FEBS Letters 208 (1998) 208–214 FEBS 21083

The murine nuclear *orphan* receptor GCNF is expressed in the XY body of primary spermatocytes

Uta-Maria Bauer[a], Stefan Schneider-Hirsch[a], Sigrid Reinhardt[a], Ricardo Benavente[b], Alfred Maelicke[a,*]

[a] *Laboratory of Molecular Neurobiology, Institute of Physiological Chemistry and Pathobiochemistry, Johannes Gutenberg-University Medical School, Duesbergweg 6, D-55099 Mainz, Germany*

[b] *Department of Cell and Developmental Biology, Theodor-Boveri Institute (Biocenter), University of Würzburg, Am Hubland, D-97074 Würzburg, Germany*

Received 22 September 1998

Abstract We have studied the expression of the nuclear *orphan* receptor GCNF (germ cell nuclear factor) on the mRNA and protein level in pubertal and adult mouse testes. We show by Northern and Western blot analyses and by in situ hybridization that GCNF is expressed in spermatocytes and round spermatids of adult mouse testis suggesting that GCNF may be a transcriptional regulator of spermatogenesis. Since the GCNF protein is accumulated in the XY body of late pachytene spermatocytes, it may be involved in transcriptional inactivation of sex chromosomes.

Key words: Germ cell nuclear factor; Neuronal cell nuclear factor; Retinoid receptor-related testis-associated receptor; Nuclear *orphan* receptor; Spermatogenesis; XY body

1. Introduction

In the mouse, the process of spermatogenesis spans a period of approximately 5 weeks [1]. At the onset of puberty, the spermatogonia begin to proliferate and, around postnatal day 9–10 (P9–P10), they give rise to the first primary spermatocytes. During the following 12 days, these proceed through the successive stages of the first wave of meiosis [2,3]. At P12, the first primary spermatocytes have entered pachytene, with the first diplotene stages appearing around P19 [1,2]. Following the first meiotic division, secondary spermatocytes emerge which almost immediately undergo the next meiotic division to become haploid spermatids (P20–P22) [1,4]. The spermatids which are initially of a round shape, elongate during terminal differentiation and transform into spermatozoa, to be released into the lumen of the testis tubules [5].

Several genes controlling spermatogenesis have been cloned, e.g. the *Spy* gene which is involved in spermatogonial proliferation [6], a region of the Y chromosome long arm which functions in sperm head development [7], the azoospermia factor AZF [8] and the CREM gene [4]. Notwithstanding the identification of these genes, the molecular mechanism of male germ cell development is not yet well understood.

Recently a nuclear receptor, designated GCNF (germ cell nuclear factor [9]) or RTR (retinoid receptor-related testis-associated receptor [10]), has been cloned from mouse testis. GCNF is strongly expressed during germ cell development. In addition, GCNF seems also to be involved in neurogenesis [11–13]. It is in this context that we cloned the GCNF cDNA from neuronal precursor cells and designated it NCNF (neuronal cell nuclear factor) [11]. In the developing mouse brain, GC/NCNF is expressed in a zone known to contain young postmitotic neurons. Such pleiotropic action is not unusual for transcription factors, e.g. the *Drosophila daughterless* gene regulates sex determination and neural development [14]. GC/NCNF belongs to the nuclear receptor subfamily of homodimeric *orphan* receptors such as COUP and HNF-4 [11,15], because (i) it binds exclusively as a homodimer to a DR0 sequence (direct repeat of the AGGTCA motif without spacing nucleotides) [9,11,12,16,17], (ii) it does not heterodimerize with other nuclear receptors, e.g. RXR, RAR, TR and VDR [11,17] and (iii) it does not contain an AF-2 subdomain in its ligand binding domain (LBD) and therefore seems to act ligand independently [11]. In co-transfection experiment, GC/NCNF silenced transcription of a reporter gene by acting on DR0 [11,18]. It is likely therefore that GC/NCNF, like other homodimeric *orphan* receptors, i.e. COUP-TF [19], also acts as a transcriptional repressor.

Since GCNF is expressed most abundantly in testis of mouse [9–11] and human [20–22] and the two receptor proteins reveal a sequence similarity of more than 98% [20,21], it seems likely that GCNF assumes identical functions in the two mammalian species. So far the possible functions of GCNF during gametogenesis have been discussed controversially in the literature. GCNF is postulated to regulate some aspects of meiosis during spermatogenesis and oogenesis [9,22,23], whereas other reports find GCNF transcripts being restricted to round spermatids suggesting involvement in the postmeiotic phase of spermatogenesis [10,24]. Interestingly the *Xenopus laevis* homologue xGCNF [12] is hardly detectable in testis.

In order to clarify the controversial issue of GCNF function in gametogenesis, in this study we investigated GCNF transcription in adult mouse testis. Prompted by the availability of a highly specific antiserum which we previously raised against GCNF [11], we report here for the first time temporal and spatial expression analysis at the protein level in mouse testes of different developmental stages. GCNF transcript and protein was detected in late pachytene spermatocytes as well as round spermatids. Immunofluorescence staining established that GCNF expression is particularly strong in the XY nuclear subregion of primary spermatocytes. Assuming a similar repressing activity as was proposed from the co-transfection experiments mentioned above, GCNF could act spermatogenically during the meiotic phase of spermatogenesis by re-

*Corresponding author. Fax: (49) (6131) 393536.
E-mail: alfred.maelicke@uni-mainz.de

PII: S0014-5793(98)01327-1

pressing the transcriptional activity of meiotic sex chromosomes in male germ cells.

2. Materials and methods

2.1. Isolation of mouse testicular cells

Testes were dissected from 6-week-old BALB/c mice. They were chopped with a razor blade and a cell suspension was prepared [25]. Centrifugal elutriation was carried out in an elutriation rotor JE-6B (Beckman, Munich, Germany) according to [26]. Pachytene spermatocytes and round spermatids were collected separately. That the spermatid fraction was not contaminated with spermatocytes was shown by the absence of immunostaining with 4c6 antibody which is directed against the XY77 protein of spermatocytes. Using a hybridization probe selective for protamine 2 RNA, we detected a minor but consistent contamination with spermatids of the spermatocyte fraction. It was too small to significantly affect the results reported.

2.2. Northern blot analyses

Total RNA of the isolated testicular cell fractions (pachytene spermatocytes and round spermatids) was prepared according to the guanidinium isothiocyanate/phenol method [27] using the TRIzol reagent (Gibco BRL, Eggenstein, Germany). 10 μg of each sample was separated on a 1.2% agarose/1.1 M formaldehyde gel and transferred to a nylon membrane. The blot was hybridized to ^{32}P-randomly labelled probes (5×10^6–1×10^7 cpm/ml) which were specific for 5′ UTR and A/B domain sequences of GCNF (nt 94–377) and cytochrome *c* oxidase at 42°C for 16 h in 50% formamide, 20 mM Tris-HCl pH 7.5, 5 mM EDTA, 750 mM NaCl, 5% (w/v) SDS and 100 μg/ml single-stranded salmon sperm DNA. Subsequently the blot was washed twice in 2×SSC (1×SSC: 0.15 M NaCl, 0.015 M sodium citrate) at room temperature for 10 min, and twice in 0.5×SSC at 65°C for 30 min and then autoradiographed by exposure to a Kodak X-AR 5 film with an intensifying screen at −80°C.

2.3. Anti-GCNF antiserum

Anti-GCNF polyclonal antibodies were raised against the hypervariable A/B domain of GCNF (nt 208–411), as described previously [11]. In short, after cloning the A/B domain into pGEX-2T vector (Pharmacia, Freiburg, Germany), the GST fusion protein (GST, glutathione *S*-transferase) was expressed in *Escherichia coli* and affinity purified using a glutathione Sepharose. 50 μg purified GST-GCNF was applied for subcutaneous immunization of two New Zealand White rabbits which were boosted twice at 1 month intervals. The polyclonal antiserum was affinity purified with the aid of a MBP-GCNF fusion protein (MBP, maltose binding protein). The polyclonal anti-GCNF antibody did not cross-react with the nuclear receptors tested, as was shown by unsuccessful immunoprecipitation of in vitro synthesized, ^{35}S-labelled RARβ2, RXRβ2, TRα1 and VDR.

2.4. Western blot analyses

The proteins obtained from purified pachytene spermatocytes (10^6 cells/slot) and round spermatids (2×10^6 cells/slot) were separated in 10% SDS-polyacrylamide gels by electrophoresis according to [28]. Thereafter the proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Eschborn, Germany) by semidry blotting [29]. The membrane was blocked by incubation in phosphate-buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) with 3% defatted milk powder for 30 min and was stained with anti-GCNF antiserum (1:100 diluted in PBS/1% BSA/0.5% Tween 20) at room temperature for 2 h. After extensive washing in PBS/3% defatted milk powder/0.5% Tween 20, bound antibodies were detected by chemiluminescence using a horseradish peroxide-conjugated goat anti-rabbit Ig antibody (1:2000 diluted; Dako, Hamburg, Germany) and Fuji medical X-ray films. In control experiments, human embryonal kidney cells HEK-293 (10^6 cells/slot) were transiently transfected with a GCNF expression plasmid or mock transfected [11] and were also analyzed by Western blot analysis using anti-GCNF antiserum.

HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and maintained in a water-jacketed incubator at 37°C, 10% CO_2. For transfection experiments, the cells were cultured onto 10 cm petri dishes and transfected subconfluently with 10 μg DNA (pCMV-GCNF or pcDNA3) in 1 ml of transfection solution using the calcium phosphate co-precipitation technique, as described in detail elsewhere [11].

2.5. In situ hybridization

Single-stranded RNA probes for in situ hybridization were synthesized in the presence of digoxigenin-UTP (Boehringer, Mannheim, Germany) and T3/T7 RNA polymerase (MBI Fermentas, St. Leon-Rot, Germany) from linearized pBluescript KS$^+$ containing 5′ UTR and A/B domain sequences of GCNF (nt 94–377). The antisense probe was generated by T7 RNA polymerase, and the complementary sense probe, which served as negative control in the hybridization, was generated by T3 RNA polymerase. Both probes did not cross-react with other nuclear receptors cDNAs, as was shown by Southern blot analysis of RARβ2, RXRβ2, TRα1 and VDR cDNA.

Cryosections were prepared from postnatal (days 10, 15, 17 and 90) BALB/c mouse testes. Frozen tissue sections (9 μm; Slee microtome, Mainz, Germany) were mounted onto glass slides coated with aminoalkylsilane [30]. For in situ hybridization they were fixed for 20 min in 4% paraformaldehyde (PFA) in PBS. In situ hybridization was performed as previously described [11].

2.6. Indirect immunofluorescence staining

For immunofluorescence staining, testis sections were fixed for 5 min in acetone at −20°C and after drying in air for 10 min in 4% PFA in PBS. Subsequently the tissues were pre-treated with dilution buffer (PBS/10% FCS/0.01% NaN_3) supplemented with 6% Triton X-100 for 15 min. The 1:5 diluted anti-GCNF antiserum was applied overnight at 4°C. After five washes in PBS, the sections were incubated with Cy3 (indocarbocyanine)-labelled anti-rabbit Ig goat antibody (1:500 diluted; Dianova, Hamburg, Germany) for 30 min in the dark. For Golgi apparatus staining the FITC (fluorescein isothiocyanate)-conjugated wheat germ agglutinin (1:400 diluted; Kem-En-Tec, Copenhagen, Denmark), which recognizes the sugar $(GlcNAc)_2$, was incubated together with the secondary antibody. Another wash followed, and then the nuclei were stained for 5 min with Hoechst 33258 dye (the stock solution of 50 μg bisbenzimide Hoechst 33258/ml methanol was diluted 1:50 in PBS). Finally the tissues were washed again and mounted in glycerol/PBS supplemented with *p*-phenylenediamine to reduce fading of fluorescence. Double staining with mouse monoclonal antibody 4c6 [31] was carried out after incubation with the secondary antibody against the anti-GCNF antiserum and three washes in PBS. The undiluted hybridoma supernatant was incubated for 1 h. After washing, the sections were stained with AMCA (7-amino-4-methylcoumarin 3-acetic acid)-labelled goat anti-mouse IgM antibody (1:100 diluted; Dianova, Hamburg, Germany) for 1 h and mounted as described above. In control experiments the staining pattern of anti-GCNF antiserum was verified by immunofluorescence staining of testis sections with anti-GCNF antiserum in the presence of MBP-GCNF fusion protein (competition experiment), with preimmune serum and with only the secondary antibody.

3. Results

3.1. GCNF is expressed in spermatids and spermatocytes of adult mouse testis

We prepared by centrifugal elutriation purified fractions of pachytene spermatocytes and round spermatids, the purity of which was tested by immunostaining with a spermatocyte-specific antiserum and in situ hybridization with a spermatid-specific hybridization probe (see Section 2 for details). According to these tests, the spermatid fraction was essentially free of any contamination by spermatocytes whereas the spermatocyte fraction contained a small but consistent contamination by spermatids. For the analysis represented below, the isolated fractions may be considered pure spermatocytes and pure spermatids. Northern (Fig. 1a) and Western analyses (Fig. 1b) were employed to investigate cell type-specific expression of GCNF in adult mouse testis. For Northern analysis 5′ UTR and A/B domain sequences were used as hybridization probe. As shown in Fig. 1a, the GCNF gene is transcribed in both testicular cell fractions, in round sperma-

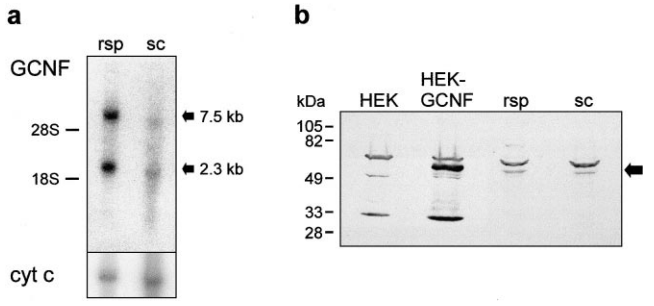


Fig. 1. Northern blot analysis (a) and Western blot analysis (b) of GCNF expression in isolated testicular cells of adult mouse. a: 10 μg total RNA of purified testicular cells (rsp: round spermatids, sc: pachytene spermatocytes) was subjected to electrophoresis in an agarose-formaldehyde gel. After transfer to membrane, the blot was hybridized at high stringency with a probe made from the 5′ UTR and A/B domain sequences of GCNF. The expression of cytochrome *c* oxidase (cyt c) was used as control and showed that each lane contained approximately the same amount of RNA. The 7.5 kb and 2.3 kb GCNF transcripts (indicated by arrows on the right) were expressed in round spermatids (rsp) as well as in pachytene spermatocytes (sc), with GCNF transcription in spermatocytes being generally weaker compared to round spermatids. As shown on the left, rRNA was used as a marker. b: Per slot were applied: 10^6 mock transfected HEK cells (HEK), 10^6 HEK cells transfected with a GCNF expression plasmid (HEK-GCNF), 2×10^6 round spermatids (rsp) and 10^6 pachytene spermatocytes (sc). After separation by SDS-PAGE, the gels were blotted onto PVDF membrane and incubated with anti-GCNF antiserum. Immunoreactivity was detected by chemiluminescence using a horseradish peroxidase-conjugated anti-rabbit-Ig antibody and X-ray films. HEK cells (as negative control) and HEK-GCNF cells (as positive control) established that the anti-GCNF antiserum recognizes the 55 kDa GCNF protein (indicated by an arrow on the right). GCNF protein was present in both round spermatids (rsp) and pachytene spermatocytes (sc). Molecular masses of reference proteins are marked in kDa on the left.

tids as well as in pachytene spermatocytes. Using the same amount of RNA for both cell types, GCNF expression in spermatids was significantly stronger than in spermatocytes, both for the large (7.5 kb) and for the small (2.3 kb) transcript. These two transcripts are generated by alternative splicing and differ only in their 3′ UTR sequence [9,10,20]. As demonstrated in Fig. 1, the two transcripts are present in each cell type in similar amounts, which disagrees with the previous suggestion that expression of the small transcript may be spermatid-specific [18,23].

GCNF expression at the protein level was studied by Western analysis of whole cells using a polyclonal rabbit antiserum that was raised against the hypervariable A/B domain of GCNF (nt 208–411) [11]. Two times more spermatids (2×10^6) than spermatocytes (10^6 cells) were analyzed per slot. A control experiment with HEK-293 cells, which were mock transfected or transiently transfected with a GCNF expression plasmid [11], established that the anti-GCNF antiserum recognizes the 55 kDa GCNF protein (Fig. 1b). Staining of GCNF protein by the antiserum could be selectively blocked by preincubation with a GCNF fusion protein (data not shown). In agreement with the Northern blot analysis of Fig. 1a, the GCNF protein was detected in round spermatids *and* in pachytene spermatocytes (Fig. 1b). However, the levels of protein expression in the two cell types appeared to be rather similar, in contrast to the transcript levels of Fig. 1a. Considering that in the Western blot of Fig. 1b twice as many spermatids than spermatocytes were used per slot, also GCNF protein expression is more pronounced in spermatids, albeit not as significantly as at the transcript level. Taking these data together, GCNF is clearly expressed in both cell types, suggesting that the receptor may be involved in the meiotic as well as postmeiotic phase of spermatogenesis.

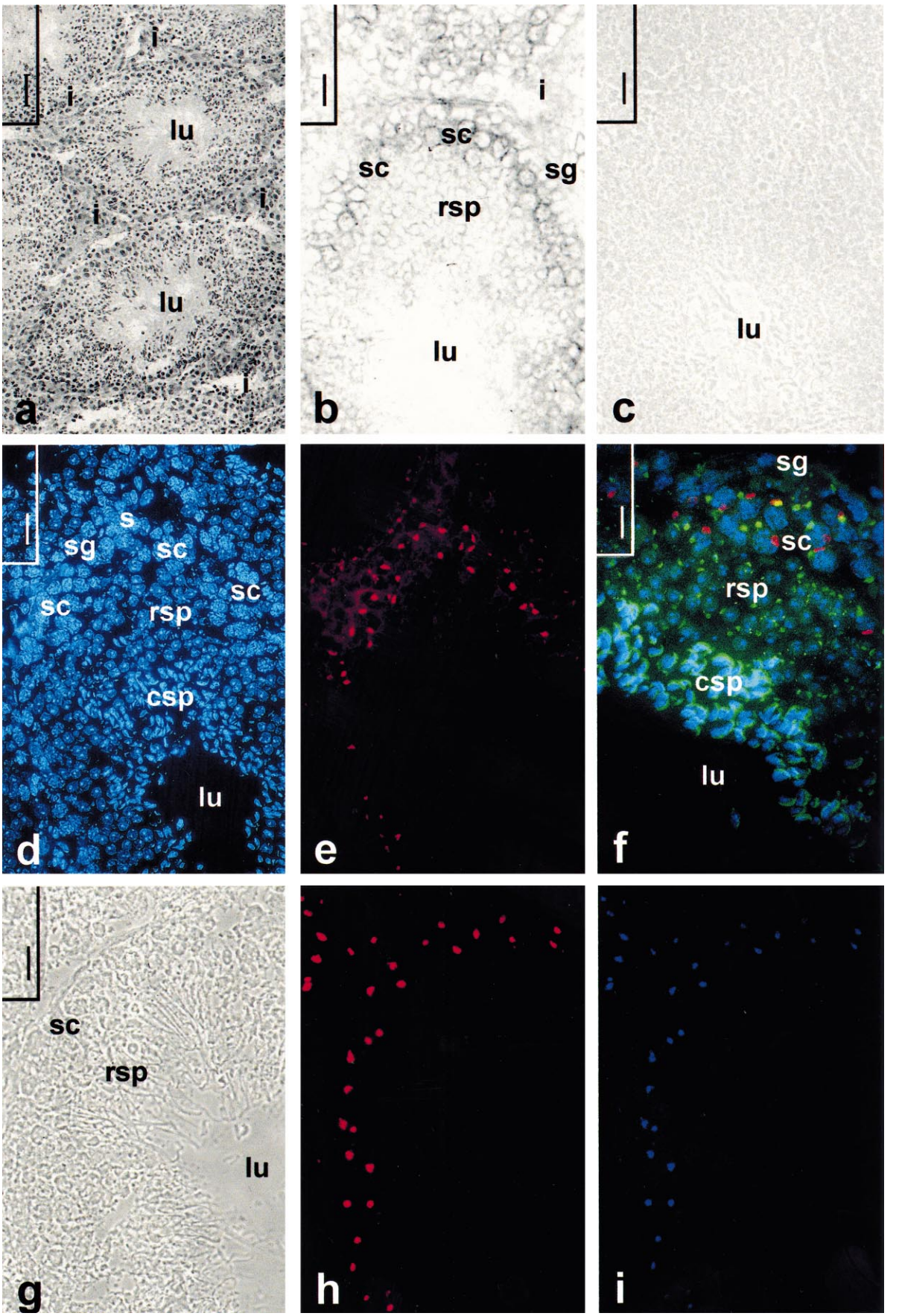
3.2. *Spatial expression pattern of GCNF in the seminiferous epithelium of adult mouse*

By using in situ hybridization and immunofluorescence staining, we investigated in cryosections of adult (P90) mouse testis tissue the spatial expression pattern of GCNF. Adult testis tissue is well suited for such studies because the full range of spermatogenic stages simultaneously occurs in this tissue, as shown by the hematoxylin-eosin stained testis sections in Fig. 2a. The seminiferous epithelium, in which germ cell development progresses radially from the periphery to the tubule lumen, can be divided into three cellular layers, the outer zone containing spermatogonia and inter-dispersed Sertoli cells, the middle zone containing the primary and secondary spermatocytes (the latter ones not being visible because they are short-lived only), and the innermost zone containing spermatids and (in the lumen) spermatozoa [5]. In agreement with our Northern blot analysis (Fig. 1a), GCNF RNA was detected in spermatocytes as well as in round spermatids (Fig. 2b). No GCNF transcripts were found in somatic testicular cells (Sertoli and Leydig cells) and in premeiotic cells (spermatogonia). Control hybridization with the sense RNA probe did not yield a signal (Fig. 2c).

In apparent contrast to the transcription data, GCNF protein (Fig. 2e) was detected exclusively in the middle zone of the P90 seminiferous epithelium, whereas the thin outer zone, the innermost zone and interstitial cells surrounding the tu-

→

Fig. 2. GCNF expression in testis cryosections of adult mouse. Cryosections of P90 mouse testis tissue were prepared and treated as described in Section 2. In situ hybridization was performed with digoxigenin-labelled antisense and sense RNA probes, and indirect immunofluorescence staining with an anti-GCNF polyclonal antibody. a: The hematoxylin-eosin stained section shows the seminiferous tubules of adult mouse testis in which spermatogenesis occurs. b: GCNF transcripts were detected in primary spermatocytes (sc) and round spermatids (rsp) with the antisense RNA probe. In contrast, no transcripts were detected in the outer zone of tubule walls, i.e. the location of spermatogonia (sg). c: Specificity of hybridization was shown by incubation with the complementary sense RNA probe, which did not yield a hybridization signal. d,e: Double staining of nuclei (d; in blue with Hoechst 33258) and of GCNF protein (e; in red with anti-GCNF antiserum). GCNF is detected in the middle zone of the P90 seminiferous epithelium where the spermatocytes (sc) are located. f: Triple staining for nuclei (in blue), GCNF protein (in red) and Golgi apparatus (in green with FITC-labelled wheat germ agglutinin) confirms these results: GCNF protein was exclusively found in nuclei of cells with very pronounced Golgi and nucleus, these being the hallmarks of primary spermatocytes. g–i: Double staining with anti-GCNF antiserum (h; in red) and with monoclonal antibody 4c6 (i; in blue). Because 4c6 selectively recognises the XY body-specific protein XY77 [31], the co-expression indicates that GCNF is co-localized with the XY77 protein in the XY bodies of late pachytene spermatocytes. The corresponding phase contrast micrograph is shown in g. Lu, lumen; i, interstitial cells; s, Sertoli cells; sg, spermatogonia; sc, spermatocytes; rsp, round spermatids; csp, condensing spermatids. Scale bars: 50 μm (a–c), 25 μm (d,e), 15.87 μm (f–i).


i
i
lu
i
i
lu
i
a
i
sc
sc
sg
rsp
lu
b
lu
c
s
sg
sc
sc
sc
rsp
csp
lu
d
e
sg
sc
rsp
csp
lu
f
sc
rsp
lu
g
h
i

bules were not labelled, as is illustrated by parallel by staining the cell nuclei (Fig. 2d). As shown by triple staining (Fig. 2f) for GCNF protein (in red), the Golgi apparatus and acrosome (in green) and nuclei (in blue), GCNF expression was restricted to the nuclei of cells with very pronounced Golgi and nucleus which are the hallmarks of primary spermatocytes [5]. In contrast, round or condensing spermatids, which have small Golgi bodies or acrosomes which overlay the condensed, crescent-shaped nuclei, were not labelled with the anti-GCNF antiserum.

At first sight, these immunofluorescence results appear to contradict our Western blot and transcriptional data. It should be noted though that nuclear receptors are regulatory proteins and hence are mostly expressed at low levels only. Our inability to demonstrate by immunostaining the presence of GCNF in cells in which we have demonstrated its presence by Western and Northern analyses, and in situ hybridization, therefore is most likely due to the low sensitivity of the method employed rather than to a real absence of the receptor. Continuing this line of argument, GCNF should then be detectable by immunostaining only under favorable conditions, such as when the receptor protein is accumulated in a small subcellular compartment, as seen in Fig. 2. Consequently, the immunofluorescence data do not appear to be at odds with the expression data of Fig. 1, to which they do not contribute. They should rather be seen as a separate set of data possibly exposing a cellular function of the nuclear *orphan* receptor.

3.3. *Subnuclear localization of GCNF in primary spermatocytes*

As shown in Fig. 2f and expected for a transcription factor, the GCNF protein was restricted to the nuclei. Moreover, the staining of primary spermatocytes was restricted to a small area of the nuclear periphery suggestive of the XY body (Fig. 2f). The XY body is a subnuclear structure in mammalian spermatocytes which contains the condensed, transcriptionally inactive sex chromosomes. These pair in the small pseudoautosomal region [32,33].

To further substantiate the localization of GCNF protein in the sex vesicle, we performed double immunofluorescence staining (Fig. 2g–i) with anti-GCNF antiserum (red staining) and the monoclonal antibody 4c6 (blue staining), which recognizes the 77 kDa XY77 protein [31]. XY77 is homogeneously distributed in the XY body and is present there only in the later pachytene stages. As shown in Fig. 2h,i, the expression patterns of GCNF and XY77 completely overlapped. We conclude from this finding that GCNF is localized in the XY body of late pachytene spermatocytes.

3.4. *Developmental regulation of GCNF expression in postnatal mouse testis*

The expression of GCNF during postnatal mouse testis differentiation was investigated by immunofluorescence staining of P10, P15 and P17 testis cryosections with the anti-GCNF antiserum (Fig. 3). Only weak unspecific staining with anti-GCNF antiserum was detected at P10 (data not shown), at the onset of mouse puberty, and the first appearance of primary spermatocytes in the seminiferous epithelium (before the pachytene stage). An overview of pubertal mouse testis histology is provided by the two serial sections that were stained with hematoxylin-eosin (Fig. 3a,d). At P15 of mouse testis development, beginning GCNF expression was indicated by faint staining within a few tubules (tubule t1 is GCNF-positive, t2 is negative in Fig. 3b,c). The GCNF expressing cells were distributed evenly throughout the whole epithelium (tubule t1 in Fig. 3c). At P17 (Fig. 3e), most testis tubule showed strong GCNF expression that coincided with the appearance of late pachytene spermatocytes ($>$P15) during the first spermatogenic cycle [1,2]. These results suggest that GCNF participates in gene regulatory events associated with the first meiotic division in male gametes.

4. Discussion

We report here that the mouse nuclear *orphan* receptor GCNF, which was identified recently as being involved in both neuronal and germ cell development [9–13], is associated with meiotic and postmeiotic events in mouse testis: during the first reductive division in primary spermatocytes as well as during spermiogenesis in round spermatids. Similar findings were recently obtained for the human homologue [22]. Our results do not agree with data suggesting either meiotic [9] or postmeiotic [10,24] expression of GCNF during mammalian spermatogenesis. As a novel observation, possibly related to the function of GCNF in spermatogenesis, we found that the GCNF protein accumulates in the XY bodies of late pachytene spermatocytes.

The expression of GCNF in round spermatids suggests that GCNF may be an important transcriptional regulator of spermiogenesis. Possibly GCNF expression induces condensation and elongation of late spermatids to sperms. Consistent with this assumption, a HRE of GCNF has been identified in the protamine 2 gene promoter [16]. Together with the overlapping expression patterns of GCNF and protamine 2 in round spermatids, this result suggests that GCNF may regulate protamine transcription, although a positive transcriptional effect of GCNF has still not been proven [11,18].

Our expression studies (Northern and Western blot analyses, and in situ hybridization) are fully consistent with each other, demonstrating doubtlessly that GCNF is expressed in both round spermatids and pachytene spermatocytes. In contrast to these very sensitive methods, immunofluorescence analysis does not appear to be sensitive enough to monitor, at the single cell level, expression of this regulatory protein. The number of GCNF molecules per nuclei is just too low to be above the level of detection by immunofluorescence analysis. For the same reason, very few immunofluorescence data exist on the local expression of other nuclear receptors. The detection by immunostaining of GCNF expression in late pachytene spermatocytes therefore is an exception to the rule. Only because GCNF protein is accumulated in a subnuclear compartment, namely the XY body, the threshold of detection is reached (Figs. 2 and 3). Clearly, GCNF expression in the XY body of late pachytene spermatocytes does not contradict the other expression data but rather adds to this intriguing information with regard to a possible cellular function of the transcription factor in pachytene spermatocytes.

During pachytene of male meiotic prophase, the X and Y chromosomes become transcriptionally inactive, they display a heterochromatic structure attached to the nuclear envelope, and they become localized in a specific area of the nucleus that is known as the sex vesicle or XY body. Because the sex chromosomes of male mammals are largely heteromorphic, their condensed structure in the XY body prevents damage

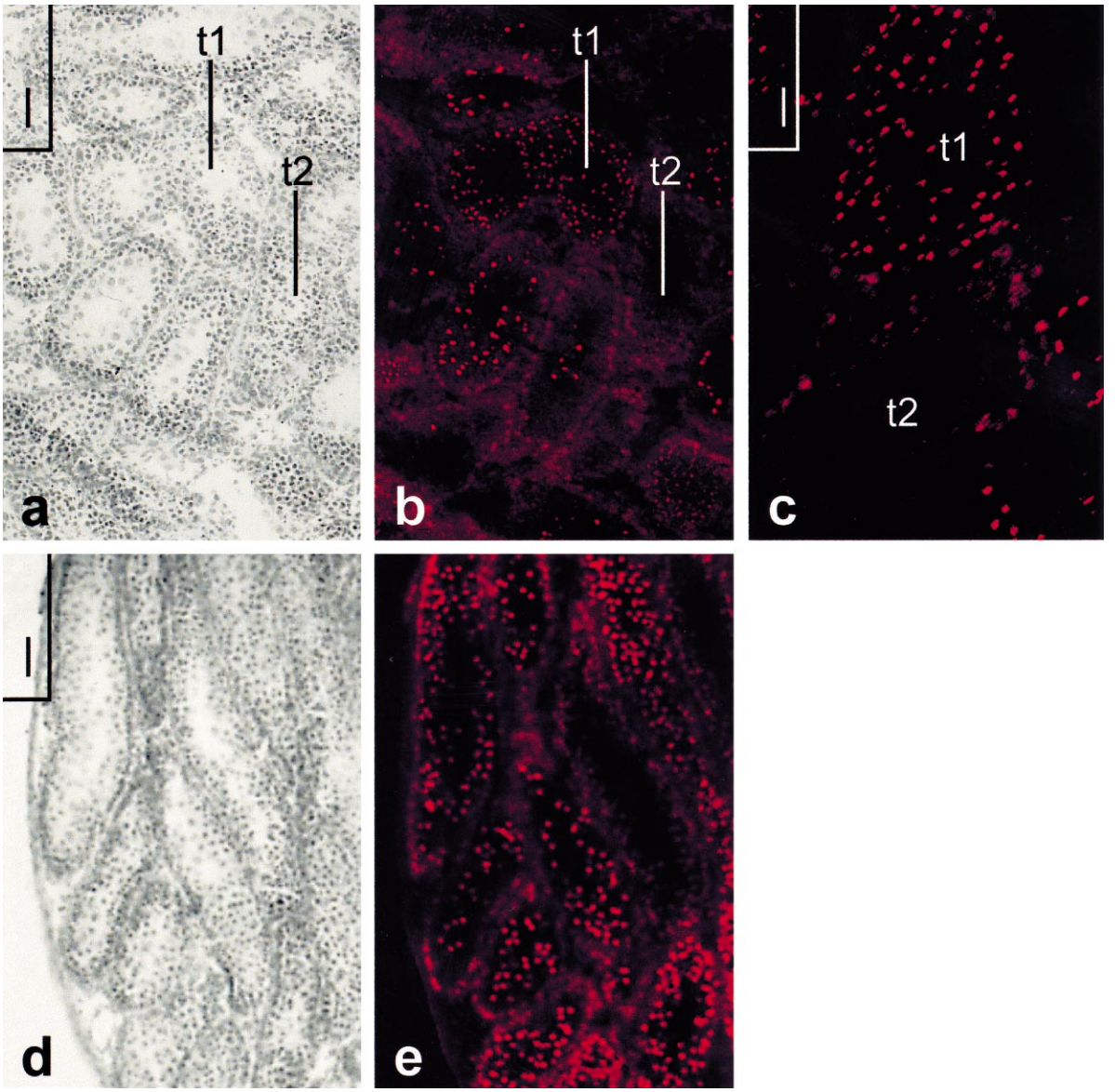


Fig. 3. GCNF expression in testis cryosections of P15 and P17 mouse. Mouse testis cryosections were prepared and stained with anti-GCNF polyclonal antibody as described in Section 2. a–c: At P15, beginning GCNF expression was indicated by faint staining within a few tubules (b,c), e.g. the marked tubule t1 shows GCNF expression, whereas tubule t2 does not. d,e: At P17, most testis tubules showed strong GCNF expression. The hematoxylin-eosin stained sections a and d are corresponding serial sections to b and e. Scale bars: 50 μm (a,b), 25 μm (c), 50 μm (d,e).

and non-homologous recombination. A further consequence of their chromatin organization is their transcriptional inactivity [32]. Because the formation of the XY body, including the limited XY pairing, plays an important role in spermatogenesis [1,7,32], it may be useful for our understanding of the underlying mechanism(s) to identify the regulatory molecules expressed in the sex vesicle.

Although in the last few years several meiosis-associated proteins have been characterized [1,34–38], of which some are exclusively expressed in the XY body [31,39], very little is known about the regulation of the sex vesicle structure. As suggested by the expression pattern of GCNF, this nuclear receptor may play a crucial role in the gene regulatory events of XY body formation. Nuclear receptors are well known to mediate activation or repression of transcription by interfering with chromatin rearrangement processes, e.g. corepressors and histone deacetylases [40,41] or TIF1 which interacts with heterochromatin-associated proteins [42,43]. Because GCNF represses gene activity by acting as homodimer via a DR0 target sequence [11,18], GCNF may participate in establishing the transcriptionally inactive state of sex chromosomes in the XY body, by producing a heterochromatin-like, condensed DNA structure. Possible partners in this activity are additional cofactors and chromatin remodelling factor.

Although it has not been proved that GCNF is a direct target gene of a retinoic acid receptor (RAR), there is strong evidence for such a regulatory cascade: in the embryonic carcinoma cell model of neural development, from which the GC/NCNF cDNA was obtained [11], GC/NCNF expression is induced shortly after retinoic acid (RA) is added to the culture, and expression begins concomitantly with that of additional RAR isoforms [11,44]. Similarly, testicular function

and structure is regulated by RA and its nuclear receptors, as shown by testis-specific RARα expression and in RARα knockout mice [45–50]. Vitamin A circulating in the plasma as protein-bound retinol is internalized and esterified by Sertoli cells, which provide RA to developing germ cells [46,47]. A requirement for RA in spermatogenesis, especially meiosis, is further indicated by the high level of CRABPI (cellular retinoic acid binding protein) expression in spermatocytes [51].

In summary, GCNF is expressed in spermatocytes as well as spermatids during mouse spermatogenesis. As one possible function, we suggest that during the meiotic phase of spermatogenesis, GCNF may be involved in XY body formation, probably by controlling chromatin condensation and transcriptional inactivation of sex chromosomes. Further studies are required to identify possible cofactors and the pathway of gene regulatory events in which GCNF is involved. GCNF may function by means of a similar molecular mechanism in spermatogenesis as in neurogenesis.

Acknowledgements: This work contains parts of the doctoral theses of U.-M.B. and S.S.-H. The study was initiated and initially supervised by Dr. Martin Rentrop, deceased. The fruitful discussions with Drs. W. Hennig (Mainz) and K. Seidel (Braunschweig) are gratefully acknowledged. We thank Mrs. Elisabeth von Glasenapp and Mrs. Ellen Fecher for the isolation of mouse testicular cells and Mrs. Vesna Pondeljak for excellent technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Re 1144/1-1) and the Stiftung Rheinland-Pfalz für Innovation.

References

[1] Calenda, A., Allenet, B., Escalier, D., Bach, J.-F. and Garchon, H.-J. (1994) EMBO J. 13, 100–109.
[2] Bellvé, A.R., Cavicchia, J.C., Millette, C.F., O'Brien, D.A., Bhatnagar, Y.M. and Dym, M. (1977) J. Cell Biol. 74, 68–85.
[3] Goetz, P., Chandley, A.C. and Speed, R.M. (1984) J. Cell Sci. 65, 249–263.
[4] Delmas, V., van der Hoorn, F., Mellström, B., Jégou, B. and Sassone-Corsi, P. (1993) Mol. Endocrinol. 7, 1503–1514.
[5] Ham, A.W. (1974) in: Histology, pp. 900–927, J.B. Lippincott, Philadelphia, PA.
[6] Sutcliffe, M. and Burgoyne, P.S. (1989) Development 107, 373–380.
[7] Burgoyne, P.S., Mahadevaiah, S.K., Sutcliffe, M.J. and Palmer, S.J. (1992) Cell 71, 391–398.
[8] Ma, K., Inglis, J.D., Sharkey, A., Bickmore, W.A., Hill, R.E., Prosser, E.J., Speed, R.M., Thomson, E.J., Jobling, M., Taylor, K., Wolfe, J., Cooke, H.J., Hargreave, T.B. and Chandley, A.C. (1993) Cell 75, 1287–1295.
[9] Chen, F., Cooney, A.J., Wang, Y., Law, S.W. and O'Malley, B.W. (1994) Mol. Endocrinol. 8, 1434–1444.
[10] Hirose, T., O'Brien, D.A. and Jetten, A.M. (1995) Gene 152, 247–251.
[11] Bauer, U.-M., Schneider-Hirsch, S., Reinhardt, S., Pauly, T., Maus, A., Wang, F., Heiermann, R., Rentrop, M. and Maelicke, A. (1997) Eur. J. Biochem. 249, 826–837.
[12] Joos, T.O., David, R. and Dreyer, C. (1996) Mech. Dev. 60, 45–57.
[13] Süsens, U., Aguiluz, J.B., Evans, R.M. and Borgmeyer, U. (1997) Dev. Neurosci. 19, 410–420.
[14] Caudy, M., Vässin, H., Brand, M., Tuma, R., Jan, L.Y. and Yan, Y.N. (1988) Cell 55, 1061–1067.
[15] Mangelsdorf, D.J. and Evans, R.M. (1995) Cell 83, 841–850.
[16] Yan, Z.H., Medvedev, A., Hirose, T., Gotoh, H. and Jetten, A.M. (1997) J. Biol. Chem. 272, 10565–10572.
[17] Borgmeyer, U. (1997) Eur. J. Biochem. 244, 120–127.
[18] Cooney, A.J., Hummelkr, G.C., Herman, T., Chen, F. and Jackson, K.J. (1998) Biochem. Biophys. Res. Commun. 245, 94–100.
[19] Cooney, A.J., Leng, X., Tsai, S.Y., O'Malley, B.W. and Tsai, M.-J. (1993) J. Biol. Chem. 268, 4152–4160.
[20] Schneider-Hirsch, S., Bauer, U.-M., Heiermann, R., Rentrop, M. and Maelicke, A. (1998) J. Recept. Signal Transduct. Res. 18, 1–13.
[21] Süsens, U. and Borgmeyer, U. (1997) Biochim. Biophys. Acta 1309, 179–182.
[22] Agoulnik, I.Y., Cho, Y., Niederberger, C., Kieback, D.G. and Cooney, A.J. (1998) FEBS Lett. 424, 73–78.
[23] Zhang, Y.-L., Akmal, K.M., Tsuruta, J.K., Shang, Q., Hirose, T., Jetten, A.M., Kim, K.H. and O'Brien, D.A. (1998) Mol. Reprod. Dev. 50, 93–102.
[24] Katz, D., Niederberger, C., Slaughter, G.E. and Cooney, A. (1997) Endocrinology 138, 4364–4372.
[25] Heyting, C. and Dietrich, A.J.J. (1991) Methods Cell Biol. 35, 177–202.
[26] Meistrich, M.L. (1977) Methods Cell Biol. 15, 15–54.
[27] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[28] Laemmli, U.K. (1970) Nature 227, 680–685.
[29] Kyhse-Andersen, J. (1984) J. Biochem. Biophys. Methods 10, 203–209.
[30] Rentrop, M., Knapp, B., Winter, H. and Schweizer, J. (1986) Histochem. J. 18, 271–276.
[31] Kralewski, M., Novelle, A. and Benavente, R. (1997) Chromosoma 106, 160–167.
[32] Solari, A.J. (1974) Int. Rev. Cytol. 38, 273–317.
[33] McKee, B.D. and Handel, M.A. (1993) Chromosoma 102, 71–80.
[34] Cunliffe, V., Koopman, P., McLaren, A. and Trowsdale, J. (1990) EMBO J. 9, 197–205.
[35] Meuwissen, R.L.J., Offenberg, H.H., Dietrich, A.J.J., Riesewijk, A., van Iersel, M. and Heyting, C. (1992) EMBO J. 11, 5091–5100.
[36] Ruppert, S., Cole, T.J., Boshart, M., Schmid, E. and Schütz, G. (1992) EMBO J. 11, 1503–1512.
[37] Trasler, J.M., Alcivar, A.A., Hake, L.E., Bestor, T. and Hecht, N.B. (1992) Nucleic Acids Res. 20, 2541–2545.
[38] Smith, A. and Benavente, R. (1995) Chromosoma 103, 591–596.
[39] Smith, A. and Benavente, R. (1992) Proc. Natl. Acad. Sci. USA 89, 6938–6942.
[40] Heinzel, T., Lavinsky, R.M., Mullen, T.-M., Söderström, M., Laherty, C.D., Torchia, J., Yang, W.-M., Brard, G., Ngo, S.D., Davie, J.R., Seto, E., Eisenman, R.N., Rose, D.W., Glass, C.K. and Rosenfeld, M.G. (1997) Nature 387, 43–48.
[41] Nagy, L., Kao, H.-Y., Chakravarti, D., Lin, R.J., Hassing, C.A., Ayer, D.E., Schreiber, S.L. and Evans, R.M. (1997) Cell 89, 373–380.
[42] Le Douarin, B., Zechel, C., Garnier, J.-M., Lutz, Y., Tora, L., Pierrat, B., Heery, D., Gronemeyer, H., Chambon, P. and Losson, R. (1995) EMBO J. 14, 2020–2033.
[43] Le Douarin, B., Nielsen, A.L., Garnier, J.-M., Ichinose, H., Jeanmougin, F., Losson, R. and Chambon, P. (1996) EMBO J. 15, 6701–6715.
[44] Hiermann, R., Rentrop, M., Lang, E. and Maelicke, A. (1993) J. Receptor Res. 13, 693–709.
[45] Giguere, V., Ong, E.S., Segui, P. and Evans, R.M. (1987) Nature 330, 624–629.
[46] Shingleton, J.L., Skinner, M.K. and Ong, D.E. (1989) Biochemistry 28, 9641–9647.
[47] Shingleton, J.L., Skinner, M.K. and Ong, D.E. (1989) Biochemistry 28, 9647–9653.
[48] Lufkin, T., Lohnes, D., Mark, M., Dierich, A., Gorry, P., Gaub, M.-P., LeMeur, M. and Chambon, P. (1993) Proc. Natl. Acad. Sci. USA 90, 7225–7229.
[49] Kastner, P., Mark, M. and Chambon, P. (1995) Cell 83, 859–869.
[50] Sassone-Corsi, P. (1997) Cell 88, 163–166.
[51] Blaner, W.S., Galdieri, M. and Goodman, D.S. (1987) Biol. Reprod. 36, 130–132.