophoresis was performed and labelled proteins or translated peptides were detected by fluorography. The pattern of peptides translated from total RNA extracted from mice of different androgenic status (Fig. 3a) showed that the major band of M_r 26 000 consisted of 6 basic spots with pH_i values varying from 6 to 9 (Fig. 3a, slab 1). The 2nd and 4th spots ($pH_i = 6.5$ and $pH_i = 7$) were not completely suppressed in 30-day-castrated males (Fig. 3a, slab 2) and the first spot ($pH_i = 6$) was not restored by testosterone in our conditions (Fig. 3a, slab 3). For the newly synthesized proteins, the band of M_r 24 000 consisted of 6 basic peptides with pH_i values varying from 7 to 9 (Fig. 3b, slab 1). The 1st and 3rd spots

Fig. 2. Androgenic regulation and influence of age on translatable mRNA(s) encoding for the protein at M_r 26 000. **(a)** Autoradiography analysis of radiolabelled peptides translated, in rabbit reticulocyte lysate, from 10 μ g total RNA from the caput epididymidis of normal adult mice (lane 1), mice castrated for 30 days (lane 2), and mice castrated for 30 days and treated with testosterone for 10 days (lane 3). Lane 4 was the reticulocyte lysate control without added RNA. Arrow indicates the position of the major androgen-dependent band of M_r 26 000. **(b)** Concentration of specific translatable mRNA(s) encoding for the band of M_r 26 000 in the caput epididymidis after castration and subsequent treatment with testosterone. Values were obtained by scanning 2 SDS-PAGE autoradiographs on a spectrophotometer. Each value is obtained by translating 10 μ g total RNA extracted from 40 pooled organs. Results are expressed as a percentage of pre-castration value of [35 S]methionine incorporated in the band of M_r 26 000 translated from caput epididymal total RNA from normal adult mice. **(c)** Developmental changes in concentration of specific translatable mRNA(s) encoding for the band of M_r 26 000 in the caput epididymidis. Each value was obtained by translating 10 μ g total RNA extracted from 40 pooled organs. Results are expressed as a percentage of the proportion of [35 S]methionine incorporated in the band of M_r 26 000 translated from the total RNA from the caput epididymidis of adult mice. Values were obtained by scanning 2 SDS-PAGE autoradiographs on a spectrophotometer.

($\text{pH}_i = 7$ and $\text{pH}_i = 8$) were not completely suppressed by castration for 30 days (Fig. 3b, slab 2) and the first spot ($\text{pH}_i = 7$) was not restored by testosterone treatment for 10 days (Fig. 3b, slab 3).

Fig. 3. Two-dimensional fluorography analyses of radiolabelled peptides. **(a)** Peptides translated, in rabbit reticulocyte lysate, from $10\ \mu\text{g}$ total RNA extracted from the caput epididymidis of normal adult mice (slab 1), mice castrated for 30 days (slab 2), and mice castrated for 30 days then treated with testosterone for 10 days (slab 3). Arrows indicate the position of the basic spots for the androgen-dependent protein of M_r 26 000. **(b)** Proteins newly synthesized *in vitro*, in the presence of $[^{35}\text{S}]$ methionine, by the caput epididymidis of normal mice (slab 1), mice castrated for 30 days (slab 2) and mice castrated for 30 days and then treated with testosterone for 10 days (slab 3). Arrows indicate the position of the basic spots corresponding to the secretory androgen-dependent protein of M_r 24 000.

Discussion

The epididymis is a complex organ with pronounced regional variations in structure and functions (Hamilton, 1975). It is now assumed that the main events of the sperm maturational changes occur in the caput epididymidis (Courot, 1981; Orgebin-Crist & Fournier Delpech, 1982) and the particular importance of androgen-regulated epididymal secretory proteins in promoting those sperm changes appears to be well established (Orgebin-Crist & Jahad, 1979). Nevertheless, in the mouse little is known about hormonal regulation of epididymal protein synthesis (Holland & Orgebin-Crist, 1988). These reasons led us to investigate whether individual mRNA(s) species corresponding to secretory proteins were regulated by testosterone in the mouse epididymis. The present results indicate an abundant mRNA(s) sequence specific to the mouse epididymis which is translatable *in vitro* into a protein band of M_r 26 000. This mRNA(s) was restricted to the caput

epididymidis, suggesting that it encoded for a major protein which could serve a specific function within the epididymal environment. The results described in this paper suggested also that the relative concentration of this mRNA(s) sequence was controlled by testosterone, making it a good marker of hormonal action that will facilitate further studies into the mechanism by which testosterone regulates specific protein synthesis in the mouse epididymis.

After castration, the rate of decrease in mRNA concentration is similar to that observed for secretory proteins in two other well studied androgen-dependent tissues, namely the prostate and seminal vesicle. In those organs, specific species of mRNAs are reduced in concentration by 2 or 3 orders of magnitude during the first week after castration (Parker *et al.*, 1980; Page & Parker, 1982; Mills *et al.*, 1987a, b, c). In the mouse epididymis we detected a 70% reduction of concentration of the mRNA(s) for the band of M_r 26 000 at 3 days after castration. A decline in mRNA(s) levels after castration indicates that the androgenic control of synthesis of the corresponding proteins is determined, at least in part, by control of the steady state level of mRNAs. It remains to be shown whether additional translation control, as observed in the seminal vesicle during sexual maturation (Kistler *et al.*, 1981), also operates. The failure to obtain full restoration of mRNA(s) levels for the band of M_r 26 000 should be emphasized. A similar situation has been described in the rat epididymis for Proteins B and C (Brooks, 1987a) and the synthesis of an epididymal protein of M_r 23 500 is not fully restored by testosterone in castrated mice (Holland & Orgebin-Crist, 1988). In the rat, circulating testosterone concentrations are not sufficient to obtain full maintenance of morphology and metabolism of the epididymal proximal parts (Brooks, 1983), suggesting that these epididymal regions are dependent on some other testicular factors. The nature of these additional factors is unknown but complex multihormonal control has been suspected (Brooks, 1987b). It will be of interest to determine whether other hormones or testicular factors are able to restore the mRNA(s) concentration. With a cDNA probe it will be possible to investigate all these control phenomena in more detail.

Translatable mRNA(s) for the band at M_r 26 000 were detectable in the epididymis of mice of 10 days of age. Then they increased dramatically, coinciding with the increase in epididymal testosterone content which occurs in mice aged 20 days (Jean-Faucher *et al.*, 1985). At this age the epididymal epithelium differentiates into its various cell types (Abou-Haila & Fain-Maurel, 1985). The concentration of the most important translatable mRNA(s) therefore appeared to increase during the prepubertal period and to correspond to the cellular differentiation step, as described for the mRNAs of the rat epididymal Proteins B, C and D (Brooks, 1987a), of the mouse prostate p12 and p25 (Mills *et al.*, 1987a, b, c) or for the rat SVSII in seminal vesicle (Dodd *et al.*, 1986). The seminal vesicle mRNA is present in early development but is apparently not efficiently translated *in vivo* into proteins (Kistler *et al.*, 1981). Such results led us to believe that a cell-free translation system would provide evidence for in-vitro translatable mRNA(s) sequences. We cannot exclude that untranslatable mRNA(s) for the protein at M_r 26 000 could be present in the early development of young mice. A cDNA probe will permit further investigations.

Using epididymal tissue slices incubated with [35 S]methionine, we were not able to identify a protein at M_r 26 000, but a major secretory protein band (M_r 24 000) regulated by testosterone was detected. No other androgen-dependent protein was detected in this molecular weight range. When analysed by 2D-PAGE the protein band of M_r 24 000 appeared to be composed of 6 basic peptides and the translated band of M_r 26 000 also consisted of 6 basic spots. These spots had different pI, values but the same pattern as those of the newly synthesized protein of M_r 24 000, and the hormonal regulation was similar. If the protein of M_r 26 000 and the protein of M_r 24 000 are related, an explanation is needed for the difference in relative mobility. Many secretory proteins have been demonstrated to be synthesized as presecretory proteins containing an N-terminal sequence of M_r $\approx$ 2000 referred to as the 'signal peptide' when their mRNA is translated in a cell-free system (Milstein *et al.*, 1972; Blobel & Dobberstein, 1975). Since the additional amino acids are usually cleaved *in vivo* but not in the reticulocyte lysate, the peptides translated *in vitro* would migrate with a reduced relative mobility. Therefore, the protein of M_r 26 000 could correspond to

the presecretory precursor incompletely processed into the authentic secretory protein of M_r 24 000. To account for the 'signal peptide' hypothesis, translation was performed in the presence of heterologous dog pancreatic microsomal membranes. Unfortunately no difference in the relative mobility of the translated protein at M_r 26 000 was detected (data not shown).

Antibodies against the proteins are currently being raised to study whether the translated protein of M_r 26 000 and the synthesized protein of M_r 24 000 are related.

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