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Original Article

Developmental and hormonal regulation of specific proteins in mouse vas deferens and seminal vesicle

CHRISTIANE JEAN-FAUCHER, CÉCILE GUILBAUD, MICHÈLE MANIN and CLAUDE JEAN*

Physiologie Comparée et Endocrinologie, CNRS URA 360, Université Blaise Pascal, Les Cézeaux, Aubière, France

ABSTRACT This paper is concerned with hormonal regulation of the developmental pattern of major proteins of the mouse vas deferens (mouse vas deferens protein: MVDP, 34.5 kD) and seminal vesicle (15.5, 120 and 140 kD) whose expression is regulated by testosterone at adulthood. The ontogeny of these proteins, studied by SDS-polyacrylamide gel electrophoresis, appeared to be uncoordinated. MVDP was not accumulated until animals were 20 days old and its concentration increased sharply from 20 to 30 days of age. In seminal vesicle, the 15.5 kD protein did not accumulate before day 30 whereas 120 and 140 kD proteins appeared and accumulated between 30 and 40 days. In 30-day-old mice castrated at birth or treated with cyproterone acetate over 29 days, MVDP levels were not abolished and were similar to those measured in 20-day-old males. Testosterone administration, from 1 to 10 days of age, did not induce precocious expression of MVDP. These results suggest that the neonatal expression of MVDP is independent of androgens. In seminal vesicle, the first expression of the 3 proteins studied was dependent upon testicular androgens as shown by neonatal castration and injection experiments. The marked increase in the levels of the 4 proteins studied, during sexual maturation, was not associated with quantitative or qualitative changes in tissular androgen concentrations, suggesting that other factors may be necessary for protein expression. Whereas thyroxine may induce a precocious accumulation of MVDP, prolactin had no stimulatory effect on the accumulation of proteins from vas deferens and seminal vesicle. The results suggest that during sexual maturation gene activation by androgens was progressive.

KEY WORDS: *ontogeny, androgen-dependence, major proteins*

Introduction

Androgens regulate many aspects of cellular development and differentiated function in reproductive organs by controlling the synthesis and secretion of some specific proteins such as androgen-binding protein secreted by the testis (French and Ritzen, 1973), prostatic-binding protein by the prostate (Heyns, 1977), specific epididymal proteins by the epididymis (Cameo and Blaquier, 1976; Brooks and Higgins, 1980; Jones *et al.*, 1980), seminal vesicle secretory proteins by seminal vesicle (Higgins *et al.*, 1976) and mouse vas deferens protein (MVDP) by vas deferens (Taragnat *et al.*, 1986). During the postnatal period, the development of sex accessory organs is androgen-dependent. Castration of neonatal mice or rats greatly inhibits continued growth of accessory sex organs such as prostate, an effect which can be reversed by administration of testosterone (Cunha *et al.*, 1987; Donjacour and Cunha, 1988). However, some aspects of neonatal accessory sex organ growth may be independent of androgens. For example, castration of neonatal mice or rats does not completely halt development of the prostate, seminal vesicle and bulbourethral glands (Cooke *et al.*, 1987; Cunha *et al.*, 1987; Donjacour and Cunha, 1988). Thus, it is possible that additional non-androgen factors may be required for full development of accessory sex organs. The possibility that prolactin may play a physiological role in their growth has been well documented (Moger and Geschwind, 1972; Negro-Vilar *et al.*, 1977). Thyroid hormones are known to modulate the activities of numerous biochemical processes and it has been reported that hypothyroid male mice are infertile (Beamer *et al.*, 1981). Identification of gene products and exploration of their regulation and possible role in cellular differentiation and organ functional development would be of great interest since changes in expression of specific genes may play a crucial role in the maturing process. The appearance of androgen-dependent proteins during the development of the seminal vesicle (Kistler *et al.*, 1981; Fawell and Higgins, 1986), prostate (Heyns *et al.*, 1978; Higgins *et al.*, 1982; Wada *et al.*, 1985; Mills

Abbreviations used in this paper: MVDP, mouse vas deferens protein; SDS, sodium dodecyl sulfate; VD, vas deferens; SV, seminal vesicle; SVS IV, seminal vesicle secretory protein; DHT, dihydrotestosterone; T, testosterone; CA, cyproterone acetate; SEM, standard errors mean; AEG, acidic epididymal glycoprotein; sc, subcutaneous; ip, intraperitoneal.

***Address for reprints**: Physiologie Comparée et Endocrinologie, CNRS URA 360, Université Blaise Pascal, Les Cézeaux, F-63177 Aubière Cédex, France. FAX: 73-40-70-42

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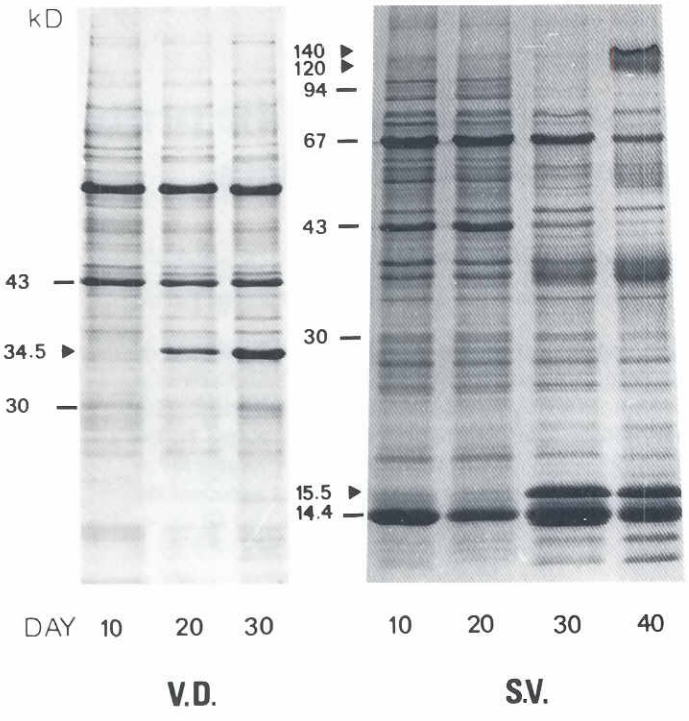

Fig. 1. Polyacrylamide gel electrophoretic analysis of proteins from vas deferens (VD) and seminal vesicle (SV) of mice aged from 10 to 40 days. *Each lane was loaded with 70μg proteins (VD) or 100μg proteins (SV). Proteins were stained with Coomassie Blue. Black arrows indicate position of the proteins studied, in vas deferens (34.5 kD: MVDP) and in seminal vesicle (15.5, 120 and 140 kD). The positions and molecular weights of standard proteins run in a parallel lane are also shown. The observations were made in 4 separate experiments.*

et al., 1987), and epididymis (Faye *et al.*, 1980; Kohane *et al.*, 1983; Charest *et al.*, 1989) seems to be quite variable, and no reports exist on the role of androgens in the first expression of these proteins.

In mouse, several proteins have been used as markers of androgen action in adult tissues. We have previously reported an androgen-dependent change of vas deferens and seminal vesicle proteins caused by castration and androgen replacement. Mouse vas deferens synthesizes and secretes an abundant androgen-dependent protein of 34.5 kD called MVDP (mouse vas deferens protein: Taragnat *et al.*, 1986). Proteins having a molecular weight of 15.5, 120 and 140 kD, which are the major protein species in the seminal vesicle, were found to be dependent on the level of androgens (Normand *et al.*, 1989). The present paper deals with studies on (1) the time at which expression of the 4 proteins described above is initiated during the course of postnatal development, and (2) the endocrine regulation of this initiating process.

Results

Influence of age on the androgen-dependent proteins of the vas deferens and seminal vesicle

The accumulation of androgen-dependent proteins, previously identified in adult males (Taragnat *et al.*, 1986; Normand *et al.*, 1989), was investigated in relation to post-natal development. Androgen-dependent secretory proteins have been previously described in seminal vesicle of adult mouse (Chen *et al.*, 1987; Normand *et al.*, 1989). Seminal vesicle secretory protein IV has been isolated and the cDNA coding for its mRNA cloned and sequenced (Chen *et al.*, 1987). Based on color staining and apparent molecular weight, the 15.5 kD protein band could be identified with SVS IV, and the 120 and 140 kD bands with the two androgen-dependent proteins of the same molecular weight described by Normand *et al.*, 1989. The present study has focussed on these proteins, which are present at high concentrations and well characterized by their electrophoretical properties. Other androgen-dependent proteins, previously described, are also present but not retained in this study.

The pubertal period can be regarded as being between 30 and 40 days, which corresponds to the time during which sperm first enters the epididymis (Jean-Faucher *et al.*, 1978). In vas deferens, the 34.5 kD band (MVDP) was detectable in 10-day-old males and accumulated in significant amounts between 10 and 20 days (Fig. 1). The concentration of MVDP rose abruptly between 20 and 30 days reaching values characteristic of adulthood. The ontogenesis of androgen-dependent proteins from seminal vesicle was quite different. The 15.5 kD protein band did not accumulate in significant amounts until animals were 30 days old, then reaching adult values (Fig. 1). Androgen-dependent proteins with high molecular weights (120, 140 kD) appeared later, between 30 and 40 days, when adult levels were reached.

Androgens and developmental expression of proteins of the vas deferens and seminal vesicle

To determine whether the accumulation of the 4 proteins studied between 10 and 40 days might be related to local changes in androgen concentrations, testosterone and DHT were measured in vas deferens and seminal vesicle during the same period. In order to compare the total androgen content of the two organs, testosterone and DHT concentrations were added (Table 1). From 10 to 40 days there were no significant variations in tissular androgen concentrations (analysis of variance). Then, the rise observed in the accumulation of androgen-dependent proteins from vas deferens and seminal vesicle during this period occurred in presence of relatively

TABLE 1

ANDROGEN (TESTOSTERONE+DHT) CONCENTRATIONS IN VAS DEFERENS AND SEMINAL VESICLE DURING POSTNATAL DEVELOPMENT

Age (days):		1	10	20	30	40
Vas deferens	ng/g:	24.0	12.7± 3.5	8.5± 2.3	6.6± 1.1	13.2± 3.1
	T/DHT:	2.0	0.10± 0.05	1.21± 0.61	0.95± 0.12	1.40± 0.41
Seminal vesicle	ng/g:	20.0	13.4± 1.9	15.3± 1.2	14.4± 1.6	15.1± 1.5
	T/DHT:	0.08	0.07± 0.01	0.05± 0.01	0.09± 0.01	0.17± 0.03

Values are means±SEM. Organs were pooled as follows: 1 day: 1 pool (60 samples); 10 days: 5 pools (20 samples in each); 20 days: 8 pools (10 samples in each); 30 days: 12 pools (4 samples in each); 40 days: 13 pools (4 samples in each). The testosterone/dihydrotestosterone ratio (T/DHT) is expressed as the mean±SEM of the individual ratios of the two hormones in the sexual target organs.

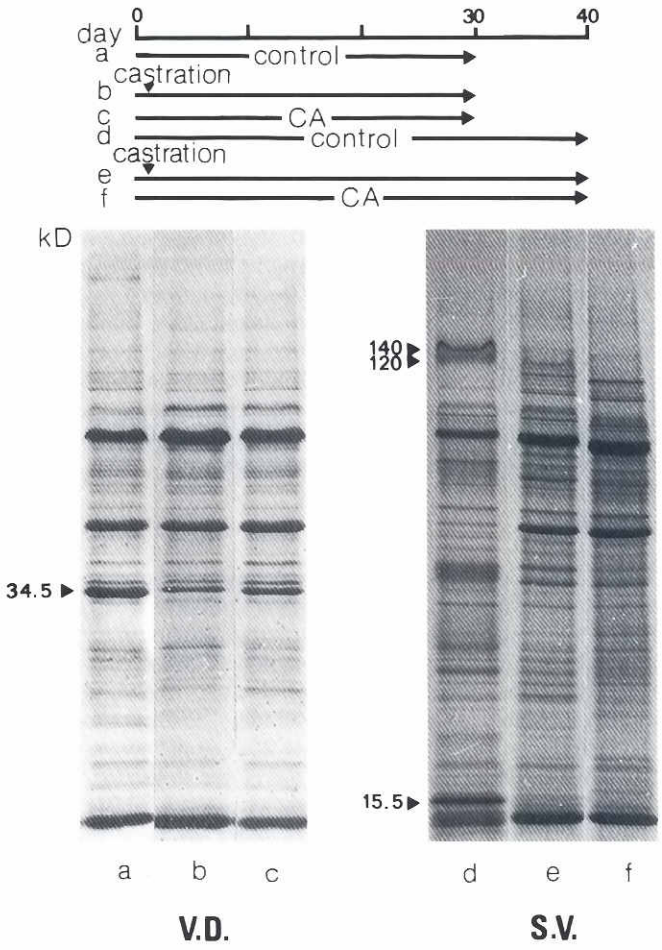

Fig. 2. Effects of castration and cyproterone acetate administration on protein levels in males aged 30 (VD) or 40 (SV) days. *Vasa deferentia and seminal vesicles from treated males were pooled (5-10 animals per condition). The experiment was made twice with two different pools and identical results were obtained with both analyses. Polyacrylamide gel electrophoretic analysis of proteins from vas deferens (VD) and seminal vesicle (SV). Each lane was loaded with 70μg proteins (VD) or 100μg proteins (SV). Proteins were stained with Coomassie Blue. Newborn mice were castrated* **(b, e)** *or treated every day with cyproterone acetate* ***(c, f)*** *until sacrifice. Untreated males aged 30* **(a)** *or 40* **(b)** *days. Black arrows indicate position of the proteins studied.*

constant androgen concentrations. In the vas deferens, with the exception of 10 days, both androgens were present in similar quantities from birth to 40 days of age (T/DHT between 0.95 and 2.0). In the seminal vesicle, the predominant androgen was DHT at all stages studied.

To determine whether or not the appearance and accumulation of the 4 proteins studied were under the control of testicular androgens, newborn males were castrated and examined at 30 (vas deferens) or 40 (seminal vesicle) days of age. As shown in Fig. 2, castration strongly inhibited the normal increase occurring between 10 and 30 days in MVDP accumulation. However, MVDP appeared and was expressed in levels similar to those observed in 20-day-old untreated males. When endogenous androgens were inhibited by CA administration from 1 to 29 days of age, MVDP levels were significantly reduced but not abolished. The levels of MVDP observed in CA-treated males were higher than those of neonatally castrated males. In contrast, both castration and CA treatment nearly abolished the appearance and accumulation of the 15.5, 120 and 140 kD protein bands in seminal vesicle (Fig. 2).

In adult males, treated with CA during sexual maturation, tissular and circulating androgen levels were equal to those of controls (Jean-Faucher *et al.*, 1984). Thus, in order to determine whether the alterations described above were reversible, males treated with CA

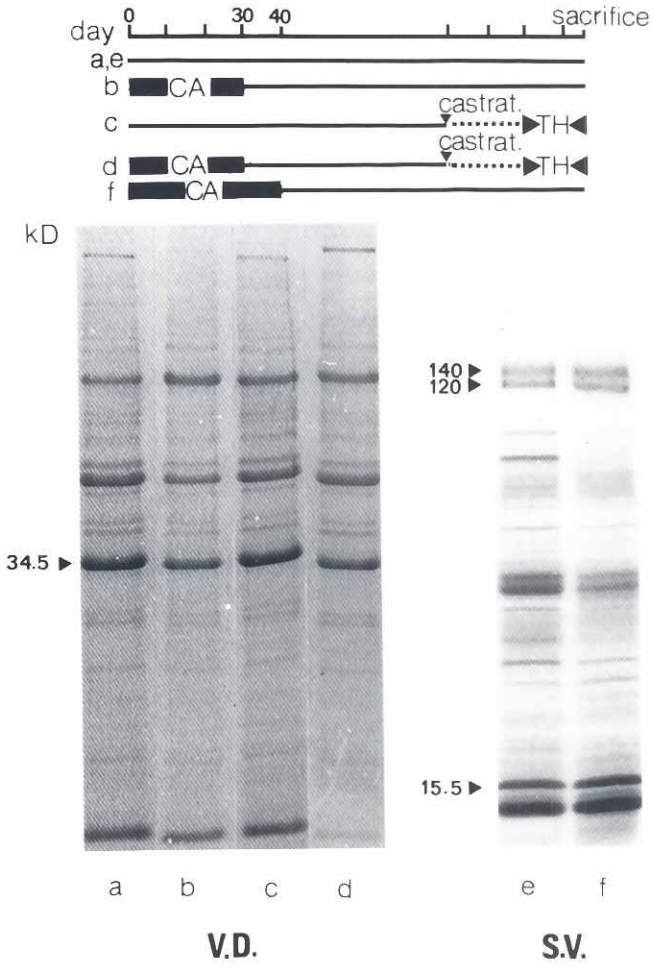

Fig. 3. Effects of cyproterone acetate during prepubertal life on protein levels in adult males. *Polyacrylamide gel electrophoretic analysis of proteins from vas deferens (VD) and seminal vesicle (SV). Each lane was loaded with 70μg proteins (VD) or 100μg proteins (SV). Proteins were stained with Coomassie Blue. Newborn mice were treated with cyproterone acetate from 1 to 30 days (VD) or from 1 to 40 days (SV) and sacrificed at 3-4 months of age* **(b, f). (a, e)** *Untreated mice.* **(c)** *Untreated mice castrated at adulthood and treated 3 weeks later with testosterone heptylate (75μg twice daily for 2 weeks).* **(d)** *Prepubertally CA-treated mice castrated at adulthood and treated 3 weeks later with testosterone heptylate (75μg twice daily for 2 weeks). Black arrows indicate position of the proteins studied. The observations were made in 4 separate experiments.*

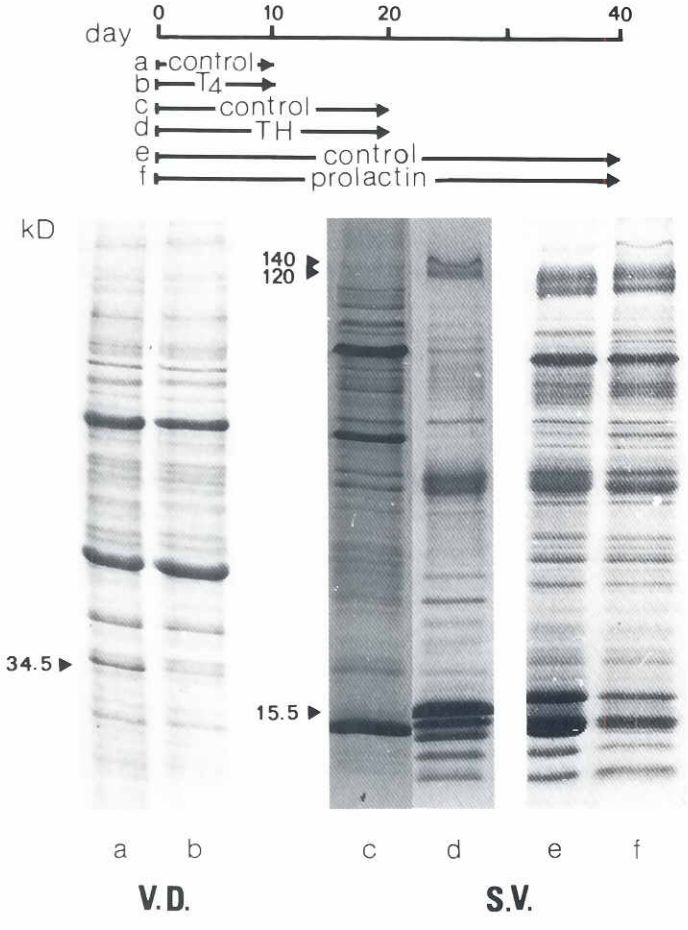

Fig. 4. Effects of thyroxine, testosterone and prolactin administration on protein levels in males aged from 10 to 40 days. *Polyacrylamide gel electrophoretic analysis of proteins from vas deferens (VD) and seminal vesicle (SV). Each lane was loaded with 70μg proteins (VD) or 100μg proteins (SV). Proteins were stained with Coomassie Blue. Mice were treated every day with thyroxine* **(b)**, *testosterone* **(d)** *or prolactin* **(f)** *and examined at 10* **(b)**, *20* **(d)** *or 40* **(f)** *days.* **(a, c, e)** *Untreated males of 10, 20 and 40 days of age, respectively. Black arrows indicate position of the proteins studied. The observations were made in 4 separate experiments.*

from 1 to 29 days were examined at adulthood (4 months). As shown in Fig. 3, MVDP levels were slightly reduced in CA-treated males compared to untreated males of the same age. To eliminate the possibility of cumulative effects of long-term impaired testosterone secretion, untreated males and neonatally CA-treated males were castrated (3 weeks) and injected (2 weeks) with testosterone heptylate (75μg twice daily). MVDP levels stayed reduced in the CA-treated group, indicating that testosterone was unable to reverse the effects of neonatal CA treatment. In males treated with CA from 1 to 39 days of age,and examined at 4 months, the levels of the 15.5, 120 and 140 kD protein-bands, in seminal vesicle, were similar to those observed in controls (Fig. 3). The same observations were made when CA-treated males were castrated at adulthood and injected with testosterone heptylate (data not shown).

Hormonal regulation of the first expression of proteins of the vas deferens and seminal vesicle

To determine whether precocious accumulation of the 4 proteins studied could be induced in immature males, newborn animals were injected with testosterone heptylate, thyroxine or prolactin. Whereas testosterone or prolactin treatment during the first 10 days of life did not significantly affect MVDP expression (data not shown), thyroxine injection increased MVDP levels substantially (Fig. 4). Testosterone treatment from 1 to 20 days of age induced precocious accumulation of the 15.5, 120 and 140 kD protein bands in the seminal vesicle (Fig. 4). Prolactin treatment from 1 to 20 or 30 days did not induce precocious accumulation of the proteins from seminal vesicle. After prolactin injection from 1 to 30 or 40 days, the 15.5 kD protein was significantly reduced while the 120 and 140 kD bands were not affected (Fig. 4). The first appearance and accumulation of the 15.5, 120 and 140 kD proteins were not modified by thyroxine administration (not shown).

Discussion

In the report we have characterized the accumulation, during postnatal development, of some major proteins from the vas deferens and seminal vesicle, which are regulated by androgens at adulthood. The developmental expression of these proteins appears to be quite different and uncoordinated. MVDP, the major androgen-dependent protein from vas deferens, does not accumulate in significant amounts until animals are 20 days old and concentrations increase sharply from 20 to 30 days of age. In seminal vesicle, an abundant protein with low molecular weight (15.5 kD) does not accumulate before day 30, whereas major proteins with high molecular weight (120, 140 kD) appear and accumulate between 30 and 40 days. However, the timing of appearance of the proteins must be reinvestigated using more sensitive techniques, *i.e.* silver staining or immunological methods. In the rat, observations made for specific proteins from the epididymis (Faye *et al.*, 1980; Kohane *et al.*, 1983; Charest *et al.*, 1989), seminal vesicle (Kistler *et al.*, 1981; Fawell and Higgins, 1986) and prostate (Heyns *et al.*, 1978; Higgins *et al.*, 1982; Wada *et al.*, 1985) showed similar marked changes as a function of age. These proteins are detectable in young animals (5-20 days) but do not begin to accumulate in significant amounts until animals are about 3-5 weeks old. Recently it has been reported that grafts of newborn (1 day) seminal vesicle grown *in vitro* for 14 days show expression of the major seminal vesicle secretory proteins (Higgins *et al.*, 1989). In mouse ventral prostate, proteins p12 and p25 are detectable at 3 weeks of age, but the maximum level of both proteins is not attained until 5 weeks of age (Mills *et al.*, 1987).

At present, the specific factors responsible for the appearance and accumulation, during development, of major proteins from vas deferens and seminal vesicle are not known. It is uncertain whether androgens are the rate-limiting factor involved in the onset of protein expression because the highest concentrations of tissular androgens are observed at birth when protein expression has not begun. On the other hand, increases in androgen receptor concentration may be a prerequisite for changes in gene expression as has been shown in rat ventral prostate (Zhang *et al.*, 1987). It is doubtful whether the apparent delay in the accumulation of major proteins from the vas deferens and seminal vesicle may be explained by a later appearance of androgen receptors since it has been shown that, at 10 days of age, the mouse vas deferens and seminal vesicle

contain very high levels of androgen receptors (Gallon *et al.*, 1989). The androgen dependency of MVDP and major seminal vesicle proteins, during postnatal development, is quite different. The first expression of MVDP is probably independent of testicular androgens since: 1) MVDP levels observed in neonatally castrated males are similar to those measured in 20-day-old untreated males, 2) attempts to induce precocious expression of MVDP by administering testosterone from 1 to 10 days were not successful. In seminal vesicle, the first expression of protein bands of 15.5, 120 and 140 kD is dependent upon testicular androgens because neonatal castration completely abolishes their appearance and because precocious expression of these proteins may be induced by testosterone administration. In adult males, exposed to CA from 1 to 30 days of life, the accumulation of MVDP is slightly reduced. This decrease in MVDP levels is also observed in CA-treated males castrated and injected with testosterone at adulthood. This suggests that androgens secreted in immature males are necessary to obtain full expression of MVDP at adulthood. In contrast, the expression, at adulthood, of the 3 seminal vesicle proteins studied is apparently not affected by CA treatment.

Since we have previously shown that the 4 proteins studied are regulated by androgens (Taragnat *et al.*, 1986; Normand *et al.*, 1989), a significant increase in androgen concentration may be expected to activate their massive accumulation during postnatal development. In both vas deferens and seminal vesicle, androgen concentrations are high at birth, decline from 1 to 10 days and remain relatively constant until 40 days. Hence, there is no change in androgen concentration at the time the accumulation of these proteins is rapidly increasing. A quite similar situation has been recently described for the AEG epididymal protein (Charest *et al.*, 1989). It is well known that prostate and other androgen-sensitive peripheral structures metabolize testosterone into DHT (Baulieu *et al.*, 1968). It has been shown that the development of measurable concentrations of specific proteins in the prepubertal rat epididymis is coincident with a transient increase in 5α-reductase and 3α-hydroxysteroid dehydrogenase activities leading to increasing levels of 5α-reduced metabolites in the tissue (Larminat *et al.*, 1980; Kohane *et al.*, 1983). In mouse, no qualitative change in androgen concentrations occurs from 1 to 40 days of age: DHT is the main steroid in seminal vesicle, and in vas deferens testosterone and DHT are present in similar quantities. The time course of induction of protein accumulation in vas deferens and seminal vesicle, which occurs without qualitative or quantitative changes in androgen levels and in presence of relatively constant androgen receptor concentrations (Gallon *et al.*, 1989), indicates that gene activation by androgens is progressive. This progressive induction may result from a proliferation of responsive cells. The first accumulation of androgen-dependent proteins in vas deferens and seminal vesicle coincides with an increase in the number of total and epithelial cells (Okamato *et al.*, 1982; Jean-Faucher *et al.*, 1989). It is also possible that additional non-androgen factors may be required to activate accumulation of some androgen-dependent proteins. Present results show that thyroxine may induce a precocious accumulation of MVDP but not that of the seminal vesicle proteins. It has been previously shown that thyroxine and testosterone regulate renin gene expression in the submaxillary gland of mice (Tronik and Rougeon, 1988). However, the influence of thyroid hormones on male reproduction has not been demonstrated (Chubb and Henry, 1988). There are a number of reports proving that prolactin has a direct action on male accessory organs (reviewed by Thomas and Keenan, 1976). Present results show that prolactin has no stimulating effect on the accumulation of androgen-dependent proteins. This agrees with previous studies showing that prolactin had no effect on general or specific protein synthesis in rat epididymis (Jones *et al.*, 1983). Alternatively, the progressive protein accumulation by androgens may also reflect developmental pattern for the genes themselves. During the differentiation of rat prostate it has been observed that specific mRNA induction is accompanied by progressive demethylation of the genes for the prostatic binding protein subunits (White and Parker, 1983). In mouse kidney, a number of mRNAs are selectively induced by administration of androgens and maximal activation requires 2 to 10 days, indicating that responding genes do not appear simply to switch from a basal state to an induced state (Watson and Paigen, 1990). Thus, it is possible that during development, gene activation by androgens may reflect an intrinsic property of each gene rather than changes in components of the androgen response system, *e.g.* androgen levels, androgen receptors concentrations. Studies with specific cDNA and genomic clones will test these possibilities.

Materials and Methods

Animals

Mice of the Swiss strain (CD-1 Charles River, France) were raised in the laboratory under standardized conditions. To investigate the role of androgens, newborn males were castrated or treated with cyproterone acetate (CA) or with testosterone heptylate. Castration was performed within the first 36h of life under cold anaesthesia. Cyproterone acetate (Schering AG, Berlin) was dissolved in a mixture of benzyl benzoate and sesame oil (10%) and administered sc as follows: 200μg/day from 1 to 10 days; 400μg from 11 to 20 days; 800μg from 21 to 30 days; 1000μg from 31 to 40 days. Testosterone heptylate (Theramex Laboratories, Monaco), 50μg dissolved in sesame oil, were injected sc every day from 1 to 10 or 20 days. Thyroxine (Sigma) was dissolved in 10mM NaOH and injected ip. Mice were treated with daily injections of 10μg. Prolactin (Sigma) was dissolved in isotonic solution adjusted to pH 9.0 with 0.1N NaOH. Mice were treated twice daily sc with 1.5 I.U. Animals were killed 24h after the last injection. Vasa deferentia and seminal vesicles were removed immediately after killing, frozen in liquid nitrogen and stored at -20°C. Seminal vesicles and vasa deferentia were homogenized for 30sec at 4°C in 400μl (immature males) or 1000μl (mature males) buffer A in a glass-glass hand-held homogenizer (Braun, Melsungen, FRG). After centrifugation at 12000g for 10 min, the supernatant fluid was retained and used as homogenate.

Polyacrylamide gel electrophoresis

One-dimensional electrophoresis was performed under denaturing conditions using the procedure of Laemmli (1970). Sodium dodecylsulphate protein samples (70-100μg) were applied to 15% resolving gels (vas deferens) or to 10-20% linear gradient resolving gels (seminal vesicle) with a 4.5% stacking gel and run at 25mA at room temperature until the tracking dye (bromophenol blue) reached the bottom of the gel (about 30 min). Gels were then stained with 0.25% Coomassie brilliant blue in an aqueous solution containing 50% (v/v) methanol and 10% (v/v) acetic acid for 45 min, and destained in the same solution without dye. The apparent molecular weight of the proteins was calculated by comparison with the mobility of standard proteins.

Radioimmunoassays

Testosterone and dihydrotestosterone (DHT) were measured as previously described (Jean-Faucher *et al.*, 1984). The sensitivity of the method was 45±10 pg for testosterone and 55±12 pg for DHT. The intra and interassay coefficients of variation were respectively 6 and 6. 1% for testosterone and 9 and 8.6% for DHT.

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