Experimental cryptorchidism induces a change in the pattern of expression of LH receptor mRNA in rat testis after selective Leydig cell destruction by ethylene dimethane sulfonate

M Tena-Sempere, J Kero, A Rannikko and I Huhtaniemi

Department of Physiology, University of Turku, 20520 Turku, Finland

(Requests for offprints should be addressed to I Huhtaniemi, Department of Physiology, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland)

(M Tena-Sempere is now at Department of Physiology, University of Córdoba, 14004 Córdoba, Spain)

Abstract

In the rat, the cytotoxic drug ethylene dimethane sulfonate (EDS) selectively eliminates mature Leydig cells (LCs) from testicular interstitium, activating a complex process of proliferation and differentiation of pre-existing LC precursors. We observed previously that after EDS treatment, the early LC precursors persistently express a truncated 1.8 kb form of LH receptor (LHR) mRNA. This prompted us to study whether experimental cryptorchidism, known to alter the process of LC repopulation, can influence the pattern of testicular LHR mRNA expression after EDS administration.

EDS treatment completely eliminated mature LCs both in control and unilaterally cryptorchid (UC) rats. This response was followed by gradual reappearance of newly formed, functionally active LCs, as evidenced by the recovery in testicular LHR content and plasma testosterone levels in both experimental groups. Noteworthy, the rate of LC repopulation was higher in the abdominal testes of UC rats, in keeping with previous findings. Interestingly, the 1.8 kb LHR transcript was persistently expressed in scrotal testes at all time-points, but undetectable upon Northern hybridization in abdominal testes at early stages after EDS administration, when low levels of

expression of truncated LHR transcripts could only be detected by semi-quantitative RT-PCR analysis. In addition, the faster LC repopulation in cryptorchid testes was associated with precocious recovery of the complete array of LHR mRNA transcripts, including the 1.8 kb species. These changes appeared acutely and irreversibly, as unilateral positioning of scrotal testes into the abdomen resulted in a rapid loss of expression of the 1.8 kb LHR transcript, whereas scrotal relocation of the UC testes failed to alter the pattern of LHR gene expression.

In conclusion, experimental cryptorchidism changes the pattern of LHR mRNA expression in rat testis after selective LC destruction by EDS. This change, i.e. repression of the 1.8 kb LHR transcript after EDS administration, is acute and irreversible, and likely related to the impairment of testicular microenvironment following cryptorchidism. However, even though at low levels, the expression of truncated forms of LHR mRNA appears to be a universal feature of proliferating LC precursors. The UC testis may represent a good model for analysis of the regulatory signals involved in the control of LHR gene expression.

Journal of Endocrinology (1999) **161**, 131–141

Introduction

In the rat and other mammals, testicular Leydig cells (LCs) show two distinct phases of proliferation. The first proliferative wave is detected *in utero* and results in the appearance of fetal-type LCs. The second phase takes place during puberty and gives rise to the adult-type LC population. Fetal- and adult-type LCs differ morphologically and functionally (for reviews see Huhtaniemi & Pelliniemi 1992, Huhtaniemi 1994). In the adult rat, the proliferative activity of mature LCs is negligible (Teerds *et al.* 1988, Gaytan *et al.* 1992). However, active proliferation of pre-existing undifferentiated precursors is triggered by destruction of the mature LC population.

Administration of a single dose of the cytotoxic drug ethylene dimethane sulfonate (EDS) induces complete elimination of mature LCs from the testicular interstitium (Molenaar *et al.* 1985, Morris *et al.* 1986), activating the proliferation and further differentiation of LC precursors with the appearance of a new population of functionally active LCs within 3 weeks (Molenaar *et al.* 1986, Kerr *et al.* 1987, Teerds *et al.* 1990, Gaytan *et al.* 1992). Considering that this repopulation process resembles the normal developmental events of adult-type LCs during puberty, the EDS-treated rat has been widely used as an experimental model for studies on LC development (Jackson *et al.* 1986, Molenaar *et al.* 1986, Kerr *et al.* 1987, Teerds *et al.* 1989a,b, Gaytan *et al.* 1992,

Tena-Sempere *et al.* 1994, Veldhuizen-Tsoerkan *et al.* 1994).

Proliferation of LCs, both during puberty and after EDS administration, critically depends on the co-ordinate action of systemic signals, mainly luteinizing hormone (LH), and locally produced factors, and it is well documented that LC development can be altered by manipulations of the endocrine and paracrine environment of the testis. For instance, hypophysectomy of immature rats severely diminished the number of functionally active LCs around puberty (Teerds *et al.* 1989c). Similarly, the blockade of the LH surge after EDS prevented the reappearance of newly formed, mature LCs (Molenaar *et al.* 1986, Tena-Sempere *et al.* 1997). In addition, modifications of the intratesticular environment can also induce changes in the proliferative activity of LCs. Selective depletion of testicular macrophages impaired LC proliferation and differentiation in prepubertal rats, and inhibited LC repopulation after EDS treatment (Gaytan *et al.* 1994a,b,c). On the contrary, experimental cryptorchidism and other manipulations known to preferentially alter the seminiferous epithelium expedited the rate of LC repopulation after EDS (Molenaar *et al.* 1986, O'Leary *et al.* 1986a,b). Overall, cross-talk between positive and negative modulators within the testis is critical in the fine-tuning of LC development.

A key developmental event during LC differentiation is the acquisition of LH receptors (LHRs). This is conceivable given the key role of LH in LC development and function. In a series of experiments, we have analysed the pattern of expression of LHR mRNA in rat testis during EDS-induced LC repopulation (Tena-Sempere *et al.* 1994, 1997). Our initial observation that early LC precursors express high levels of a truncated 1.8 kb form of LHR mRNA much before the acquisition of the mature features of adult-type LCs (Tena-Sempere *et al.* 1994) prompted us to evaluate whether experimental manipulations known to alter LC repopulation influence the pattern of LHR gene expression in differentiating LC precursors. The present paper reports our findings on the pattern of expression of LHR mRNA in abdominal and scrotal testes of unilaterally cryptorchid (UC) rats after EDS administration.

Materials and Methods

Animals and experimental designs

Adult male (75- to 90-day-old) Sprague-Dawley rats were used. The animals were caged under controlled conditions of light (14 h light:10 h darkness; lights on at 0700 h) and temperature (21 °C) with free access to standard laboratory animal food and tap water. All the experimental procedures were approved by the Turku University Committee on Laboratory Animal Care and were conducted in

accordance with the European Union normative for care and use of experimental animals.

In experiment 1, the pattern of testicular LHR mRNA expression after EDS administration was evaluated in UC rats. Adult male rats were rendered unilaterally cryptorchid by anchoring the upper pole of the testes to the abdominal wall using a suture passing through the connective tissue of the caput epididymis, as previously described (Jégou *et al.* 1984). Sham-operated rats served as controls. One month after surgery, the animals were injected i.p. (day 0) with a single dose of EDS (75 mg/kg body weight) or vehicle (DMSO:H₂O; 1:3). Groups of animals were sequentially killed 0, 5, 15, 20 and 40 days after EDS. An additional sampling time-point (day 30 after EDS) was included for UC animals, given the expected faster rate of LC repopulation in this group (O'Leary *et al.* 1986a,b). Trunk blood, testes, and ventral prostates were taken, and the weights of the organs recorded. Sera were separated from blood samples and stored at -20 °C until used for hormone measurements. After removal, the testes were immediately frozen in liquid nitrogen and stored at -70 °C until used for LHR binding assays and RNA analyses.

In experiment 2, the time-course and reversibility of the observed cryptorchidism-induced changes in the pattern of expression of testicular LHR mRNA after EDS were assessed. UC rats and their paired controls were injected i.p. (day 0) with a single dose of EDS (75 mg/kg body weight). Five days later, a surgical switch was performed in the 'testicular status' of the experimental groups. Control rats were rendered UC as described above. Inversely, orchidopexy was performed on the UC rats. The positioning of the abdominal testes into scrotal location was achieved by releasing the former suture to the abdominal wall, followed by gentle pulling of the testes down to the scrotum and fixation through the connective tissue of cauda epididymis to the scrotal skin (Jégou *et al.* 1984). Blood and testicular samples were taken before EDS administration (day 0), on the day of surgery (day 5), and 7, 10, 15, 20 and 40 days after EDS. Samples were collected and processed as in experiment 1.

Hormone and LHR-binding measurements

Serum testosterone levels were measured by RIA, after diethyl ether extraction of the samples (Huhtaniemi *et al.* 1985), using ¹²⁵I-labeled testosterone (Orion-Farmos Diagnostica, Turku, Finland) as tracer, and testosterone antiserum kindly donated by Professor R Vihko (Department of Clinical Chemistry, University of Oulu, Finland). Serum LH levels were measured using a supersensitive immunofluorometric assay (Wallac Oy, Turku, Finland), based on the Delfia principle (Haavisto *et al.* 1993), and the results were expressed in terms of the reference preparation LH-RP-2 (NIDDK, Bethesda, MD,

USA). The sensitivity of the assay was 0.75 pg/tube, the intra-assay coefficient of variation (C.V.) was 7%, and the interassay C.V. was 10%.

Testicular LHR binding was measured as previously reported (Huhtaniemi *et al.* 1981). Briefly, testes were homogenized in Dulbecco's PBS (10 ml/g tissue), and the homogenates centrifuged at 2000 *g* for 10 min. The supernatants were collected, and the membrane fractions pelleted by centrifugation at 20 000 *g* for 20 min. Finally, the pellets were dissolved in Dulbecco's PBS+0.1% (w/v) BSA (Sigma Chemical Co., St Louis, MO, USA) (10 ml/g original tissue), and used for binding assay. [¹²⁵I]Iodo-human chorionic gonadotropin (hCG) binding was measured by incubation of 100 µl aliquots of testicular homogenates in the presence of 150 000 c.p.m. (~4 ng) highly purified iodinated hCG (NIH CR-125; 13 000 IU/mg). Non-specific binding was evaluated in matched samples in the presence of 1000-fold molar excess of unlabeled hCG (Pregnyl, Organon, Oss, Netherlands). The incubation time was 16 h at room temperature (22–24 °C). Bound and free hormones were separated by 15-fold dilution with ice-cold Dulbecco's PBS-BSA and centrifugation of the samples at 3000 *g* for 20 min. As the concentration of hCG used in the incubations was near saturating (Huhtaniemi *et al.* 1981), the assays gave a reliable estimate of the testicular LHR content.

Northern hybridization analysis

Total RNA was isolated from testicular samples using the single-step acid guanidinium thiocyanate–phenol–chloroform extraction method, as described previously (Chomczynski & Sacchi 1987). For Northern analyses, RNA samples (20 µg/lane) were resolved on 1.2% denaturing agarose gels and transferred onto Hybond-N⁺ nylon membranes (Amersham International plc, Aylesbury, Bucks, UK). Only membranes showing even loading of the samples, as assessed by the amount of 18S ribosomal RNA transferred per lane, estimated under ethidium bromide staining, were used. The membranes were pre-hybridized for 4–6 h at 64 °C in a solution containing 50% deionized formamide (Sigma), 3 × SSC, 5 × Denhardt's solution, 0.1 g/l heat-denatured calf thymus DNA, 1% SDS, and 0.1 g/l yeast transfer RNA. For hybridization, a ³²P-labeled cRNA probe for the rat LHR was generated using a Riboprobe system II kit (Promega, Madison, WI, USA), and a template composed of a fragment of the LHR cDNA, spanning nucleotides 441–849 of its extracellular domain, subcloned into the pGEM-4Z plasmid (LaPolt *et al.* 1990). Hybridization was carried out at 66 °C for 20 h in the same prehybridization solution after addition of the cRNA probe. After hybridization, the membranes were washed in 2 × SSC and 0.1% SDS at room temperature for 20 min, and, to completely remove non-specific hybridization, they were treated with ribonuclease-A (3 µg/l in 2 × SSC) for 15 min at room temperature,

followed by three washes in 0.2 × SSC and 0.1% SDS at 64 °C for 20 min. The membranes were exposed to Kodak X-ray films (Kodak XAR-5; Eastman Kodak, Rochester, NY, USA) at –70 °C for 5–15 days. The molecular sizes of the mRNA species were estimated by comparison with mobility of the 18S and 28S ribosomal RNAs.

RT-PCR and Southern hybridization

RT-PCR, optimized for semi-quantitative detection, was used to identify the expression of truncated LHR mRNA species in UC testes at early stages after EDS administration. For amplification of LHR transcripts, encoding areas of the extracellular domain, the following oligonucleotide primers, synthesized according to the rat LHR cDNA (McFarland *et al.* 1989), were used: LHR1 (nucleotides –6 to 17), 5'-CGG GCC ATG GGG CGG CGA GTC CC-3', and LHR4 (nucleotides 342 to 322) 5'-GTT TTT GGT GTT CTG GAT CAG-3'. In addition, to provide an appropriate internal control, co-amplification of a 395 bp fragment of the L19 ribosomal protein mRNA was carried out in each sample using the primer pair: L19s, 5'-GAA ATC GCC AAT GCC AAC TC-3', and L19as, 5'-TCT TAG ACC TGC GAG CCT CA-3', generated according to the rat L19 ribosomal protein cDNA (Chang *et al.* 1987).

For the amplification of the target genes, RT and PCR were run in two separate steps. Furthermore, to enable appropriate amplification in the exponential phase for both targets, PCR amplification of LHR and L19 ribosomal protein transcripts was carried out in separate reactions with different numbers of cycles, but using similar amounts of the corresponding cDNA templates, generated in single RT reactions. Briefly, equal amounts of total testicular RNA (2 µg) were heat-denatured and reverse-transcribed by incubation at 42 °C for 2 h with 12.5 U AMV RT (Promega), 20 U ribonuclease inhibitor RNasin (Promega), 200 nM deoxy-NTP mixture, and 1 nM anti-sense primers LHR4 and L19as, in a final volume of 30 µl 1 × AMV-RT buffer. The reactions were terminated by heating at 96 °C for 5 min and cooling on ice, and the samples were extracted using phenol–chloroform. Finally, the cDNAs were precipitated overnight at –20 °C with absolute ethanol and 3 M sodium acetate, pH 5.2, and dissolved in 20 µl sterile water. For semi-quantitative PCR, 5 µl aliquots of the cDNA samples were amplified in 50 µl 1 × PCR buffer in the presence of 2.5 U Dynazyme-DNA polymerase (Finnzymes, Espoo, Finland), 200 nM deoxy-NTP mixture, and the corresponding primer pairs (1 nM) LHR1–LHR4 or L19s–L19as. The PCR reactions consisted of a first denaturing cycle at 97 °C for 5 min, followed by a variable number of cycles of amplification defined by denaturation at 96 °C for 1.5 min, annealing at 55 °C for 1.5 min, and extension at 72 °C for 3 min. A final extension cycle of 72 °C for

10 min was included. The number of cycles was optimized to ensure amplification in the exponential phase of PCR. Different numbers of cycles were tested for LHR (ranging between 30 and 45) and L19 ribosomal protein (ranging between 15–30), and 37 and 25 cycles respectively were chosen for further analyses. In all RT-PCR reactions, negative (liver RNA) and liquid controls were run in parallel with testicular RNA samples (data not shown).

Southern hybridization was used to confirm the specificity of the transcripts amplified by RT-PCR. The DNA fragments generated were resolved on 1.5% agarose gels and transferred onto nylon membranes using the capillary method. The membranes were prehybridized for 2 h at 42 °C in a total volume of 25 ml containing 5 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 0.1 g/l heat-denatured calf thymus DNA. Hybridization was performed overnight at 42 °C by adding the corresponding ³²P-labeled antisense oligo-probe (LHR probe: 5'-CTT CAG CAG CAG CAC TGC CAG CAC CAG CAG-3'; L19 probe: 5'-GAC CTT CAG GTC CAG GCT GTG ATA CAT ATG-3') to the prehybridization solution. The probes were labeled at the 5' end by means of [γ -³²P]ATP (Amersham International plc) and T4 polynucleotide kinase (Promega). The blots were washed once for 15 min at room temperature in 2 × SSC–0.1% SDS, and twice with 1 × SSC–0.1% SDS for 15 min at 42 °C. The membranes were then exposed to Kodak X-ray films for 2–24 h. The molecular sizes of the RT-PCR products were determined by comparison with mobility of molecular size markers run together with the DNA fragments.

Statistical analysis

The data are expressed as mean ± S.E.M. Statistically significant differences between groups were determined by one-way ANOVA, followed by Duncan's new multiple range test. $P < 0.05$ was considered statistically significant.

Results

UC alters the pattern of testicular LHR mRNA expression after EDS treatment

UC did not modify basal LH and testosterone levels (Fig. 1). However, cryptorchid testes showed reduced weight and LHR content (~50% and 70% respectively of those of contralateral scrotal testes), in keeping with earlier observations (de Kretser *et al.* 1979, Huhtaniemi *et al.* 1984). Both in control and UC rats, administration of EDS induced complete elimination of mature LCs from testicular interstitium as indicated by the drop in serum testosterone levels to the range measured in orchidectomized rats (<0.5 nmol/l, data not shown), and the disappearance of hCG-binding sites in scrotal and cryptorchid testes, 5 days after EDS administration (Fig. 2A). In line with

Figure 1 (A) Serum testosterone (T) and (B) LH levels in the experimental groups indicated, before (day 0) and at different time-points after treatment with a single dose of EDS (75 mg/kg body weight). The experimental groups were: (○) controls, (□) UC rats, (■) UC rats with relocation of abdominal testis to the scrotum 5 days after EDS administration (UC-des), and (●) control rats rendered UC 5 days after EDS administration (Control-UC). Data points with different superscripts are significantly different (means ± S.E.M.; 6–10 samples/group).

previous findings (O'Leary *et al.* 1986a,b), the rate of repopulation of LCs after EDS was higher in abdominal testes of the UC rats as demonstrated by the faster recovery of serum testosterone levels (compared with control animals) and LHR content (compared with scrotal testes). On the contrary, newly formed LCs, as estimated by testicular hCG binding, repopulated to a lower extent the contralateral scrotal testes (Fig. 2A). Serum LH levels increased in control and UC rats after EDS administration (Fig. 1B). The changes in serum LH inversely correlated with those in testosterone levels, i.e. the faster recovery in testosterone concentrations in UC animals resulted in a concomitant precocious decline in serum LH levels. By day 40 after EDS, LH values had returned to the basal range in both control and UC rats.

Figure 2 Testicular [^{125}I]iodo-hCG binding in the experimental groups indicated, before (day 0) and at different time-points after treatment with a single dose of EDS (75 mg/kg body weight). The experimental groups were: (○) controls, (□) UC rats, (△) scrotal testes of UC rats (USc), (■) UC testes with relocation to the scrotum 5 days after EDS administration (UC-des), and (●) scrotal testes rendered UC 5 days after EDS administration (Control-UC). Data points with different superscripts are significantly different (means $\pm$ S.E.M.; 6–8 samples/group).

The pattern of testicular LHR mRNA expression in control rats after EDS administration resembled that previously described by us (Tena-Sempere *et al.* 1994, 1997), i.e. persistent expression of the 1.8 kb species in the absence of longer LHR transcripts in early stages after EDS, followed by the gradual recovery of a 'mature' pattern of LHR mRNA expression along with the repopulation of LCs (Fig. 3). This 1.8 kb transcript has been previously reported to correspond to a truncated form of LHR mRNA encoding regions of the extracellular domain (LaPolt *et al.* 1991). In addition, the lack of

Figure 3 Northern hybridization analysis of total RNA (20 $\mu\text{g}/\text{lane}$) isolated from individual testes of control rats (bilateral scrotal testes) before (day 0) and at different time-points (5, 15, 20 and 40 days) after administration of a single dose of EDS. Two samples per time-point are presented. Northern hybridization was carried out using a specific LHR cRNA probe. The molecular sizes of the major LHR mRNA transcripts, as well as the location of the 28S and 18S ribosomal RNAs are indicated.

expression of longer LHR transcripts at early stages after EDS administration was further confirmed by Northern hybridization of poly (A) $^{+}$ RNA isolated from rat testes 5 days after EDS treatment (data not shown). A similar pattern was found in scrotal testes of the UC rats (Fig. 4, upper panel), although 40 days after EDS the level of expression of the full-length LHR mRNA species was lower than in controls, in line with our findings on testicular hCG binding (Fig. 2A). On the contrary, the pattern of LHR mRNA expression in UC testes differed, as the 1.8 kb transcript was not discernible upon Northern hybridization 5 days after EDS (Fig. 4, lower panel). However, the level of expression of this species was not altered by unilateral cryptorchidism prior to EDS administration, in contrast to a previous report (Iizuka *et al.* 1996). In addition, the faster rate of LC repopulation in the abdominal testes after EDS resulted in precocious recovery of the 'mature' pattern of LHR mRNA expression, which was visible already on day 15 after EDS, although variability between animals was also noted. Interestingly, the expression of full-length LHR mRNA transcripts was associated with the reappearance of the truncated 1.8 kb species.

Cryptorchidism-induced changes in the pattern of testicular LHR mRNA expression after EDS are acute and non-reversible

The time-course and reversibility of the cryptorchidism-associated changes in the pattern of LHR mRNA expression were assessed by switching the 'testicular status' in control and UC rats 5 days after EDS administration. Induction of UC in control rats did not alter the pattern of response to EDS in terms of LH and testosterone secretion.

Figure 4 Northern hybridization analysis of total RNA (20 µg/lane) isolated from scrotal and cryptorchid testes of UC rats before (day 0) and at different time-points (5, 15, 20, 30 and 40 days) after administration of a single dose of EDS. For comprehensive presentation of data, control RNA samples from bilateral scrotal testes (C) were included in both blots. In addition, RNA samples from scrotal testes of UC rats prior to EDS administration (Sc0) were run together with those from cryptorchid testes. Two samples per time-point are presented. Northern hybridization was carried out using a specific LHR cRNA. The molecular sizes of the major LHR mRNA transcripts, as well as the location of the 28S and 18S ribosomal RNAs are indicated.

Similarly, repositioning of the UC testes to the scrotal location did not change the pattern of LH and testosterone secretion observed in UC rats. Overall, rats subjected to UC 1 month before EDS administration showed a faster recovery in testosterone production as serum testosterone levels 15 days after EDS were significantly higher ($P < 0.01$) than those of control animals, regardless of the actual status (scrotal or UC) of the testes at this time-point (Fig. 1A). Accordingly, the normalization of LH levels after EDS was faster in animals subjected to UC 1 month before EDS administration, even in those where the relocation of the abdominal testes to the scrotal position was performed (Fig. 1B).

The rate of LC repopulation in these experimental paradigms was evaluated by monitoring testicular hCG binding, in line with previous references (Jackson *et al.* 1986, O'Leary *et al.* 1986a,b, Tena-Sempere *et al.* 1994, 1997). Figure 2B depicts the changes in the pattern of LC repopulation in abdominal testes after their relocation to the scrotal position (termed UC-des testes) on day 5 after EDS administration. The rate of repopulation of LCs was lower in UC-des testes than in UC ones, yet it was higher than in controls. A significant decrease in the repopulation rate by testicular descent was evident already on day 15

Figure 5 Northern hybridization analysis of total RNA (20 µg/lane) isolated from scrotal and cryptorchid testes after surgical switch of testicular location 5 days after EDS administration. Testicular samples were obtained before EDS administration (day 0), on the day of the surgery (day 5) and 7, 10, 15, 20 and 40 days after EDS. Data from scrotal testes rendered UC on day 5 after EDS are presented in the upper panel. In the lower panel, a representative blot from UC testes relocated to the scrotum is shown. Two samples per time-point are presented. Northern hybridization was carried out using a specific LHR cRNA probe. The molecular sizes of the major LHR mRNA transcripts, as well as the location of the 28S and 18S ribosomal RNAs are indicated.

after EDS, and remained all through the study period. Figure 2C shows the changes in the pattern of LC repopulation in scrotal testes after relocation to the abdominal position (termed Control-UC testes) on day 5 after EDS administration. The rate of repopulation of LCs was higher in Control-UC testes than in controls, although it was lower than in UC testes. A significant increase in the repopulation rate by experimental cryptorchidism was evident already on day 20 after EDS, and persisted thereafter.

The pattern of testicular LHR mRNA expression after EDS administration in UC-des testes was similar to that of UC testes as (1) no discernible 1.8 kb LHR mRNA was found in Northern blots in the early stages after EDS, and (2) the recovery of a complete pattern of LHR transcripts, including the 1.8 kb species, was faster than in controls (Fig. 5, upper panel). On the contrary, in Control-UC testes the level of expression of the 1.8 kb LHR transcript rapidly declined after the testicular switch, and became undetectable by day 10 after EDS, i.e. 5 days after relocation of the testes into the abdominal position. The reappearance of the longer LHR mRNA species was delayed when compared with UC testes but, as in their case, it was associated with the expression of the truncated 1.8 kb band (Fig. 5, lower panel).

Figure 6 Southern hybridization analysis of RT-PCR products generated using the primer pairs LHR1–LHR4 and L19s–L19as and RNA samples obtained from the different experimental groups, as indicated in previous figures. The oligoprimers LHR1 and LHR4 are located in position – 6 to 17 and 342 to 322 bp respectively of the rat LHR sequence; amplification using this pair gives rise to a 350 bp species (termed LHRe) encompassing exons 1 to 4 of the extracellular domain of the receptor. The primer pair L19s–L19as allows coamplification of a 395 bp fragment of L19 ribosomal protein mRNA (termed L19), used as internal control. Southern hybridizations were performed using specific oligo-probes, as described in Materials and Methods.

Truncated form(s) of LHR mRNA are expressed at low levels in UC testes after EDS treatment

To evaluate whether experimental cryptorchidism is able to completely prevent LHR gene expression in early LC precursors, the presence of LHR mRNAs was assessed in the different experimental models by means of semi-quantitative RT-PCR (Fig. 6). In keeping with our previous findings (Tena-Sempere *et al.* 1994), persistent

expression of LHR mRNA transcripts, encoding areas of the extracellular domain, was detected in control testes at all time-points after EDS administration. In addition, lack of expression, 5 days after EDS, of LHR transcripts encoding the transmembrane and cytoplasmic domains was demonstrated (data not shown), using RT-PCR conditions similar to those previously reported (Tena-Sempere *et al.* 1994). This confirms that the early LC precursors express truncated LHR mRNA species. In UC testes, despite low level of expression, positive amplification of an area of LHR mRNA, coding for a region of the extracellular domain (exons 1 to 4), was detected 5 days after EDS. This finding suggests that the expression of truncated forms of LHR mRNA is a universal phenomenon in proliferating LC precursors. As in the case of control testes, no expression of mRNA species encoding areas of the transmembrane and cytoplasmic domains of the LHR was detected at this time-point (data not shown).

Using a similar approach, the expression of LHR mRNA species encoding areas of the extracellular domain was investigated in UC-des and Control-UC testes. Descent of UC testes, 5 days after EDS administration, did not result in major changes in the pattern of LHR mRNA amplification. Low levels of expression of LHR transcripts encoding areas of the extracellular domain were detected in early stages, i.e. on days 5, 7 and 10 after EDS administration. Along with the LC repopulation, the level of expression of such transcripts normalized to control values. Positioning of control testes into the abdomen, 5 days after EDS administration, resulted in a rapid and profound decline in the expression of LHR transcripts, encoding areas of the extracellular domain, in agreement with data from Northern hybridization. As described above, LC repopulation was associated with normalization of expression of this species.

Discussion

In most mammalian species, testes are located in the scrotum. This ensures that testicular temperature is a few degrees below that of the core body, thus providing an optimal environment for complete testicular function. Retention of testes inside the abdomen results in disruption of spermatogenesis (Clegg 1963a,b), impairment of steroidogenesis (Huhtaniemi *et al.* 1984), and higher rate of testicular malignancies (Batata *et al.* 1991). Although all cell types of the testis are affected by cryptorchidism, the most prominent damage occurs in the seminiferous epithelium (Clegg 1963a,b, Kerr *et al.* 1979). Experimental cryptorchidism is a useful paradigm for studies on testicular physiology and pathophysiology (O’Leary *et al.* 1986a,b, Shikone *et al.* 1994, Eskola *et al.* 1995, Peltola *et al.* 1995, Iizuka *et al.* 1996). As a continuation of our previous work on the characterization of the pattern of LHR mRNA expression in the rat testis during LC regeneration, in the

present study we compared testicular LHR expression and LC repopulation after EDS administration in scrotal and abdominal testes of UC rats. Two major questions were asked: (a) is the pattern of LHR mRNA expression in repopulating LCs altered by changes in the paracrine/autocrine signals in the cryptorchid testis, and (b) is there a direct correlation between the higher rate of LC repopulation in cryptorchid testes and the observed changes in the pattern of LHR gene expression?

Noteworthy, the UC rat constitutes a good model to analyse the differential role of endocrine signals and locally produced factors in the control of testicular functions, as in this experimental paradigm both testes share similar endocrine background but with different intratesticular environment.

Concerning the LHR mRNA expression, two major differences were noted between scrotal and abdominal testes. First, the 1.8 kb transcript, persistently expressed in scrotal testes at all time-points, was absent upon Northern hybridization in abdominal testes at the early stages (i.e. 5 days) after EDS administration. Secondly, the faster repopulation rate in cryptorchid testes, as estimated by hCG binding, was associated with precocious reappearance of the complete pattern of LHR mRNAs with four major transcripts, including the 1.8 kb one. Apparent differences in the pattern of expression of longer LHR transcripts in abdominal testes from EDS-treated rats were noted between this and a previous study (Veldhuizen-Tsoerkan *et al.* 1994). This discrepancy can be attributed, at least partially, to the use of long-term hypophysectomized, EDS-treated animals and poly (A)⁺ RNA for Northern hybridization in the latter. However, as a whole, our data clearly indicate that intratesticular factors actively influence the pattern of LHR gene expression in repopulating LCs, and that expression of the 1.8 kb LHR transcript is selectively repressed in the cryptorchid testis during early stages after EDS administration. Interestingly, unilateral cryptorchidism *per se* did not induce major changes in LHR gene expression as shown by Northern analysis of RNA samples from UC testes prior to EDS administration. In fact, the apparent increase in the level of expression of LHR transcripts in abdominal testes was likely due to LC 'enrichment' following testicular shrinkage after major damage of spermatogenesis. This is in contrast to the data reported by Iizuka *et al.* (1996) showing that unilateral cryptorchidism reduces the level of expression of the 1.8 kb LHR species. The basis for this discrepancy is unclear, given the similarities in rat strain and experimental procedures used. The only apparent differences are noted in the age of the animals (6–7 months vs 2–3 months in the present study) and the use of poly (A)⁺ RNA instead of total RNA for Northern hybridization. Whether they explain the reported discrepancies remains to be elucidated.

A conspicuous feature of gonadotropin receptor gene expression is the presence of several splice variants of the

coding mRNAs. Alternative splicing may be a potential mechanism for production of isoform diversity at the protein level (Smith *et al.* 1989), and it has been proposed that such receptor variants may play a role in the generation of pleiotropic cellular responses to gonadotropins (Tsai-Morris *et al.* 1990, Vu-Hai *et al.* 1992, Sairam *et al.* 1997). However, complete characterization of the functional role of the different gonadotropin receptor splice variants has remained elusive. Concerning the LHR, the 1.8 kb mRNA species has been previously identified as a truncated form encoding areas of the extracellular domain of the receptor (LaPolt *et al.* 1991); however, its function is still unclear. It has been suggested that it may encode a soluble LH-binding protein, thus allowing a modulatory role for such a receptor variant in LH responsiveness of target cells (Tsai-Morris *et al.* 1990). However, the translational efficiency of this transcript *in vivo* is low (Tena-Sempere *et al.* 1997), and most *in vitro* studies point out that the translated protein, if produced, is trapped intracellularly (Segaloff & Ascoli 1993). In the EDS-treated control rat testes, this truncated transcript was persistently expressed throughout LC regeneration, making conceivable a functional role for this species in the control of such an event. Assumedly, our recent data suggested that a potential role of the 1.8 kb transcript in driving/modulating LH actions in early LC precursors is unlikely (Tena-Sempere *et al.* 1997). However, this species may contribute, alternatively, to the regulation of the expression of functional LHRs. In this sense, the 6.8 kb LHR mRNA is the likely candidate to encode the functional holoreceptor (Koo *et al.* 1994), and it is possible that its rates of transcription and/or translation are affected by the presence of other LHR splice variants.

Our initial findings on changes in the pattern of testicular LHR mRNA expression after EDS administration in the context of the UC rat prompted us to evaluate whether a relationship exists between the faster rate of LC proliferation and alterations in LHR gene expression in the cryptorchid testis. To this end, we analysed the time-course and reversibility of the aforementioned changes after switching the 'testicular status' in control and UC rats 5 days after EDS administration. Unilateral positioning of the scrotal testes into the abdomen resulted in a rapid decline in the level of expression of the 1.8 kb LHR species to undetectable levels on day 10 after EDS. In addition, the rate of LC repopulation in this model was accelerated from day 20 after EDS onwards, but remained lower than that of UC testes. On the contrary, relocation of the abdominal testes of UC rats into the scrotum failed to induce major changes in the pattern of LHR gene expression, despite the significant reduction in the rate of LC repopulation detected from day 15 after EDS onwards. As a whole, our data indicate that the switch from scrotal to abdominal background results in a rapid and irreversible change in the pattern of LHR gene expression in repopulating LCs. This is likely due to the

fixed impairment of testicular microenvironment in the adult cryptorchidism, as suggested before (Jégou *et al.* 1983). Further, our results indicate that, in this model, the pattern of LHR gene expression and the rate of LC repopulation are, at least partially, dissociated. However, it is apparent from the semi-quantitative RT-PCR analysis (Fig. 6) that the expression of truncated form(s) of LHR mRNA is qualitatively a universal phenomenon in proliferating LC precursors. Such transcript(s) may be an early sign of LC differentiation, as proposed previously (Tena-Sempere *et al.* 1994). Whether additional functions are attributable to this species remains to be determined.

The severe damage of seminiferous epithelium in the cryptorchid testis has been pointed out as the major cause for the increased rate of proliferation and/or differentiation of repopulating LCs after EDS administration (O'Leary *et al.* 1986a,b). However, the potential involvement of testicular temperature cannot be ruled out. The change in temperature associated with testicular descent has been implicated in the appearance of functional G_i proteins in developing rat LCs (Eskola *et al.* 1995). In addition, evidence for a facilitatory role of lower temperature in proper folding of the LHR protein has been presented (Jaquette & Segaloff 1997). Our data from cryptorchid testes prior to EDS administration suggest, however, that a direct, primary role of temperature in the generation of the reported changes in the pattern of LHR gene expression in repopulating LCs of abdominal testes is unlikely. In keeping with this concept, a change in the culture temperature of mouse Leydig tumor cells (mLTC-1) from 32 °C (scrotal temperature) to 37 °C (abdominal temperature) or vice versa did not alter the pattern of expression of the truncated forms of LHR mRNA in this cell line (our unpublished results). Taken together, these data suggest that cryptorchidism-induced modifications in the expression of LHR gene are likely mediated through changes in the paracrine inputs on LC precursors, and not in testicular temperature. Noteworthy, a partial decrease in the repopulation rate, as estimated by hCG-binding assay, was observed in cryptorchid testes shortly after their relocation into the scrotum, despite the fixed damage in the seminiferous epithelium (Jégou *et al.* 1983). This points out that both temperature and cell-damage are involved in the generation of the higher rate of LC proliferation in the cryptorchid testis, in line with previous references (Wu & Muroso 1994, 1996).

A puzzling finding was that the recovery in LHR content after EDS administration was dramatically decreased in the scrotal testes of UC rats, as compared with controls. Interestingly, other unilateral experimental manipulations of the testis such as efferent duct ligation (Risbridger *et al.* 1981) or testicular torsion (Baker & Turner 1995) have been reported to variably alter the contralateral testis. In our model, the reported phenomenon was obviously not dependent on changes in testicular temperature or paracrine signals upon repopulating LCs,

and different mechanisms may be involved. However, on the basis of the current data, the likely explanation is that the faster decline in serum LH levels in UC rats, due to the higher rate of LC repopulation in the abdominal testes, resulted in a lower gonadotropic input on proliferating LC precursors in the scrotal testes. In this sense, it is well established that normal LC development critically depends on the tropic action of LH (Molenaar *et al.* 1986, Teerds *et al.* 1989a, Tena-Sempere *et al.* 1997).

In conclusion, the data presented herein indicate that experimental cryptorchidism, likely through changes in the intratesticular environment, induces quantitative changes in the pattern of LHR gene expression in rat testis after selective LC elimination by EDS treatment. This experimental model may thus prove useful for further analysis of the regulatory role of locally produced factors in the control of LHR gene expression.

Acknowledgements

We thank Dr A J W Hsueh (Stanford University School of Medicine, Stanford, CA, USA) for the rat LHR cDNA template. The skilful technical assistance of Ms Aila Metsävuori and Ms Tarja Laiho is gratefully acknowledged. M T-S is indebted to Dr E Aguilar and Dr L Pinilla for helpful discussions during the preparation of this manuscript. This work was supported by a research contract from the Academy of Finland and grants from the Sigrid Jusélius Foundation and the Ahokas Foundation. M T-S was supported by a post-doctoral grant from DGICYT (Ministerio de Educación y Cultura, Spain).

References

- Baker LA & Turner TT 1995 Leydig cell function after experimental testicular torsion despite loss of spermatogenesis. *Journal of Andrology* **16** 12–17.
- Batata MA, Whithmore JR Jr, Chu FCH, Hilaris BS, Loh J, Grabstald H & Golbey R 1991 Cryptorchidism and testicular cancer. *Journal of Urology* **124** 382–387.
- Chang Y-L, Lin A, McNally J, Pelleg D, Meyuhos O & Wool Y 1987 The primary structure of rat ribosomal protein L19. *Journal of Biological Chemistry* **262** 1111–1115.
- Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159.
- Clegg EJ 1963a Studies on artificial cryptorchidism: morphological and quantitative changes in the Sertoli cells of the rat testis. *Journal of Endocrinology* **26** 567–575.
- Clegg EJ 1963b Studies on artificial cryptorchidism: degenerative and regenerative changes in the germinal epithelium of the rat testis. *Journal of Endocrinology* **27** 241–251.
- Eskola V, Paukku T, Warren DW & Huhtaniemi IT 1995 A novel role for testicular descent; temperature-sensitive induction of pertussis toxin-sensitive G_i protein function in postnatal rat Leydig cells. *Endocrinology* **136** 4659–4664.
- Gaytan F, Aceitero J, Lucena C, Aguilar E, Pinilla L, Garnelo P & Bellido C 1992 Simultaneous proliferation and differentiation of mast cells and Leydig cells in rat testis. Are common regulatory factors involved? *Journal of Andrology* **13** 387–397.

- Gaytan F, Bellido C, Morales C, Reymundo C, Aguilar E & van Rooijen N 1994a Selective depletion of testicular macrophages and prevention of Leydig cell repopulation after treatment with ethylene dimethane sulfonate in rats. *Journal of Reproduction and Fertility* **101** 175–182.
- Gaytan F, Bellido C, Aguilar E & van Rooijen N 1994b Requirement for testicular macrophages in Leydig cell proliferation and differentiation during prepubertal development in rats. *Journal of Reproduction and Fertility* **102** 393–399.
- Gaytan F, Bellido C, Morales C, Reymundo C, Aguilar E & van Rooijen N 1994c Effects of macrophage depletion at different times after treatment with ethylene dimethane sulfonate (EDS) on the regeneration of Leydig cells in the adult rat. *Journal of Andrology* **15** 558–564.
- Haavisto A-M, Pettersson K, Bergendahl M, Perheentupa A, Roser JF & Huhtaniemi I 1993 A supersensitive immunofluorometric assay for rat luteinizing hormone. *Endocrinology* **132** 1687–1691.
- Huhtaniemi IT 1994 Fetal testis – a very special endocrine organ. *European Journal of Endocrinology* **130** 25–31.
- Huhtaniemi IT & Pelliniemi LJ 1992 Fetal Leydig cells: cellular origin, morphology, life span, and special functional features. *Proceedings of the Society of Experimental Biology and Medicine* **201** 125–140.
- Huhtaniemi IT, Katikineni M & Catt KJ 1981 Regulation of luteinizing hormone receptors and steroidogenesis in neonatal rat testis. *Endocrinology* **109** 588–595.
- Huhtaniemi I, Bergh A, Nikula H & Damber J-E 1984 Differences in the regulation of steroidogenesis and tropic hormone receptors between the scrotal and abdominal testes of unilaterally cryptorchid adult rats. *Endocrinology* **115** 550–555.
- Huhtaniemi IT, Nikula H & Rannikko S 1985 Treatment of prostatic cancer patients with a gonadotropin-releasing hormone analog: acute and long term effects on endocrine function of testis tissue. *Journal of Clinical Endocrinology and Metabolism* **61** 698–704.
- Iizuka A, Park MK & Mori T 1996 Effects of unilateral cryptorchidism on the expression of gonadotropin receptor mRNA. *Biochemical and Biophysical Research Communications* **221** 290–294.
- Jackson AE, O'Leary PC, Ayers MM & de Kretser DM 1986 The effects of ethylene dimethane sulphonate (EDS) on rat Leydig cells: evidence to support a connective tissue origin of Leydig cells. *Biology of Reproduction* **35** 425–437.
- Jaquette J & Segaloff DL 1997 Temperature sensitivity of some mutants of the lutropin/choriogonadotropin receptor. *Endocrinology* **138** 85–91.
- Jégou B, Laws AO & de Kretser DM 1983 The effect of cryptorchidism and subsequent orchidopexy on testicular function in adult rats. *Journal of Reproduction and Fertility* **69** 137–145.
- Jégou B, Peake RA, Irby DC & de Kretser DM 1984 Effects of the induction of experimental cryptorchidism and subsequent orchidopexy on testicular function in immature rats. *Biology of Reproduction* **30** 179–187.
- Kerr JB, Rich KA & de Kretser DM 1979 The effects of experimental cryptorchidism on the ultrastructure and function of the Sertoli cell and peritubular tissue of the rat testis. *Biology of Reproduction* **21** 823–838.
- Kerr JB, Bartlett JMS, Donachie K & Sharpe RM 1987 Origin of regenerating Leydig cells in the testis of the adult rat. An ultrastructural, morphometric and hormonal assay study. *Cell and Tissue Research* **249** 367–377.
- Koo YB, Ji Y & Ji TH 1994 Characterization of different sizes of rat luteinizing hormone/chorionic gonadotropin receptor messenger ribonucleic acids. *Endocrinology* **134** 19–26.
- de Kretser DM, Sharpe RM & Swanson A 1979 Alterations in steroidogenesis and hCG binding in the cryptorchid rat testis. *Endocrinology* **105** 135–138.
- LaPol PS, Oikawa M, Jia X-C, Dargan C & Hsueh AJW 1990 Gonadotropin-induced up- and down-regulation of rat ovarian LH receptor message levels during follicular growth, ovulation, and luteinization. *Endocrinology* **126** 3277–3279.
- LaPol PS, Jia X-C, Sincich C & Hsueh AJW 1991 Ligand-induced down-regulation of testicular and ovarian luteinizing hormone (LH) receptors is preceded by tissue-specific inhibition of alternatively processed LH receptor transcripts. *Molecular Endocrinology* **5** 397–403.
- McFarland KC, Sprengel R, Phillips HS, Köhler M, Roseblit N, Nikolic K, Segaloff DL & Seeburg PH 1989 Lutropin-choriogonadotropin receptor: an unusual member of the G protein-couple receptor family. *Science* **245** 494–499.
- Molenaar R, de Rooij DG, Rommerts FFG, Reuvers PJ & van der Molen HJ 1985 Specific destruction of Leydig cells in mature rats after *in vivo* administration of ethane dimethyl sulfonate. *Biology of Reproduction* **33** 1213–1222.
- Molenaar R, de Rooij DG, Rommerts FFG & van der Molen HJ 1986 Repopulation of Leydig cells in mature rats after selective destruction of the existent Leydig cells with ethylene dimethane sulfonate is dependent on luteinizing hormone and not follicle-stimulating hormone. *Endocrinology* **118** 2546–2554.
- Morris ID, Phillips DM & Bardin CW 1986 Ethylene dimethane sulfonate destroys Leydig cells in the rat testis. *Endocrinology* **118** 709–719.
- O'Leary PC, Jackson AE, Averill S & de Kretser DM 1986a The effects of ethane dimethane sulphonate (EDS) on bilaterally cryptorchid rat testes. *Molecular and Cellular Endocrinology* **45** 183–190.
- O'Leary PC, Jackson AE & de Kretser DM 1986b Evaluation of testicular hCG binding in unilaterally cryptorchid rats following administration of ethane dimethane sulphonate (EDS). *Molecular and Cellular Endocrinology* **48** 51–58.
- Peltola V, Huhtaniemi I & Ahotupa M 1995 Abdominal position of the rat testis is associated with high level of lipid peroxidation. *Biology of Reproduction* **53** 1146–1150.
- Risbridger GP, Kerr JB, Peake RA & de Kretser DM 1981 An assessment of Leydig cell function after bilateral or unilateral efferent duct ligation: further evidences for local control of Leydig cell function. *Endocrinology* **109** 1234–1241.
- Sairam MR, Jiang LG, Yarney TA & Khan H 1997 Alternative splicing converts the G-protein coupled follitropin receptor gene into a growth factor type I receptor: implications for pleiotropic actions of the hormone. *Molecular Reproduction and Development* **48** 471–479.
- Segaloff DL & Ascoli M 1993 The lutropin/choriogonadotropin receptor ... 4 years later. *Endocrine Reviews* **14** 324–347.
- Shikone T, Billig H & Hsueh AJW 1994 Experimentally induced cryptorchidism increases apoptosis in rat testis. *Biology of Reproduction* **51** 865–872.
- Smith CWJ, Patton JG & Nadal-Ginard B 1989 Alternative splicing in the control of gene expression. *Annual Reviews in Genetics* **23** 527–577.
- Teerds KJ, de Rooij DG, Rommerts FFG & Wensing CJG 1988 The regulation of the proliferation and the differentiation of rat Leydig cell precursors after EDS administration or daily hCG treatment. *Journal of Andrology* **9** 343–351.
- Teerds KJ, de Rooij DG, Rommerts FFG, van der Hurk R & Wensing CJG 1989a Proliferation and differentiation of possible Leydig cell precursors after destruction of the existing Leydig cells with ethane dimethyl sulphonate: the role of LH/human chorionic gonadotrophin. *Journal of Endocrinology* **122** 689–696.
- Teerds KJ, de Rooij DG, Rommerts FFG, van der Hurk R & Wensing CJG 1989b Stimulation of the proliferation and differentiation of Leydig cell precursors after the destruction of existing Leydig cells with ethane dimethyl sulphonate (EDS) can take place in the absence of LH. *Journal of Andrology* **10** 472–477.

- Teerds KJ, Closset J, Rommerts FFG, de Rooij DG, Stocco DM, Colenbrander B, Wensing CJG & Hennen G 1989c Effects of pure FSH and LH preparations on the number and function of Leydig cells in immature hypophysectomized rats. *Journal of Endocrinology* **120** 97–106.
- Teerds KJ, de Rooij DG, Rommerts FFG & Wensing CJG 1990 Development of a new Leydig cell population after the destruction of existing Leydig cells by ethane dimethane sulphonate in rats: an autoradiographic study. *Journal of Endocrinology* **126** 229–236.
- Tena-Sempere M, Zhang F-P & Huhtaniemi I 1994 Persistent expression of a truncated form of the luteinizing hormone receptor messenger ribonucleic acid in rat testis after selective Leydig cell destruction by ethylene dimethane sulfonate. *Endocrinology* **135** 1018–1024.
- Tena-Sempere M, Rannikko A, Kero J, Zhang F-P & Huhtaniemi IT 1997 Molecular mechanisms of reappearance of luteinizing hormone receptor expression and function in rat testis after selective Leydig cell destruction by ethylene dimethane sulfonate. *Endocrinology* **138** 3340–3348.
- Tsai-Morris CH, Buczko E, Wang W & Dufau ML 1990 Intronic nature of the rat luteinizing hormone receptor gene defines a soluble receptor subspecies with hormone binding activity. *Journal of Biological Chemistry* **265** 19385–19388.
- Veldhuizen-Tsoerkan MB, Ivell R & Teerds KJ 1994 hCG-induced changes in LH/CG receptor mRNA transcript levels in the testis of adult hypophysectomized, ethane dimethyl sulphonate-treated rats. *Molecular and Cellular Endocrinology* **105** 37–44.
- Vu-Hai MT, Misrahi M, Houllier A, Jolivet A & Milgrom E 1992 Variant forms of the pig lutropin/choriogonadotropin receptor. *Biochemistry* **31** 8377–8383.
- Wu N & Muro EP 1994 A Sertoli cell-secreted paracrine factor(s) stimulates proliferation and inhibits steroidogenesis of rat Leydig cells. *Molecular and Cellular Endocrinology* **106** 99–109.
- Wu N & Muro EP 1996 Temperature and germ cell regulation of Leydig cell proliferation stimulated by Sertoli cell-secreted mitogenic factor: a possible role for cryptorchidism. *Andrologia* **28** 247–257.

Received 3 August 1998

Revised manuscript received 26 October 1998

Accepted 9 November 1998