Expression of orphan receptor TR2 mRNA in rhesus monkey (*Macaca mulatta*) testis

MU Xiaomin and LIU Yixun*

State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, *China*

* *Corresponding author*

Abstract The cellular localization and expression of TR2 mRNA in rhesus monkey testis were investigated by the methods of *in situ* and Northern hybridization using the digoxigenin labeled *in vitro* transcripted cRNA probe from a TR2 cDNA fragment template and α-^{32}P-dCTP labeled cDNA probe from the same TR2 cDNA fragment. It is demonstrated that TR2 mRNA was localized specifically in the germ cells, and was predominantly expressed in the meiotic more advanced cells. The expression level was various in different seminiferous tubules, and there was stronger expression in the tubules where spermatogenesis was vigorously going on. The expression level of TR2 mRNA was low in the immature rhesus monkey testis and increased dramatically in the adult and varied with a seminiferous cycle. These data suggest for the first time that orphan receptor TR2 may have an important function in regulating spermatogenesis at later stages of germ cell development in rhesus monkey.

Keywords: **orphan receptor TR2, hybridization, localization, expression, spermatogenesis, rhesus monkey.**

NUCLEAR receptor superfamily consists of steroid receptor, thyroid receptor, vitamin D3 receptor, retinoic acid receptor and a large number of orphan receptors. The receptors of this superfamily all have highly conserved DNA binding domain consisting of two zinc fingers. They can bind to response element on specific genomic DNA, thus regulate the expression of the specific DNA, and exert a function in regulating a wide variety of biological processes, such as cell growth, differentiation and development[1]. Steroid receptor, thyroid receptor and vitamin D3 receptor have been studied widely, and their physiological function is much clear. Retinoic acid receptor and orphan receptor have been highlights of biological studies in recent years. It has been known that retinoic acid and its recepor compose a signal transduction pathway, which has a close relationship with the occurrence process of some cancers[2]. Orphan receptor is a category of receptors cognate ligand of which is still unknown. It has been reported that orphan receptors are transcription factors and may play an essential role in regulating some important physiological processes, especially in the reproductive and nervous system[3, 4]. Because the possible ligand of orphan receptor may be small molecular substances, they are potentially important in finding new medicine or new method for human birth control. Human testicular TR2 is one of the first orphan receptors identified. TR2 cDNA was isolated from human prostate and testicular cDNA libraries with a probe homologous to a highly conserved DNA-binding domain common to steroid hormone receptor. The TR2 orphan receptor cDNA encodes a protein of 603 amino acids with a calculated molecular mass of 67 ku. TR2 has been detected in various cell lines and tissues, and it has been known that TR2 plays a role in regulating the expression of some genes[5, 6]. Up to date, however, little is known about TR2 expression, expecially the cell-specific and stage-specific expression in testes. In this study we investigated the expression of TR2 mRNA in the testis of rhesus monkey by *in situ* and Northern hybridization.

1 Materials and methods

(ⅰ) Materials. Rhesus monkey. Infant (aged 18—24 months) were provided by Beijing Biomedical Institute. Adult rhesus monkeys were purchased from Kunming Institute of Zoology, the Chinese Academy of Sciences.

Plasmid and reagent. The plasmid containing TR2 cDNA was provided by Prof. Chawnshang Chang, University of Wisconsin, the USA. Dig-RNA labeling kit was purchased from Bohringer Mannheim (Gmbh, Germany). Random primers labeling kit, T7 and T3 polymerase and restriction en-

zyme *Xba* I and *Eco*RV were purchased from Promega (Madison, WI, USA). Nylon membrane (Zeta probe blotting membrane) was purchased from Bio-Rad Laboratories (Richmond, CA). Paraformaldehyde was purchased from Sigma Chemical Company (USA). α-^{32}P-dCTP was purchased from Yahui Biotechnological Company (Beijing). APES was purchased from Beijing Zhongshan Biotechnology, Inc. All the other chemicals were local products of analytical grade.

(ii) Methods. Dig-cRNA probe preparation. A 300 bp fragment including initiator codon of TR2-cDNA 5'-end was cut and subcloned into poly-clonal site bewteen T7 and T3 promotor in Bluscript SK, thus recombinant plasmid Bluscript-TR2 was constructed. After the plasmid Bluscript-TR2 was linerized by the digestion of restriction enzyme *Xba* I, antisense cRNA probe was transcribed with T7 polymerase. After the plasmid Bluescript-TR2 was linerized by the digestion of *Eco*RV, sense cRNA probe was transcribed with T3 polymerase using cRNA Dig RNA labeling kit according to the manufacturer's recommendation. ^{32}P-labeled cDNA probe was prepared by degisting the plasmid Bluscript-TR2 with restriction enzyme *Eco*RV and *Xba* I and TR2 fragment was recovered by low-melting agrose electrophoresis and labeled by the random primer method according to the manufacturer's recommendation.

α-^{32}P cDNA probe preparation. ^{32}P-labeled cDNA probe was prepared by degisting the plasmid Bluscript-TR2 with restriction enzyme *Eco*R V and *Xba* I and recover TR2 fragment by low-melting agrose electrophoresis and labeled by the random primer method according to the manufacturer's recommendation.

In situ hybridization and detection. The testes were collected from freshly killed adlut rhesus monkey and were immerse in Bouin's solution fixative for 24 h at room temperature, hyalinizated in xylene, embedded in paraffin, cut into 8 μm sections, and mounted onto APES coated microscope slides. Slides were then dried at 45℃ for 12 h, stored at room temperature. After deparaffinization and rehydration, slides were digested in 0.2 mol/L HCl for 25 min, incubated in 0.3% Triton X-100/PBS for 15 min to demembranize, whshed in PBS for 3 × 5 min. Slides were then digested in 1 μg/mLprotease K solution for 10 min at room temperature, washed in PBS for 3 × 5 min, post-fixed in 4% paraformaldehyde in PBS for 5 min, washed in PBS for 3 × 5 min. Slides were then acetylated in 0.25% acetic anhydride in 0.1 mol/L triethanolamine for 15 min. Prehydridization were performed in a solution of 50% formamide, 2 × SSC 250 μg/mL yeast tRNA, 2 × Denhardt's solution, 10 mol/L Tris. HCl (pH 7.5) for 2 h at 40℃, and hybridization was performed in the same solution containing 400 ng/mL anti-sense dig-cRNA probe or sense digcRNA probe for 20 h at 45℃. After hydridization, slides were washed in different strength of SSC solution as the following order: 2 ×, 1 ×, 0.2 × for 0.5 h at room temperature, individually, 0.1 × SSC for 2 × 15 min at 40℃. The hybridized probes were detected according to the manufacturer's recommendation. Slides were examined and recorded in pictures by a brightfield light microscope. Black staining represented positive reaction.

Total RNA extraction. Testes were collected from infant and adult rhesus monkey which have been freshly killed. Testes were snap-frozen in liquid nitrogen and homogenized in 4 mol/L guadinine isothiocynate using polytron (Sweden). The homogenate was centrifuged at 15 000 r/min for 20 min. The spernatant was transferred to a fresh tube and then layered onto a cushion of 1.66 mL 5.7 mol/L CsCl in a Beckman ultracentrifuge tube (11 × 34 mm), centrifuged at 55 000 r/min, 20℃ for 20 h in a Beckman Optima™ Ultra-centrifuger. The pellet at the bottom of the tube was dissolved in TE containing 0.1% SDS. The RNA solution was preciptated with ethanol and dissolved in the steriled water without RNase. The amount and purity of RNA was assessed using absorption at 260 and 280 nm on a spectrophotometer.

Northern blotting. Purified RNA samples (40 μg) were size-fractioned on 1% agrose gel containing 3.3% formaldehyde, transferred to nylon membrane by standard vaccum transfer in 20 × SSC. The RNA was fixed to membrane by UV crosslinking in a Bio-Rad GS Linker™, and prehybridized for 3 h at 42℃ in a sulotion containing 5 × SSC, 5 × Denhardt's solution, 50% formamide, 250 μg/mL yeast tRNA, 0.1% SDS. Hybridization was performed for 18 h at 42℃ in the same solution containing denatured (100℃, 5 min) TR2 cDNA probe labeled with α-^{32}P-deoxycytidine triphophate by random primers. Membranes were washed under highly stringent conditions in 2 × SSC-0.1%SDS and 1 × SSC-0.1%SDS

for 2 × 20 min, and washed in 0.2 × SSC-0.5% SDS at 0.1 × SSC-0.5% SDS for 2 × 20 min at room temperature and washed with 0.1 × SSC-0.5% SDS at 68℃ for 20 min. Autoradiography was performed at −80℃ with RX Fuji medical X-ray film for 18 h.

2 Results and discussion

TR2 mRNA was expressed and localized specificaly in germ cells, and was not found in sertoli cells of rhesus monkey testis, leydig cells and peritubular cells (fig. 1(b)). Positive staining was located

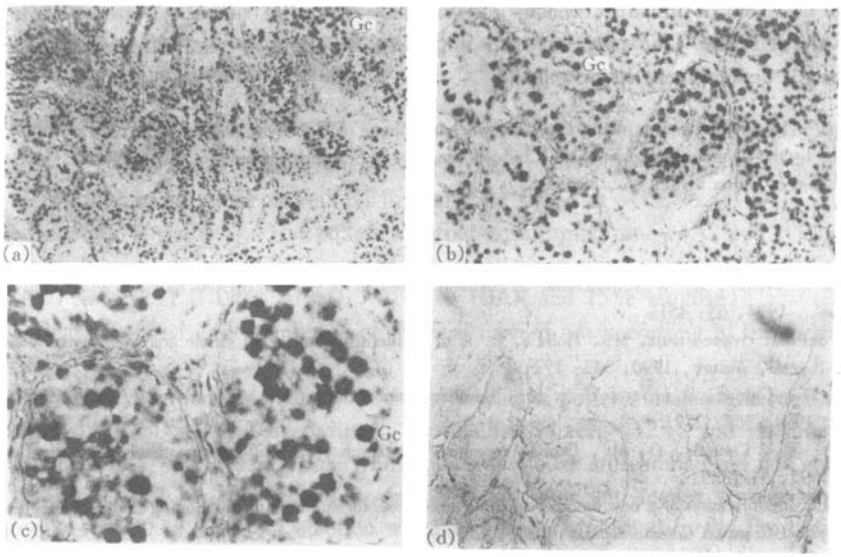

Fig. 1. Cellular localization and specific expression of TR2 mRNA in rhesus monkey testes. (a) Difference in staining intensity is observed in different seminiferous tubules × 100. (b) Positive staining for TR2 mRNA is observed specifically in germ cells (Gc,) × 200. (c) Positive staining is observed mainly in adluminal compartment of seminiferous tubules, where advanced germ cells reside. (d) No detectable staining is observed when sense cRNA prob is used (control).

mainly in adluminal compartment of seminiferous tubules, where advanced germ cells reside. Taking this with cell morphology together, it was indicated that TR2 mRNA was predominantly expressed in meiotic more advanced germ cells (fig. 1(c)). TR2 mRNA was not significantly expressed in spermatogonia, and was not found in mature spermatozoa in the testis of rhesus monkey. Expression level was greatly different in different seminiferous tubules, and there was stronger expression in the seminiferous tubules where spermatogenesis is vigorously going on (figure 1(a)).

The expression level of TR2 mRNA was low in the testis of immature rhesus monkey, and it increased dramatically in the adult testis as indicated by Northern blotting.

These data suggest that TR2 mRNA was localized specifically in germ cells, mainly in the more advanced miototic germ cells, and the expression level varied with a seminiferous cycle. There was a stronger expression in seminiferous tubules where spermatogenesis was vigorously going on. The expression level was low in immature rhesus monkey testes and was increased dramatically in the adult.

In most mammals, spermatogenesis is a complicate cyclic physiological process. Spermatogenesis starts from the specific stage of individual development. It is a multiple cell differentiation process with multiple stages. Spermatogonia is transformed into spermatocyte

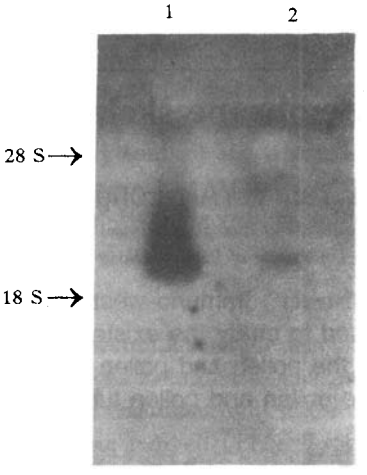

Fig. 2. Northern blotting analysis of total RNA from infant and adult rhesus monkey testes with TR2 cDNA probe. Experimens were performed with 40 μg total RNA loaded in each line and hybridized with TR2 cDNA. 1, Adult; 2, infant.

through mitosis. Spermatocyte is transformed into round spermatid through meiosis. The mature spermatozoa is transformed through morphological changes from the round spermatid. Spermatogenesis is associated with a series of specific gene expression[7]. It is of theoretical and practical importance, therefore, to investigate the expression of these genes during spermatogenesis at the molecular level. It has been shown that TR2 is involved in many regulatory processes *in vivo*, especially in the male reproductive system[8]. In this study we showed that TR2 was expressed in a significant amount in rhesus monkey testis, and TR2 mRNA was specifically localized, mainly in the more advanced miotic germ cells. The expression level of TR2 mRNA was low in inmature rhesus monkey testis, and it increased dramatically in the adult. These results showed that TR2 may have an important function in regulating spermatogenesis at later stages of germ cell development in primate.

Acknowledgement The authors wish to thank Prof. Chawnchang Chang of University of Wisconcin, for providing TR2 cDNA. This work was supported by the Climbing Program of the Chinese Academy of Sciences (Grant No. KJ952-J1-603) and the National Natural Science Foundation of China (Grant No. 39670285).

References

1 Tsai, M. J., O'Malley, B. W., Molecular mechanism of action of steroid/thyroid receptor superfamily members, *Annu. Rev. Biochem.*, 1994, 63: 451.
2 de The. H, del Mar vivanco-Ruiz, M., Tiollais, P. *et al.*, Identification of a retinoic acid responsive element in the retinoic acid receptor β gene, *Nature*, 1990, 343: 177.
3 Woronicz, J. D., Calnan, B., Ngo, V. *et al.*, Requirement for the orphan receptor Nur 77 in apoptosis of T-cell hybridomas, *Nature*, 1994, 367: 277.
4 O'Malley, B. W., Connely, O. M., Orphan receptor: In search of a unifying hypothesis for activation, *Molecular Endocrinology*, 1992, 6: 1359.
5 Chang, C. K., Identification of a new member of the steroid receptor superfamily by cloning and sequence analysis, *Biochemical and Biophysical Research Communication*, 1988, 155: 971.
6 Lee, H. J., Chang, C., Identificationn of human TR2 orphan receptor response element in the transcriptional initiation site of the simian virus 40 major late promotor, *J. Biol. Chem.*, 1995, 270: 5334.
7 Kelwyn, T., Jesus, D. M., Pam, E. *et al.*, Developmental regulation of expression of the lactate dehydrogenase (LDH) multigene family during mouse spermatogenesis, *Development*, 1990, 109: 483.
8 Lee, C. H., Chang, L. M., Wei, L. N., Molecular cloning and characterization of a mouse nuclear orphan receptor expression in embryos and testis, *Molecular Reproduction and Development*, 1996, 44: 305.
9 Liu, Y. X., Du, Q., Zhou, H. M. *et al.*, Regulation of tissue-type plaminogen activator and plasminogen activator inhibitor type-1 in cultured rat sertoli and leydig cells, *Science in China*, Ser. C, 1996, 39: 31.

(*Received September* 17, 1998)