Zur Transkriptions- und Translationskontrolle des Gens für Transitionsprotein 2

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Özlem Topaloglu
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Referent:	Prof. Dr. W. Engel
Korreferent:	Prof. Dr. U. Grossbach
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ABBREVIATIONS

BCP	1-bromo-3-chloropropane
BODIPY	difluoride fluorophore (borondipyrromethane)
BSA	Bovine serum albumin
CAT	Chloramphenical acetyltransferase
CBB	Coomasie brilliant blue
cpm	counts per minutes
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
Dnase	deoxyribonuclease
DNTP	deoxynucleotide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
HEPES	N-2-hydroxyethyl piperazine N'-2-ethane sulphonic acid
IPTG	Isopropyl-ß-thiogalactopyranoside
NaAc	Sodium acetate
NBT	Nitro-blue tetrazolium
NTP	Nucleotide
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fuoride
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecylsulfate
SV 40	Simian Virus 40
TEMED	Tetramethylethylene diamine
TLC	Thin layer chromatograhy
UV	Ultra violet
X-Gal	5-bromo-4-chloro-3-indolyl-ß-galactosidase

1. INTRODUCTION

Spermatogenesis is the sequence of cytological events that result in the formation of highly specialized spermatozoa from undifferentiated stem cells, namely spermatogonia (Bellve, 1979). This process takes place within the seminiferous tubule throughout the reproductive life-span of the male. Spermatogenesis can be divided into four distinct phases: (1) the proliferation and renewal of undifferentiated spermatogonia; (2) the differentiation of spermatogonia; (3) the process of meiosis; (4) spermatid development (spermiogenesis) and release of spermatozoa (spermiation). During the final step which is called spermiogenesis, round spermatids undergo several morphological, biochemical and physiological modifications which result in the formation of mature spermatozoa. These include the nuclear reorganization, the development of the acrosomic system originating from the Golgi apparatus, the assembly of tail structures and cytoplasmic reorganization whose final phase results in the release of spermatozoa in the lumen of the seminiferous tubules (Daduone *et al.,* 1993; Russel *et al.,* 1990). The sequence of morphological events has been divided into different successive steps which vary in number with the species: 8 in man, 10 in the baboon, 15 in the ram, bull and boar, 16 in the mouse and 19 in the rat (Oakberg, 1956).

Nuclear reorganization in haploid spermatids involves the change of nucleosomal chromatin to the highly condensed chromatin found in the sperm nucleus. In mammals, early spermatids, which have nucleosomal chromatin, contain a mixture of somatic histones as well as testis-specific variants of H1 and H2B and sometimes H2A and H3 (Meistrich, 1989). About midway through spermatid development, the nucleus begins to condense, to elongate, and to become resistant to mechanical disruption. These nuclear changes coincide with the replacement of both the somatic-type and testis-specific histones with a set of spermatid-specific chromosomal proteins which are referred to as „transition proteins“. Transition proteins are small lysine- and arginine-rich proteins that play role in the transformation of the nucleosomal chromatin into smooth condensed chromatin fibers. In the final stages of nuclear restructuring, the transition proteins are replaced by the distinctive cysteine-rich protamines to produce the tightly compacted nucleus of the spermatozoon (Balhorn *et al.,* 1984; Balhorn, 1989; Bellve *et al.,* 1983; Dadoune *et al.,* 1993).

During spermatogenesis, stringent temporal and stage-specific gene expression is a prerequisite for the correct differentiation of male germ cells. There are two levels of transcriptional regulation: Methylation and binding of the trans-acting factors to the TATA-box, and other specific DNA sequences in the promoter region of nucleoproteins (Goldberg, 1996). The dogma which dictates that methylation of specific cytosines in DNA leads to a reduction or cessation of gene transcription is not valid for all spermatid-specific genes. The opposite occurs in the chromosomal locus on mouse chromosome 16, which contains the protamine 1 and 2, transition protein 2 genes which are located in a large methylated region in their expressing cell type, namely round spermatids (Choi *et al.,* 1997). On the other hand, the gene for transition protein 1 reveals a cell-specific demethylation associated with gene activity (Trasler *et al.,* 1990). Another evidence that general transcription may be differentially regulated in germ cells is the accumulation of the TATA-binding protein (TBP) in early haploid germ cells at much higher levels than in any other somatic cell types. It has been calculated that adult testis contains 7.9 and 11.4 fold more molecules of TBP per haploid genome equivalent than adult spleen and liver cells, respectively (Schmidt and Schibler, 1997). Additionally, TFIIB and RNA polymerase II were also found to be overexpressed in testis (Schmidt and Schibler, 1995).

Studies in transgenic mice have shown that relatively short 5‘ upstream sequences can direct tissue- and stage-specific expression of haploid expressed genes in testis (Howard *et al.,* 1993; Peschon *et al.,* 1987; 1989; Reddi *et al.,* 1999). Zambrowicz *et al*. have demonstrated that a 113 bp region from –150 to –37 of the mouse protamine 1 can successfully direct spermatid-specific transcription in transgenic mice (Zambrowicz *et al.,* 1993). A number of ubiquitous and testis-specific proteins bind to this region (Zambrowicz *et al.,* 1994), including Tet-1 which is a testis-specific trans-acting nuclear protein that recognizes the 11-mer sequence at –64 in 5‘UTR of mouse protamine 1 (Tamura *et al.*, 1992). Analysis of the mouse protamine 2 promoter by *in vitro* transcription assays have identified a potential positive regulatory region from –140 to –23 (Bunick *et al.,* 1990). Mobility shift assays revealed binding of both ubiquitious and testis-specific proteins (Johnsons *et al.,* 1991). Furthermore, there is evidence of binding for a novel orphan nuclear factor and CRE at positions –64/-48 and –84/-72, respectively (Enmark and Gustafsson, 1996; Delmas *et al.,* 1993). The cAMP response element (CRE)-like sequences have been found in a number of genes that are transcribed in the spermatids. Expression of cAMP response element modulator (CREMτ) appears to be restricted to the testis. There is direct evidence that CREMτ is one of the transcription factors responsible for the postmeiotic expression of the calspermine gene (Sun *et al.,* 1995) and the

transition protein 1 gene (Kistler *et al.,* 1994). In two recent reports (Blendy *et al.,* 1996 and Nantel *et al.,* 1996) the specific role of CREM in spermiogenesis was addressed using CREM-mutant mice which showed postmeiotic arrrest at the first stage of of spermiogenesis. Absence of the CREM gene leads to the lack of expression of CREM-dependent genes such as genes for protamine 1 and 2, and calspermin.

Post-transcriptional control is especially important towards the end of spermatogenesis since the global transcription ceases several days before the completion of spermiogenesis. Thus, mRNA storage and translational activation play important roles in the expression of many spermatid and spermatozoon proteins which are synthesized in late stages of germ cell maturation. For example, although the genes for transition proteins and protamines are transcribed in round and elongating spermatids, their mRNAs are stored as ribonucleoprotein (RNP) particles in a translationally repressed state for several days until they are translated in elongating and elongated spermatids (Dadoune *et al.,* 1995; Eddy *et al.,* 1993; Hecht *et al.,* 1989; 1990a; 1990b; Morales *et al.,* 1991; Kleene *et al.,* 1996). The need for the translational regulation was demonstrated in transgenic mice where the mouse protamine 1 mRNA was prematurely translated by exchanging the 3'UTR of protamine 1 with the 3'UTR of human growth hormone (hGH). Premature accumulation of Prm1 mRNA resulted in dominant male sterility accompanied by a complete arrest in spermatid differentiation, early condensation of spermatid nuclear DNA, abnormal head morphogenesis, and incomplete processing of Prm2 protein in transgenic mice (Lee *et al.,* 1995). Deadenylation and the interaction between RNA-binding proteins and both/either 5' untranslated regions and/or 3' untranslated regions are the main suggested mechanisms to achieve the translational regulation of testis-specific genes. It was shown that the length of poly-A tails of transition proteins and protamine mRNAs correlates with translational activity (Heidaran and Kistler, 1987; Kleene *et al.,* 1984; Kleene, 1989; 1993). The mRNAs for protamines are subjected to a shortening process before or during translation, namely from 0.62 kb to 0.45 kb for protamine 1 and from 0.9 kb to 0.7 for protamine 2 (Domenjoud *et al.,* 1991). This reduction in poly (A) length of mRNAs appears restricted to the haploid cells of the testis, because mRNAs expressed in premeiotic or meiotic male germ cells encoding ornithine decarboxylase, lactate dehydrogenase c, and cytochrome c show increases in polyadenylation (Hecht, 1998). One of the best characterized RNA-binding protein is the 70 kDa poly-A binding protein (PABP) whose mRNA level increases as germ cells enter meiosis (Gu *et al.,* 1995). The high level of PABP in round spermatids and in mRNPs suggests that it may have a role in the storage of developmentally regulated mRNAs in mammalian testis. It has been suggested that PABP migrates from the

poly (A) tail to the AU-rich segments in the 3'UTR, leaving the poly (A) tail naked and vulnerable to degradation (Bernstein and Ross, 1989).

Translational repression in round spermatids is achieved by the binding of sequence-specific RNA-binding proteins to the 3'UTRs, whereas translation in elongating spermatids is achieved by the covalent modification of the mRNP complex and release of translatable protamine 1 mRNA (Braun, 1990). The studies of Braun et al. have demonstrated that the 3' UTR of mouse protamine 1 gene contains all of the elements required for proper translational regulation of protamine 1 mRNA in transgenic mice (Braun *et al.,* 1989). Subsequent studies identified cis-acting elements in the 3'UTRs of protamine 1 and 2 mRNAs and trans-acting factors that recognize them. All of the cis-acting sequences required for the translational regulation of protamine 1 has been mapped to 62 nucleotides in the 3' most region of the protamine 1 3' UTR (Fajardo *et al.,* 1997). A growing number of RNA-binding proteins that influence the translation through binding to the 3'UTR of mouse protamine 1 and 2 mRNAs have been identified with the help of gel mobility shift assays and UV-crosslinking experiments. Screening of male germ cell cDNA expression libraries with 3' UTR has also yielded several clones encoding RNA-binding proteins like protamine 1 RNA-binding protein (Prbp) which was later shown to be required for the activation of repressed protamine 1 mRNA (Lee *et al.,*1996; Zhong *et al.*, 1999), testis-brain-RNA-binding protein (TB-RBP) which supresses the translation of protamine 1 and 2 in vitro and attaches mRNAs to microtubules by binding to conserved elements in the 3' untranslated regions of specific mRNAs, spermatid perinuclear RNA binding protein (Spnr) which is highly expressed in elongating haploid germ cells, is localized to a spermatid-specific microtubule array called the manchette and thereby might play a role in the putative subcellular localization of protamine mRNA molecules that are destined to be activated for translation at the nuclear periphery (Schumacher *et al*, 1995), and finally Tenr which is an RNA-binding protein, is localized in a lattice-like network within the spermatid nucleus (Schumacher *et al*., 1995).In this study, the transcriptional and translational regulation of rat transition protein 2 (Tnp2) were investigated. Transition proteins are quite variable with regard to size and amino acid compositions. They are generally more basic than histones but less basic than protamines. They appear to be species- or perhaps class-specific proteins. In mammals, during elongation and condensation of the spermatid nucleus, several transition proteins have been characterized. In boar, man, mouse, ram and rat, this family consists of four proteins, Tnp1-4, of which Tnp1 and Tnp2 are best characterized. Tnp2 is about the molecular size of a core histone and is characterized by a large amount of basic residues (~32%), serine (~22%) and proline (~13%) and by the

presence of cysteine (5%) (Wouters-Tyrou *et al.,* 1989). Tnp2 consists of 115 amino acids in rat and has characteristic domains (Luerssen *et al.,* 1989). The carboxyl terminal is enriched in basic residues and is likely to be the major site of DNA-binding (Cole *et al.,* 1987). The amino terminal region binds zinc and has two proposed zinc finger-like structures (Kundu *et al.,* 1994). The transition protein 2 mRNA is first detectable in step 7 round spermatids, persists at high levels through step 13, and is degraded before step 14 in mouse (Shih and Kleene, 1992). On the other hand, the Tnp2 protein can be firstly detected in step 12 spermatids, is strongly present in step13, and finally disappears in step 14 spermatids in the mouse (Alfons and Kistler, 1993).

Aim of the study

In the framework of this study, the transcriptional and translational regulation of rat Tnp2 gene was to be investigated by means of both *in vitro* and *in vivo* systems. The rat Tnp2 promoter region should be characterized by primer extension analysis and analysis of transgenic promoter/reporter strains. Based on prior information of a transgenic line, carrying 525 bp 5’ untranslated region, the relevant region should be narrowed down by further transgenic mice. The role of 3’UTR in translational repression of Tnp2 mRNA should be investigated by further transgenic lines. In analogy to a reported mouse protamine 1 transgene, where the 3' UTR of protamine was replaced by human growth hormone 3' UTR, a similar transgenic line for a precocious Tnp2 expressing transgenic strain was planned. By RNA-affinity chromatography RNA-binding proteins that are specifically bind to the Tnp2 3’ UTR mRNA was planned to be enriched, isolated, and cloned.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Acetyl CoA	Sigma, Deisenhofen
Acrylamide	Gibco/BRL, Eggstein
Ammonium sulfate	Sigma, Deisenhofen
Aprotinin	Sigma, Deisenhofen
Agar	Difco, Detroit, USA
Agarose	Gibco/BRL, Eggstein
Ampicillin	Sigma, Deisenhofen
Aprotinin	Sigma, Deisenhofen
BSA	Biomol, Hamburg
CAM Substrate	Strategene, Heidelberg
Choloroform	Roth, Karlsruhe
Dextran sulfate	Pharmacia, Freiburg
Dithiothreitol	Biomol, Hamburg
DNA Markers	GibcoBRL, MBI
DNA Ligase	GibcoBRL, MBI
Dnase	Boehringer, Mannheim
dNTPs	Boehringer, Mannheim
Dye Terminator Mix	Applied Biosystems
Ethidium bromide	Sigma, Deisenhofen
Formamide	Merck
Ethanol	Roth, Karlsruhe
Glycine	Biomol, Hamburg
JETPREP-Plasmid Midi Kit	Genomed, Bad Oeynhausen
Pepstatin	Sigma, Deisenhofen
Phenol	Biomol, Hamburg

Picric acid	Fluka, Neu Ulm
Poly (dI.dC)(dI.dC)	ICN, Cleveland, USA
PMSF (Phenylmethylsulfonylfluoride)	Sigma, Deisenhofen
Proteinase K	Boehringer; Mannheim
Protein marker	Biorad, Sigma
SDS	Serva, Heidelberg
Spermidin	Sigma, Deisenhofen
Urea	ICN, Cleveland, USA
Triton X-100	Serva; Heidelberg
tRNA	Boehringer; Mannheim
Tween-20	Fluka; Neu Ulm
X-Gal	Boehringer; Mannheim

2.1.2 Solutions and buffers

Ampicillin	50 ug/mL H_2O
Denaturing Solution	1.5 M NaCl 0.5 M NaOH
Denhardt Solution (50x)	1 % BSA 1 % Polyvinylpyrrolidone 1 % Ficoll 400
E-Buffer (10x)	300 mM NaH_2PO_4 pH 7.0 50 mM EDTA
Hybridization Solution	12.5 % Denhardt Solution 5 % Dextran sulfate 0.25 % SDS 5 % SSC
LB-Agar pH 7.2	LB- Medium with

	1.5 % Bacto-Agar
LB-Medium pH 7.2	1 % Bacto-Tryptone 0.5 % Bacto-Yeast-Extract 1 % NaCl
Sodium acetate	3 M Sodium acetate pH 5.3
Neutralization solution	1 M Tris pH 5.5 3 M NaCl
SSC (20x)	0.3 M Tri-sodium acetate pH 7.0 3 M NaCl
TBE Buffer (5x)	225 mM Tris pH 8.3 225 mM Boric acid 10 mM EDTA
TE Buffer (10x)	100 mM Tris pH 8.0 10 mM EDTA

2.1.3 Sterilization of solutions and equipments

All solutions, which are not heat sensitive, were sterilized at 121°C, 10^5 Pa for 60 min in an autoclave (Webeco, Bad Schwartau). Heat sensitive solutions were filtered through a disposable sterile filter (0.2 to 0.45 μm pore size). Plasticware was autoclaved, as above. Glassware was sterilized overnight in an oven at 220°C.

2.1.4 Bacterial strains

E.coli HB 101	(Boliver and Beckman, 1979)
E.coli JM 109	(Messing, 1983)

E.coli XL1 Blue	(Young and Davis, 1983)

2.1.5 Plasmids

Bluescript II SK (-)	Stratagene, La Jolla, USA
pCAT3	Promega, Wisconsin, USA
pGEM-T, pGEM-T EASY	Promega, Wisconsin, USA

2.1.6 Synthetic oligonucleotide primers

The synthetic oligonucletide primers used in this study were ordered from either NAPS (Göttingen, Germany) or Roth (Karlsruhe, Germany).

Tppro1-F	5‘	GGG GCG AGC TCT GCC ATA CCT GTC ACC	3‘
RTP2-5A	5‘	CAT GGG ATC CCA CAC TCG GAG GG	3‘
RTP2-PE1	5‘	GTG GTG GCT GCA GGC ACA CTG	3‘
RTP2-KpnI-F	5‘	GAA GGT ACC AAG TGA CAC AC	3‘
Tp2-E2-R	5‘	GGA ATT CTC ACT TGT ATC TTC GTC C	3‘
hGH-3‘UTR-F	5‘	GGA ATT CCT GCC CGG GTG GCA TCC C	3‘
hGH-3‘UTR-R	5‘	CCC AAG CTT CAA ACC ACC CCC CTC CAC	3‘
EcoRI-$(T)_{30}$-R	5‘	CGG AAT TCT TTT TTT TTT TTT TTT TTT TTT	
		TTT TTT TTC CAG GGT ACA AGC	3‘
Tp2-Ex1-F	5‘	GAC ACC AAG ATG CAG AGC CTT	3‘

2.1.7 Antibodies

For the detection of chloramphenicol acetyltransferase (CAT) protein in Tnp2-147 transgenic line, the polyclonal rabbit anti-CAT antibody from BioTrend, Köln, Germany was used.
The polyclonal rabbit anti-Tnp2 antibody was kindly provided by S.K. Kister, University of South Carolina, Columbia, USA (Alfons and Kistler, 1993).
To carry out immunodetection in hGH-Tnp2 transgenic line, the rabbit peroxidase-anti-peroxidase complex from Dako, Hamburg, Germany was used.

2.1.8 Animals

In this study NMRI and CD-1 mice lines were used for both RNA and protein preparations which are bred at Institute of Human Genetics, Göttingen, Germany.
SIV 50 rat line was utilized for the preparation of nuclear and cytoplasmic proteins from rat.

2.2 Methods

2.2.1 Isolation of nucleic acids

2.2.1.1 Isolation of genomic DNA from tissue samples

Lysis Buffer	50 mM Tris-HCl pH 8.0
	100 mM EDTA
	0.5 % SDS

The method employed was the same as that of Laird *et al*. (1991). 1 to 2 cm of the tail from a mouse was incubated in 700 µl of lysis buffer containing 35 µl proteinase K (10 ug/ul) at 55°C overnight. Equal volume of phenol was added, mixed by inverting, and centrifuged at 8000 xg at room temperature for 10 min. After transferring the aqueous layer into a new tube, the same procedure was repeated, but this time with 0.5 Vol phenol and 0.5 Vol chloroform. Then, the DNA was precipitated with 2.5 Vol 100 % ethanol, and fished-out with a pipette tip. Usually, it was dissolved in 100-200 µl of 1 x TE buffer.

2.2.1.2 Isolation of total RNA from tissue

TRI Reagent is an improved version of the single-step method for total RNA isolation. The composition of TRI Reagent includes phenol and guanidine thiocyanate in a mono-phase solution.
100-200 mg tissue sample was homogenized in 1-2 ml of TRI Reagent by using a glass-teflon homogenizer. The sample volume should not exceed 10 % of the volume of TRI Reagent used for the homogenization. The homogenate was incubated at room temperature for 5 min to

permit the complete dissociation of nucleoprotein complexes. Then, 0.1-0.2 ml of BCP was added which is a less toxic chemical than chloroform, shaked vigorously, and stored at room temperature for 15 minutes. After centrifugating the sample at 12000 xg for 15 min at 4°C, the colorless upper aqueous phase was transferred into a new tube. The RNA was precipitated by adding 0.5 ml of isopropanol. Finally, the pellet was washed with 75 % ethanol, and dissolved in 80-100 µl of DEPC-H_2O.

2.2.1.3 Isolation of poly(A)-enriched RNA

To isolate polyadenylated mRNA, the Qiagen Oligotex kit was employed. The purification procedure makes use of oligo-dT coated latex particles that provide a hybridization carrier on which nucleic acids containing polyadenylic acid sequences can efficiently immobilized and easily recovered.
400 µg total RNA was mixed with 400 µl of 2x binding buffer and 30 µl of oligotex suspension and incubated for 3 minutes at 65°C to distrupt the secondary structure of the RNA. It was then further incubated for 10 min at room temperature to allow the hybridization between the oligo (dT)30 linked to the latex particles and the poly (A) tail of the mRNA. The oligotex resin containing the RNA was pelleted by centrifugation for 2 min at full speed. After removing the supernatant, the pellet was resuspended in 400 µl of wash buffer QW2, then transferred to a spin column. The column was washed 2 times with wash buffer QW2, and finally the RNA was eluted with 100 µl of H_2O.

2.2.1.4 Isolation of plasmid DNA

2.2.1.4.1 Small-scale isolation of plasmid DNA

5 ml of LB medium with the appropriate antibiotic was inoculated with a single *E. coli* colony and incubated overnight at 37°C with shaking. 1.5 ml of this culture was centriguted at 5000 xg for 10 minutes. The pellet was resuspended in 200 µl of solution E1. After adding equal volumes of solutions E2 and E3, respectively, the pellet was incubated on ice for 15 minutes, and centrifuged at full speed at 4°C. The supernatant was transferred into a new tube, and 1 ml of 100 % ethanol was added to precipitate the DNA. It was then stored at –20°C for 30

minutes, centrifuged at full speed for 30 minutes, and finally the pellet was dissolved in 30 µl of H_2O.

2.2.1.4.2 Large-Scale isolation of plasmid DNA

80 ml of LB medium was inoculated with a single E.coli colony and incubated overnight at 37°C with shaking. In order to pellet the cells, it was centrifuged at 5000 rpm for 10 min. The pellet was resuspended in 4 ml of solution E1. To lyse the cells 4 ml of solution E2 was added, mixed gently, and incubated for 2-3 minutes at room temperature. Equal amount of solution E3 was added to the tube, and mixed immediately by inverting. The mixture was centrifuged at 4000 rpm for 30 minutes at 20°C. Meanwhile, the column that was provided by the kit was equilibrated with 10 ml of solution E4. The clear lysate after the centrifugation was applied to the equilibrated column. The column then was washed twice with 10 ml of solution E5. Finally, the DNA was eluted with 5 ml of solution E6. To precipitate the DNA, 0.7 Vol of isopropanol was added, and centrifuged at 4000 rpm for an hour at 12°C. The DNA was usually dissolved in 100 ul of H_2O.

Solution E1	50 mM Tris/HCl pH 8.0 10 mM EDTA 100 µg/ml RNase	Solution E4	600 mM NaCl 100 mM NaAc pH 5.0 0.15 % Triton X-100
Solution E2	200 mM NaOH 1% SDS	Solution E5	800 mM NaCl 100 mM NaAc pH 5.0
Solution E3	3.1 M Potassium acetate pH 5.5	Solution E6	1250 mM NaCl 100 mM Tris/HCl pH 8.5

2.2.1.4.3 Isolation of DNA fragments after agarose gel electrophoresis

For the isolation of DNA fragments which are 300-4000 bp in length from agarose gels, the Geneclean kit from Biomol 101 (Biomol, Hamburg) was employed. The principle of this method depends on the binding capacity of DNA to silica in high salt concentrations and elution in low salt solutions. After separation of DNA on an agarose gel, the DNA band to be isolated was excised with a razor blade, and weighed. 3 Vol of 6 M NaI was added to the tube, and the agarose slice was melted at 55°C. Depending on the DNA amount, required amount of GLASSMILK which is an aqueous suspension of silica matrix was added and the tube was placed on ice for 30 min. After centrifuging it at full speed for 2 min, the pellet was washed 2 times with „New Wash“, and allowed to dry at room temperature. To elute the DNA, the pellet was resuspended in 30 µl of H_2O and incubated at room temperature for 10 min. After the final centrifugation at 14000 rpm for 5 min, the supernatant containing the DNA was transferred into a new tube.

2.2.1.4.4 Isolation of DNA fragments from acrylamide gels

For the isolation of small DNA fragments (50-300 bp), the DNA was first separated on a polyacrylamide gel. After staining the gel with ethidium bromide, the desired band was cut out and transferred into a 1.5 ml centrifuge tube. 500 µl TE buffer was added to the gel slice and incubated at 50°C overnight, with shaking. After centrifugation at 12000 xg at room temperature for 15 minutes, the supernatant containing DNA was precipitated by adding 1/10 Vol 3 M NaAc and 2.5 Vol 100 % of ethanol.

2.2.2 Determination of the nucleic acid concentration

The concentration of nucleic acids was determined photometrically by measuring absorption of the samples at 260 nm. DNA quality i.e. contamination with salt and protein was checked by the measurements at 230, 280, and 320 nm. The concentration can be calculated according to the formula:

C = (E 260 – E 320) x f x c

C = concentration of sample (ug/ul)
E 260 = absorption at 260 nm
E 320 = absorption at 320 nm
f = dilution factor
c = concentration (standard) / absorption (standard)
for double stranded DNA : c = 0.05 ug/ ul
for RNA : c = 0.04 ug/ul
for single stranded DNA : c = 0.03 ug/ul

2.2.3 Enzymatic modifications of DNA

2.2.3.1 Restriction of DNA

Restriction enzyme digestions were performed by incubating double-stranded DNA molecules with an appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier, and at the optimal temperature for that specific enzyme. Typical digestions include 2-10 U enzyme per microgram of starting DNA, and one enzyme unit usually (depending on the supplier) is defined as the amount of enzyme needed to completely digest one microgram of double-stranded DNA in one hour at the appropriate temperature. These reactions were usually incubated for 1-3 hrs to insure complete digestion at the optimal temperature for enzyme activity which was typically 37°C.

2.2.3.2 Dephosphorylation of 5‘ ends of DNA

To prevent the recircularization of vector plasmid without insertion of foreign DNA, alkaline phosphatase treatment was peformed. Alkaline phosphatase catalyses the hydrolysis of 5'-phosphate residues from DNA. The followings were mixed,

1-5 µg vector DNA
5 µl 10 x reaction buffer

1 µl alkaline phosphatase (1 U)
in a total volume of 50 µl

and incubated at 37°C for 30 min. Then the reaction was stopped by heating at 85°C for 15 min.

2.2.3.3 Ligation of DNA fragments

The ligation of an insert into a vector was carried out in the following reaction mix:

30 ng vector DNA
50-100 ng insert DNA
1 µl ligation buffer (10 x)
1 µl T4 DNA ligase (5U/µl)
in a total volume of 10 µl.

Blunt-end ligations were carried out at 16°C overnight whereas sticky-end ligations were carried out at room temperature for 2-4 hrs.

2.2.3.4 TA-Cloning

Taq and other polymerases seem to have a terminal transferase activity which results in the non-templated addition of a single nucleotide to the 3'-ends of PCR products. In the presence of all 4 dNTPs, dA is preferentially added. This complicates cloning, as the supposedly blunt-ended PCR product often is not. This terminal transferase activity is the basis of the TA-cloning strategy. For the cloning of PCR products, pGEM-T Easy Vector system which has 5‘ T overhangs was employed. The followings were mixed,

50 ng of pGEM-T or pGEM-T Easy Vector
PCR product (3:1 vector : insert ratio)
1 µl T4 DNA Ligase 10x buffer
1 µl T4 DNA Ligase

in a total volume of 10 µl

The contents were mixed by pipetting and the reaction was incubated overnight at 4°C. To transform the ligation reaction JM 109 competent cells were used.

2.2.3.5 Filling-up reaction

0.1-4 µg of digested DNA was mixed with 0.05 mM dNTPs and 1-5 U of Klenow fragment. The reaction was incubated at 37°C for 10 min, then stopped by heating at 75°C for 10 min.

2.2.4 Gel electrophoresis

Gel electrophoresis is the technique by which mixtures of charged macromolecules, especially nucleic acids and proteins, are rapidly resolved in an electrical field.

2.2.4.1 Agarose gel electrohoresis of DNA

Agarose gels are used to electrophorese nucleic acid molecules from as small as 150 bases to more than 50 kilobases, depending on the concentration of the agarose and the precise nature of the applied electrical field (constant or pulse).
1 g of agarose was dissolved in 100 ml 0.5 x TBE buffer, and boiled in the microwave, then cooled down to about 50°C before adding 3 µl ethidium bromide (10 mg/ml). The gel was poured into a horizantal gel chamber.

2.2.4.2 Agarose gel electrophoresis of RNA

Single-stranded RNA molecules often have small regions that can form base-paired secondary structures. To prevent this, the RNA should be run on a denaturing agarose gel which contains formaldehyde, and additionally is pre-treated with formaldehyde and formamide.

1.25 g of agarose was dissolved in 100 ml of 1 x E-Buffer, after cooling it to about 50°C, 25 ml of formaldehyde (37 %) was added, stirred and poured into a vertical gel chamber. To 10-20 µg of RNA,

2 µl 10 x E-Buffer
3 µl Formaldehyde
8 µl Formamide (40%)
1.5 µl Ethidium bromide

were added and the samples were denatured at 65°C for 10 min, and chilled on ice before applying to the gel. The gel was run at 80 V at 4°C for about 3-4 hrs.

2.2.4.3 Polyacrylamide gel electrophoresis (PAGE) of DNA fragments

Polyacrylamide gel electrophoresis was employed to analyze the small DNA fragments which were 50-500 bp and to separate the gel retardation samples. The percentage of acrylamide (6-12 % w/v) determines the resolving property of the gel. A 10 % of PAG was prepared as follows:

2.5 ml 40% PAA stock solution
2.5 ml 5 x TBE buffer
150 µl APS (10 % w/v)
15 µl TEMED
in a total volume of 10 µl

APS and TEMED were used to initiate the polymerization of the gel. The gel was poured vertically between two clean glass plates, ensuring no air bubbles. After completion of the electrophoresis, DNA was visualized by staining the gel in an ethidium bromide solution.

2.2.4.4 SDS-PAGE for the separation of proteins

(Laemmli, 1970)

Sample buffer (2 x)	0.5 M Tris/HCl pH 6.8 20%Glycerol 4% SDS 10 % ß-Mercaptoethanol
Running buffer (5 x) (Tris/Glycine buffer)	25 mM Tris/HCl pH 8.3 192 mM Glycine 0.1 % SDS
Stacking gel buffer (4 x)	0.5 M Tris/HCl pH 6.8 0.4 % SDS
Separating gel buffer (4 x)	1.5 M Tris/HCl pH 8.8 0.4 % SDS

SDS gel electrophoresis is a method for separating proteins within a sample for analysis and molecular weight detemination. The proteins are denatured and rendered monomeric by boiling in the presence of reducing agents (2-merceptoethanol or dithiotheitol) and negatively charged detergent (SDS). The proteins which normally differ according to their charges are all coated with the SDS which is negatively charged. Hence, all the proteins in the sample become negatively charged. In this way, the separation is according to the size of the proteins. A SDS-PAG consists of two gels; firstly, usually 10 % separating gel was poured. In order to achieve a smooth boundary between separating and stacking gel, the separating gel was covered with a layer of water saturated butanol. After the polymerization of the separating gel completed, a 4 % stacking gel was poured. The samples were boiled in sample buffer for 10 minutes at 95°C before applying to the gel. The gel was run at 15 mA for one hour then at a constant current of 30 mA.

2.2.4.5 Acid-Urea gel electrophoresis for the separation of small proteins

(Panyim and Chalkey, 1969)

This gel system is a SDS- free PAGE and applied specially for the separation of basic proteins which have small molecular weight like histones, transition protein 1 and 2 and protamines. In the absence of SDS, the proteins would still be separated essentially on the basis of their sizes, but their charges would vary according to their amino acid contents. This is because of the charge on a protein at any particular pH is the sum of the charges prevailing on the side chain groups of it s constituent amino acid residues, and the free amino and carboxyl groups at it s termini. Thus, in an ionic detergent-free gel electrophoretic system, both the molecular size and charge act as bases for effective protein separation. The pH prevailing in such a system might be anything, but it is commonly about pH 3.0. At pH 3.0, all proteins are likely to be positively charged and to travel towards the cathode in an electrical field. In an acid-polyacrylamide gel electrophoresis system, two proteins of similar size but different charge maybe separated from each other. In this work, this gel system was employed method for the separation of small basic proteins such as histones, transition protein 1 and 2, and protamines. For this purpose, 15 % polyacrylamide with 2.5 M urea and 5 % acetic acid was prepared as follows:

3 g Urea
7.5 ml 40 % PAA
1 ml 100 % Acetic acid
75 µl TEMED
500 µl APS
in a total volume of 20 ml

The electrophoresis was carried out for 4 hours at 15 mA. The gel was run in the direction from anode to cathode. A stop mix containing 2.5 M urea, 5 % acetic acid, and 40 % saccharose was used.

2.2.5 Labelling of nucleic acids

2.2.5.1 „Random Prime“ method for generation of ^{32}P labelled DNA

(Feinberg and Vogelstein, 1989)

Ready-To-Go DNA labelling kit (Pharmacia) was employed for labelling of DNA fragments radioactively. The method relies on the random priming principle developed by Feinberg and Vogelstein. The reaction mix contains dATP, dGTP, dTTP, Klenow fragment (4-8 units) and random oligodeoxyribonucleotides, primarily 9-mers. 25-50 ng of DNA was denatured in a total volume of 46 µl at 95°C for 15 minutes. It is then transferred to Ready-To-Go reaction cup, mixed thoroughly by vortexing, and finally 4 µl of [α-^{32}P] dCTP (3000 uCi/mmol) was added to the reaction mixture. The labelling reaction was carried out at 37°C for 1-3 hours.

2.2.5.2 5‘ End-Labelling of oligonucleotides

The oligonucleotides which were used in electrophoretic mobility shift assay were labelled as follows:

20-50 ng ds DNA
2 ul 10 x Buffer for Klenow Fragment
1 ul Klenow Fragment
3 ul [α-^{32}P] dCTP
in a total volume of 20 ul

The labelling reaction was carried at room temperature for 10 min. Then, the labelled DNA was purified with the use of MicroSpin G-25 Columns (Amersham Pharmacia Biotech Inc., NJ, USA).

2.2.5.3 Labelling of in vitro transcripts

The RNA which was used in Northwestern analyses was *in vitro* transcribed from a lineralized vector in the presence of [α-^{32}P] UTP.

2-5 µg lineralized plasmid
1 µl RNasin (20-40 U)
1 µl 10 mM ATP; CTP; GTP
1 µl 1 mM UTP
4 µl 5 x Transcription buffer
2 µl 0.1 M DTT
1 µl [α-^{32}P] UTP (800 µCi/mMol; 16 µCi)
1 µl RNA Polymerase (T7, T3 or SP6)
in a total volume of 20 µl

The contents were mixed, and incubated at 37°C for an hour. In order to remove the DNA template, 1 µl of RNase-free DNase I (1 U/µl) was added and incubated further for 15 min. Finally, the *in vitro* transcribed RNA was precipitated by adding 1/10 Vol of 3 M NaAc and 2.5 Vol of 100 % ethanol, and dissolved in 100 µl of DEPC-H_2O.

2.2.6 Non-radioactive *in vitro* transcription

Ambion MEGAshortscript kit was used to obtain high yields of *in vitro* transcription products in the 20-500 nucleotide range. These high yields were achieved by optimizing reaction conditions for RNA synthesis in the presence of high nucleotide and polymerase concentrations.

10 ug template DNA in a volume not to exceed 8 ul
2 µl 10x Transcription buffer
2 µl ATP solution (75 mM)
2 µl CTP solution (75 mM)
2 µl GTP solution (75 mM)
2 ul µl UTP solution (75 mM)
2 µl T7 MEGAshortscript enzyme mix
-- µl RNase-free dH_2O
in a 20 µl of total volume

The contents were mixed, and incubated at 37°C for 4 hrs. The removal of template DNA and the precipitation of the *in vitro* transcribed RNA were achieved as described in section 2.2.5.3.

2.2.7 Non-Radioactive dye terminator cycle sequencing

The non-radioactive sequencing was achieved with Dye Terminator Cycle Sequencing-Kit (ABI, Weiterstadt) and the reaction products were analyzed with automatic sequencing equipment, namely 373 A DNA Sequencer (ABI, Weiterstadt). For the sequencing reaction, four different dye labelled dideoxy nucleotides were used, which, when exposed to an argon laser, fluorescent emitting light which could be detected and interpreted. The reaction was carried in a total volume of 10 µl containing 1 µg plasmid DNA or 100-200 ng purified PCR products, 10 pmol primer and 4 µl reaction mix (contains dNTPs, dideoxy dye terminators and *Taq* DNA polymerase). Elongation and chain termination takes place during the following program in a thermocycler: 5 min denaturing followed by 25 cycles 95°C 30 sec, denaturing; 55°C 15 sec, annealing; 70°C 4 min, elongation. After the sequencing reaction, the DNA was precipitated with 1/10 Vol 3 M NaAc and 2.5 Vol 100 % ethanol. The pellet was dissolved in 4 µl of loading buffer, denatured at 95°C for 3 min, and finally loaded onto the sequence gel.

2.2.8 Blotting techniques

2.2.8.1 Dot blotting of DNA onto nitrocellulose filters

Dot-blotting is probably the simplest and least laborious method for monitoring the transgene and hence the routine maintenance of breeding lines. However, dot-blotting does not give any information on the physical integrity of the transgene which must be checked by Southern blotting.

After assembling the dot-blot apparatus according to the manufacturer's instruction, the vacuum pump was switched on, and each well was washed with 20 x SSC. Meanwhile, 10 ug of genomic DNA was denatured at 95°C for 10 min., and placed immediately on ice. To each sample, 150 µl of ice-cold 20 x SSC was added. Then the samples were applied to a separate

well of the dot-blot apparatus. When all the wells were emptied, they were washed with 20 x SSC again. Then the apparatus was dismantled, and the filter was baked for 2 hrs at 80°C.

2.2.8.2 Southern blotting of DNA onto nitrocellulose filters

(Southern, 1975)

In the Southern blotting, the transfer of denatured DNA from agarose gels to nitrocellulose membrane is achieved by capillary flow. 20 x SSC buffer, in which nucleic acids are highly soluble, is drawn up through the gel into the nitrocellulose membrane, taking with it the single-stranded DNA which becomes immobilized in the membrane matrix. After electrophoresis of the DNA, the gel was shaken in 0.25 M HCl for the depurination. It was followed by shaking it further in denaturing solution for 30 min, and eventually 45 min in neutralizing solution. The gel was layed on a Whatman filter paper whose ends were in reservoir of 20 x SSC and an equilibrated nitrocellulose filter was placed on the gel. After laying 2 more Whatman filter papers and paper towels, a 500 g weight was also put on the top of the blot. The transfer was carried out overnight. Finally, after disassembling of the blot, the filter was washed shortly in 2 x SSC and the DNA was fixed onto the filter by either baking it at 80°C for 2 hours under vacuum or by UV-crosslinking (120 J; UV Stratalinker 1800, Stratagene, USA).

2.2.8.3 Northern blotting of RNA onto nitrocellulose filters

For the transfer of RNA onto a nitrocellulose filter, the same procedure as above (2.2.6.3) was performed. In this case, however, the gel does not need to be denatured, but is transferred directly onto the filter.

2.2.8.4 Western blotting of protein onto nitrocellulose filters

(Gershoni and Palade, 1982)

Towbin Buffer	25 mM Tris pH 8.3
	192 mM Glycine
	20 % Methanol

After the electrophoresis of proteins on a SDS polyacrylamide gel, the gel and the membrane which was cut at the size of the gel were equilibrated in Towbin buffer for 10 minutes. 2 sheets of Whatman 3MM filter paper were cut and soaked in the transfer buffer too. The gel was placed on these filter papers, and the membrane on the gel avoiding any air bubbles. Another 2 sheets of filter paper were also wet to complete the sandwich model, and it was placed between the pre-wetted fibre pad, and the cassette was closed and placed into the tank. The transfer was carried out either at 90 mA at 4°C overnight or at 200 mA at room temperature for 2-3 hours.For the Western blotting of acid-urea gel, the same procedure was employed except from the transfer buffer which was 0.1 M glycine, pH 3.0.

2.2.9 Hybridization of nucleic acids

The membrane to be hybridized was first equilibrated in 2 x SSC, then transferred to a hybridization bottle. After adding 10 ml of hybridization solution, it was incubated for 2 hours in the hybridization oven at an appropriate temperature which was usually 65°C. Then, the labelled probe and salmon DNA were denatured at 95°C for 10 min and added to the hybridization solution. The hybridization was carried out overnight in the oven. Next day, the filter was washed firstly for 10 minutes with 2 x SSC at room temperature, then with 2 x SSC and 0.2 x SSC at the hybridization temperature. When further washing was needed, finally it washed with 0.2 x SSC containing 0.1 % SDS at the hybridization temperature. After drying the filter, it was sealed in Saran wrap, and exposed to an autoradiogram overnight at -80°C.

2.2.10 Isolation of proteins

2.2.10.1 Isolation of total proteins for CAT assay

100 mg of tissue was homogenized in 500 µl of 0.25 M Tris pH 7.8 with a teflon-glass headed pestle. The cell membrane was destroyed by freezing in liquid nitrogen and thawing at 37°C, repeating three times. The samples were centrifuged at 8000 xg for 10 min at 4°C. The proteins which might interfere with the CAT assay were denatured by incubation at 65°C for 10 min. A final centrifugation at 8000 xg for 10 min at 4 °C was carried out, and eventually the supernatant was distributed in several e-cups, frozen in liquid nitrogen, and stored at – 80°C.

2.2.10.2 Isolation of nuclear proteins

(Deryckere et al. 1994)

Solution A	0.6 % Nonidet P-40
	150 mM NaCl
	10 mM HEPES pH 7.9
	1 mM EDTA
	0.5 mM PMSF (0.5 M stock solution in methanol)
	0.5 mM DTT
Solution B	25 % Glycerol
	20 mM HEPES pH 7.9
	420 mM NaCl
	1.2 mM $MgCl_2$
	0.2 mM EDTA
	0.5 mM DTT
	0.5 mM PMSF
	2 mM Benzamidine
	5 µg/µl Aprotinin
	5 µg/µl Leupeptin
	5 µg/µl Pepstatin

Between 100 and 500 mg of tissue was homogenized in 15-25 ml of solution A with the use of a 50 ml Dounce tissue homogenizer. It was then centrifuged for 1 minute at 800 xg at 4°C to get rid of any unbroken tissue. The supernatant was centrifuged again for 8 min at 3200 xg. The pellet which contained nuclei was dissolved in 50-500 µl of Buffer B, and incubated on ice for 20 minutes in order to lyse the nuclei. After a final centrifugation at 14000 xg for 2 minutes, the nuclear protein extract was distributed into tubes and stored at –80°C.

2.2.10.3 Isolation of nuclear basic proteins

(Alfonso and Kistler, 1993)

2-4 freshly prepared testis were homogenized in 800 µl of 0.25 M HCl in a 1.5 ml microcentrifuge tube with a teflon-glass headed pestle. Proteins were allowed to extract for 20 min on ice, and 24 µl of TCA was added. The precipatate was removed by centrifugation for 5 min at full speed, and the supernatant was transferred to a new tube. Transition proteins were precipitated by the addition of 200 µl of 100 % TCA, and collected by centrifugation as before for 10 min. The faint precipitate was washed with 700 µl of acetone, and let it dry completely. The pellet then was dissolved in 40-60 µl of 0.5 % acetic acid. This fraction was designated the 3-20 % TCA preparation.

2.2.10.4 Isolation of cytoplasmic S-100 proteins

(Dignam *et al*., 1983, modified)

Buffer A	10 mM HEPES pH 7.9
	1.5 mM $MgCl_2$
	10 mM KCl
	0.5 mM PMSF
	0.5 mM DTT
Buffer B	0.3 M HEPES pH 7.9
	1.4 M or 2 M KCl
	0.3 mM $MgCl_2$

Buffer D	20 mM HEPES pH 7.90.
	1 M KCl0.2 mM EDTA
	20 % Glycerol
	0.5 mM PMSF
	0.5 mM DTT

The testes from 5 adult SIV 50 rats were freshly prepared, and shortly washed in cold PBS. 2 testes at a time were homogenized in two Vol of Buffer A by 10 strokes of a B type pestle. The homogenate was distributed into Eppendorf cups, and centrifuged at 2000 rpm for 10 min at 4°C in order to remove the cell debris. The supernatant was transferred this time to ultracentrifuge e-cups, and a salt shock by adding 0.11 Vol Buffer B was applied. After centrifugation for one hour at 45000 xg at 4°C, the supernatant was collected and dialyzed for 2-4 hrs against 20 Vol of Buffer D. Finally, after a short centrifugation at full speed, the proteins were distributed into several e-cups, freezed in liquid nitrogen, and stored at –80°C.

2.2.11 Determination of protein concentration

(Bradford, 1976)

To determine the protein concentration, Bio-Rad protein assay was employed which is a dye binding assay based on the differential color change of a dye in response to various concentrations of protein. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Blue G-250 shifts from 495 to 595 nm when the binding to protein occurs. The BSA stock solution of 1 mg/ml was diluted in order to obtain standart dilutions in the range of 10 μg/ml to 100 μg/ml. The Bio-Rad's color reagent was diluted 1:5 with H_20, and filtered through 0.45 μm filters. In a 96-well microtiter plate, 20 ul of from each standard dilutions and from the samples to be measured were pipetted with 280 μl of the color reagent. The absorption of the color reaction was measured at 595 nm in a microplate reader (Microplate Reader 450, Bio-Rad, Munich).

2.2.12 Reverse transcriptase PCR (RT-PCR)

1-5 ug total RNA was mixed with 1 ul of oligo (dT)18 primer (10 pmol/ul) in a total volume of 11 ul. To avoid the possible secondary structure of the RNA which might interfere with the synthesis, the mixture was heated to 70°C for 10 minutes, and then quickly chilled on ice. After a brief centrifugation, the followings were added to the mixture:

4 ul 5 x First Strand buffer
2 ul 0.1 M DTT
1 ul 10 mM dNTPs
1 ul Rnasin (10 U/ ul)

The content of the tube was mixed gently and incubated at 42°C for 2 min. Then, 1 ul of reverse transcriptase enzyme (SUPERSCRIPT II RNase H - Reverse Transcriptase, GibcoBRL Life Technologies, USA) was added, and further incubated at the same temperature for 1 hr for the first strand cDNA synthesis. Next, the reaction was inactivated by heating at 70°C for 15 minutes. 10 % of the first strand reaction was used for the PCR reaction.

10 ul 10 x PCR buffer
3 ul 50 mM KCl
2 ul 10 mM dNTP mix
1 ul primer 1 (10 pmol/ ul)
1 ul primer 2 (10 pmol/ ul)
1 ul Taq DNA polymerase (5 U/ul)
2 ul cDNA (from first strand reaction)
80 ul H_2O

The reaction was first heated to 94°C for 3 min. for the denaturation of the DNA, then 35 cycles of PCR amplification was performed with appropriate annealing and extension conditions depending on the primers.

2.2.13 Non-radioactive CAT assay

The role of CAT in bacteria is to detoxify the antibiotic chloramphenicol by mono- and diacetylation. CAT activity can therefore be measured by following the conversion of chloramphenicol to its 1-acetyl and 3-acetyl derivates. The mono- and diacetyl derivates of chloramphenicol are separated from unmodified compound by thin layer chromatography on silica gel. Stratagene's Flash CAT nonradioactive CAT assay based on the use of a fluorescent chloramphenicol (CAM) substrate. 50 µg of total protein extract was mixed with 15 µl of BIODIPY CAM substrate reagent and 10 µl of 4 mM acetyl CoA in a total volume of 80 µl, and incubated at 37°C for 4 hrs. Then the reaction was terminated by adding 1.0 ml of ice-cold ethyl acetate. The samples were vortexed and centrifuged at 10000 xg for 5 min. After removing the supernatant, the rest was evaporated for about 90 min. in a vacuum drier. The remaining yellow residue was dissolved in 30 µl of ethyl acetate. Only 5 µl of sample was spotted about 1.5 cm from bottom of the TLC plate. The thin layer chromatography was performed in a closed preequilibrated TLC tank with an 87:13 mixture of chloroform-methanol to depth of 1.0 cm. The result of TLC was visualized under long-wavelength UV light (366-nm).

2.2.14 Primer-extension analysis

(Domenjoud, 1990, modified)

Primer extension analysis is used to determine the location of the 5´-end of specific RNAs. An end-labeled oligonucleotide is hybridized to RNA and is utilized as a primer by reverse transcriptase in the presence of deoxynucleotides. The RNA is thus reverse transcribed into cDNA, which is analyzed on a denaturing polyacrylamide gel. The length of the generated cDNA reflects the number of bases between the labeled nucleotide of the primer and the 5´-end of the RNA.

2.2.14.1 Labelling of the primer

A primer which was estimated to be about 50-200 bp from the transcription start point was labeled with [γ-^{32}P] as follows:

50 pmoles of primer
1.2 µl of Kinase Buffer (10x)
1 µl of T4-Polynucleotide kinase
5 µl of [γ-^{32}P] ATP
in a total volume of 12 µl

The reaction was incubated at 37°C for 45 min, and then stopped with 2 µl of 0.5 M EDTA. After precipitation, the pellet was dissolved in 15 µl of H_2O.

2.2.14.2 Annealing of the primer to RNA

Poly A^+-RNA which was isolated from 100 µg of total RNA was mixed with 2-3x10^6 cpm of labeled primer and 6 µl of hybridization buffer in a total volume of 30 µl. The reaction was immediately placed in a thermoblock which was 85°C for 15 min. It was allowed to cool down till 42°C slowly, and further incubated at this temperature for 2 hrs. Finally, it was precipated by adding 1/10 Vol of 3 M NaAc and 2.5 Vol of 100 % ethanol.

2.2.14.3 Primer extension reaction

To the RNA/oligonucleotide pellet,

12 µl of 5x Reverse transcriptase buffer
12 µl of 5 mM dNTPs
1 µl of RNasin (20 U/ml)
0.5 µl of DTT
2.5 µl Reverse transcriptase (Gibco/BRL)
36 µl $H_2O$was added.

The reaction was incubated at 42°C for 30 min. Then, the samples were precipitated and dissolved in 10 µl of formamid stopmix.

2.2.14.4 Gel electrophoresis

The samples were denatured at 95°C for 5 min. before they were applied on a 8 % sequencing gel. A sequence reaction of a M13 single-stranded DNA was also run so that the size of the reaction product could be determined. The gel was dried and exposed to an X-ray film.

2.2.15 Analysis of DNA-Protein interaction

2.2.15.1 Electrophoretic mobility shift assay (EMSA)

(Johnson et al., 1991, modified) (Fried and Crothers, 1981; Garner and Revzin, 1981)

The electrophoretic mobility shift assay provides a simple and rapid method for detecting DNA-protein binding. The assay is performed by incubating nuclear or cell extract preparations with a ^{32}P end-labeled DNA fragment containing the putative protein binding sites. The reaction products are then analyzed on a nondenaturing polyacrylamide gel. The principle of the procedure is that DNA-protein complexes have different mobilities from uncomplexed DNA during PAGE.

Footprinting buffer	24 mM HEPES pH 7.6
	80 mM KCl
	2 mM EDTA
	1.2 mM DTT
	16 % Glycerol

To avoid unspecific binding, 10 µg nuclear extract was first incubated with 3 µg poly dI.dC on ice for 15 min. In the case of competition experiments, the unspecific and/or specific competitor DNA was added to the mixture, and further incubated for 15 min. Next, 10000 cpm of labelled oligonucleotide was added and the incubation on ice was continued for 30 min. Finally, the samples were applied to 8 % polyacrylamide gel. After electrophoresis of about 3 hrs, the gel was dried for 45 min and exposed to an autoradiogram overnight at -80°C.

2.2.15.2 Southwestern analysis

(Dai *et. al*., 1990, Miskimins, *et. al*., 1985, modified)

Binding Buffer	10 mM HEPES pH 8.0
	50 mM NaCl
	10 mM $MgCl_2$
	0.1 mM EDTA
	1 mM DTT
	0.25 % Dry milk

40-60 μg of nuclear proteins were separated on 10 % SDS-polyacrylamide gel, and transferred overnight at 4 °C. The filter was incubated in 5 % dry milk in 10 mM HEPES, pH 8.0, for 2 hrs at room temperature. The binding reaction of radioactively labelled oligonucleotides with immobilized proteins was carried out in 15 ml of binding buffer which contains 3 μg of poly dI.dC as the unspecific competitor and 10^5 cpm of ^{32}P labelled oligonucleotide for 2-3 hrs at room temperature. Finally, the filter was washed two times for 5 min in 10 ml of binding buffer.

2.2.16 Analysis of RNA-Protein interaction

2.2.16.1 RNA-affinity chromatography

(Gu *et. al*., 1996 ; Wu *et. al*., 1997, modified)

RNA Binding Buffer	25 mM HEPES pH 7.5
	100 mM KCl
Washing Buffer A	20 mM HEPES
	40 mM KCl
	5 mg/ml Heparin
	50 μg/ml tRNA
Washing Buffer B	20 mM HEPES
	40 mM KCl
	0.5 % Nonidet P-40

Washing Buffer C	20 mM HEPES 40 mM KCl
Washing Buffer D	20 mM HEPES 100 mM KCl
Elution Buffers	0.5 M / 1 M / 2 M / 3 M KCl in 20 mM HEPES
Dialyse Buffer	10 mM HEPES 40 mM KCl 3 mM Mg_2Cl_2 1 mM DTT 0.5 mM PMSF

Polyadenylated RNA transcripts were generated *in vitro* with the T7 MEGAscript kit (Ambion, USA) from the lineralized plasmid carrying the insert to be transcribed. 500 µl of poly(U)-agarose beads (type 6; Pharmacia) was resuspended in 1 ml RNA binding buffer and packed into a 2-ml column. 40 µg of the *in vitro* transcribed polyadenylated transcript was added to the column at 4°C and recycled at least 5 times. The efficiency of the binding of RNA to the poly(U)-agarose beads was determined by analyzing the flow-throughs on an agarose gel. Then the column was equilibrated with the protein extraction buffer, followed by addition of about 30-40 mg of testicular cytoplasmic extract. The column was then stored and shaked gently at room temperature for 1 hr to enhance the binding of cytoplasmic proteins to the given transcript. The protein extract contained 60 U/ml RNasin to inhibit endogenous RNase activity, and to minimize the nonspecific binding, heparin and yeast tRNA at final concentrations of 5 mg/ml and 40 µg/ml, respectively, were added. To pellet the agarose beads, the column was centrifuged at 1000 rpm for 5 min. The following steps of washing of the column and elution of the proteins were again carried at 4°C. The column was washed extensively with 2 ml of washing buffer B, buffer C, and finally buffer D. Bound proteins were step eluted with 200 µl of 0.5, 1, 2, and 3 M KCl. The eluted fractions were dialyzed for 2 hrs at 4°C.

2.2.16.2 Northwestern analysis

(Houman et al., 1990; Kwon and Hecht, 1991, modified)

Renaturation Buffer	50 mM Tris, pH 7.6
	50 mM NaCl
	1 mM EDTA
	1 % BSA
	1 mM DTT
Binding Buffer	10 mM Tris, pH 7.6
	50 mM NaCl
	1 mM EDTA
	1 mM DTT

After separation of proteins on a 10 % SDS polyacrylamide gel, and transferring onto a nitrocellulose filter, the filter was gently shaked in renaturation buffer at room temperature for 4 hours. It was then shortly equilibrated with the binding buffer, and hybrized with 100000 cpm radioactively labelled in vitro transcript at room temperature for one hour. Finally, the filter was washed 2 times for 10-20 min. again in binding buffer, dried, and exposed to an X-ray film at –70°C overnight.

2.2.17 Immunodetection

AP Buffer	100 mM Tris/HCl pH 9.5
	100 mM NaCl
	5 mM $MgCl_2$

After Western blotting, the membrane was incubated in 15 ml of 140 mM NaCl, 10 mM Tris/HCl, pH 7.5, 0.05 % Tween containing 5 % Milk powder for 2 hrs at room temperature. Primary serum which was diluted 1:10000 was added to the mixture and incubation continued for 2 hrs, then the membrane was washed 2 times for 10 min in the same buffer. Upon washing, the blot was incubated with the appropriate alkaline phosphatase-conjugated second antibody which was goat anti-rabbit Ig G in the case of Tnp2 for 2 hrs. The second antibody

was also diluted 1:10000. Finally, the blot was washed again for 10 min. in blocking buffer. The blot was developed in 10 ml of AP buffer containing 132 ul of NBT 50 mg/ml and 66 ul of BCIP 50 mg/ml.

2.2.17 Histological techniques

2.2.17.1 Tissue preparation for transmission electron microscopy (TEM)

Fixation Solution	0.01 M $NaH_2PO_4.H_20$
	0.04 M $Na_2HPO_4. H_20$
	2.5% Glutaraldehyde

The freshly prepared tissue was incubated in 5 ml of fixation solution overnight at 4°C.

2.2.17.2 Tissue preparation for paraffin-embedding

Bouin's Solution	15 ml Picrin acid
	5 ml 37% Formaldehyde
	1 ml Acetic acid

The freshly prepared testis was fixed in Bouin's solution for 2-4 hrs to prevent the alterations in the cellular structure. The tissue to be embedded in paraffin should be free of water. The dehydration process was accomplished by passing the tissue through a series of increasing alcohol concentrations. For this purpose, the tissue was let in 30%, 70%, 90%, and 100% (2x) ethanol for one hour at room temperature. Later, the alcohol was removed from the tissue by incubating it in methyl benzoat overnight. It was then incubated in 5 ml of roti-clear (Xylol) for 10-30 min at room temperature. The second roti-clear was not discarded but 5 ml of paraplast was added and the incubation was continued at 60°C for another 30 min. The roti-clear and paraffin mixture was discarded, and the tissue was further incubated in 5 ml of paraplast at 60°C overnight. Before embedding, the paraffin was changed at least three times. Finally, the tissue was placed in embedding mold and melted paraffin was poured into the mold to form a block. The block was allowed to cool and was then ready for sectioning.

2.2.17.3 Peroxidase anti-peroxidase technique (PAP)

Enzyme anti-enyzme soluble complexes have been routinely employed in histochemical techniques. The soluble complex of peroxidase anti-peroxidase (PAP) consists of three peroxidases and two anti-peroxidase subunits in a cyclic structure.The sections were shortly heated to 60°C in order to melt paraffin, and placed in xylol bath for 30 min at 60°C. Before successive ethanol concentrations, they were again incubated in xylol for 30 min at room temperature. Then, the sections were placed in 100% ethanol for 10 min twice, 95%, 80%, 70% ethanol for 5 min. After they were washed in 5-10 min in TBS, the endogenous peroxidase was blocked by the incubation in 3% H_2O_2 in TBS for 15 min. After washing them once more in TBS for 5-10 min, they were incubated with 7.5 ug Proteinase K for 10 min, and then with 0.1 M Glycin in TBS for 5 min. The incubation with primary antibody which is anti-Tnp2 antibody (1:100 diluted) in our case was accomplished at 37°C for 30 min. After washing shortly with TBS, the sections were this time incubated with the 'bridge antibody' for 30 min at 37°C. The incubation with PAP complex (1:150 diluted) was carried out at 37°C for 30 min. The substrate DAB was added to allow the detection of formed complexes.

3. RESULTS

3.1 Transcriptional regulation of rat Tnp2 gene

To investigate the promoter sequence responsible for the testis- and spermatid-specific expression of rat Tnp2 gene, transgenic mice with 147 nts genomic fragment of the 5' regulatory region fused with the *E. coli* CAT gene as the reporter gene were generated. For the further analysis of this region, *in vitro* studies such as primer extension analysis and southwestern analysis were carried out.

3.1.1 Primer extension analysis

To determine the transcription start point for rat Tnp2 gene, we carried out primer extension analysis by using a synthetic oligonucleotide with both testis and brain total and poly $(A)^+$ RNA. For the cDNA synthesis, a reverse primer RTP2-PE1 (2.1.5) which is located 78 nts downstream of the translational start codon (ATG) was chosen, and the reaction was carried out on RNA from testis and brain. To estimate the length of the product, a sequencing reaction of M13 phage clone was also run on the gel. The reaction with testis RNA yielded a single product which was 148 nts in length, corresponding to a transcription start site of 70 nts upstream of ATG. As expected, with brain RNA no product was detected as the gene is not expressed in brain but only in testis. A consensus TATA box (TATATAA) could be identified in an expected distance from the transcription start point, which is 24 nts upstream of the transcription start point.

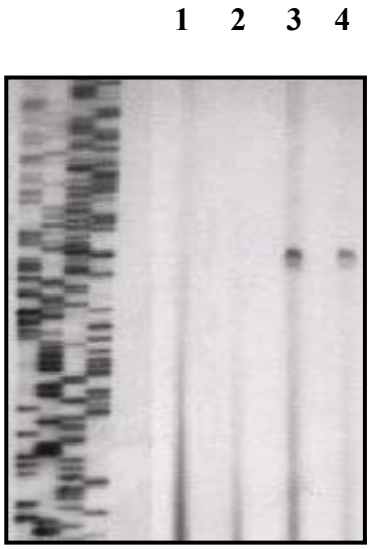


Fig.1 Determination of the transcription start point by primer extension analysis.
Primer extension reactions were carried out with total RNA and poly $(A)^+$ RNA from testis and brain. The reactions on total RNA and poly $(A)^+$ RNA from brain served as the negative controls. A product of 148 nts was detected with the total (3) and poly $(A)^+$ RNA (4) from testis but not with the total (1) or poly $(A)^+$ RNA (2) from brain.

3.1.2 Generation of Tnp2-147 transgenic mouse line

3.1.2.1 Plasmid construction for the transgenic mice

The fusion construct of 1.8 kb which contained 147 nts of 5‘ untranslated region of Tnp2 gene, 650 nts of CAT reporter gene, 3' UTR of rat Tnp2 gene which is 150 nts, and a 850 nts genomic fragment of the 3' flanking region of the Tnp2 gene was used for the transgenic mice.
The 147 nts genomic fragment was amplified by PCR reaction by using modified forward and reverse primers, Tppro1 and RTP2-5A (2.1.5), which contained Sst I and Bam HI recognition sites, respectively. After amplification, the product was digested with Sst I and Bam HI restriction enzymes. For the cloning of this fragment, the RTnp2-525 vector which was kindly provided by Dr. G. Schlüter, Institute of Human Genetics, Göttingen, was used. This construct already contained the CAT gene, 3' UTR and 3' flanking region from rat Tnp2 gene, therefore it was partially digested with Sst I and Bam HI to exchange the 525 nts 5' UTR fragment with

the shortened promoter sequence of 147 nt Sst I/Bam HI fragment. For the microinjection, 1.8 kb Sst I/Kpn I fragment was used.

By using this construct, the region of –74 to + 73 of the 5' untranslated region of rat Tnp2 gene could be analyzed in transgenic mice.

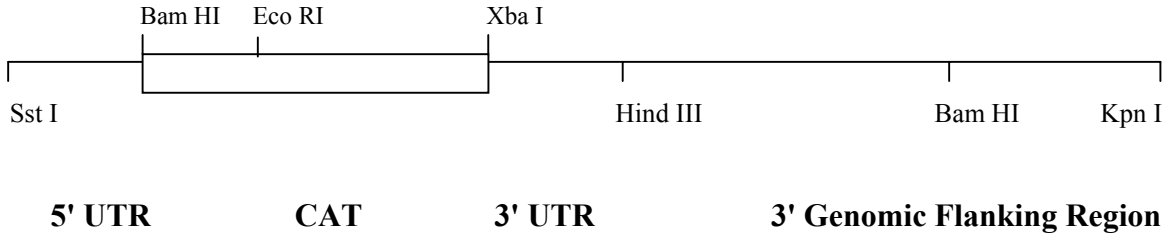


Fig.2 Structure and the restriction map of the microinjected Tnp2-147 fusion construct.
The construct consists of 147 bp 5' UTR of Tnp2 gene relative to translation start site, CAT gene as the reporter gene, followed by 3' UTR and 3' flanking region of rat Tnp2 gene, and enabled us to analyze the region of -74 to +73 of Tnp2 gene in transgenic mice.

GGGGCAGGCTTTGCCATACCTGTCACCCTCTGTGGCCTGTGCCATCATAAT
Tppro1-F
-24 +1
TGGCCCAGCTATATAACTAGGGGCTCAGAGGACTCCCCAAAGCGAGGCCTGCTG
TATA-Box Tsp
GGAGAAGAGGAGGAGGAGGAAGTCTCTGCCCCTCCGAGTGTGGCCTCCCATGG
RTP2-5A ATG
ACACCAAGATGCAGAGCCTTCCCACCACTCATCCCCACCCGCACAGCTCCTCT
CGGCCTCAAAGTCACACCAATAACCAGTGTGCCTGCAGCCACCAC
RTP2-PE1

Fig.3 Nucleotide sequence of 5' regulatory region of rat Tnp2 gene (Accesion no. Z46939).
The reverse primer, RTP2-PE1, which is located 78 nts downstream of the translation start codon was chosen for the primer extension analysis. By means of primer extension analysis, a transcription start point at -70 position relative to translational start codon could be identified.

A consensus sequence of TATA box could be found 24 bp upstream of the transcription start point. Tppro1-F and RTP2-5A primers were used to amplify the region of -74 to +73 according to the result of primer extension analysis to be used in transgenic mice.

3.1.2.2 Genomic integration of the transgene

Southern blot analysis of genomic DNA isolated from tail biopsies from microinjected mice yielded 2 animals that were positive for the transgene. 10 μg of genomic DNA was digested with Eco RI, separated on an 1% vertical agarose gel, blotted and probed with radioactively labeled CAT probe. The expected 1.3 kb band due to 2 Eco RI sites in the construct and a variable band could be detected for 2 founder animals. Transgenic lines were established by breeding male founders with non-transgenic females. However, only one line of transgenic mice could be established as in the other line the transgene was not transmitted to the next generation. After breeding heterozygous female transgenic mice with heterozygous male mice, homozygous mice were obtained. The homozygosity of the offsprings were tested by back-cross test which is the breeding of selected offsprings that were identified by a strong signal by dot-blotting with wild type mice. The offprings from this breeeding were also analyzed by dot-blotting, and the parent mice which produced offsprings which were all positive for transgene were identified as homozygous mice.

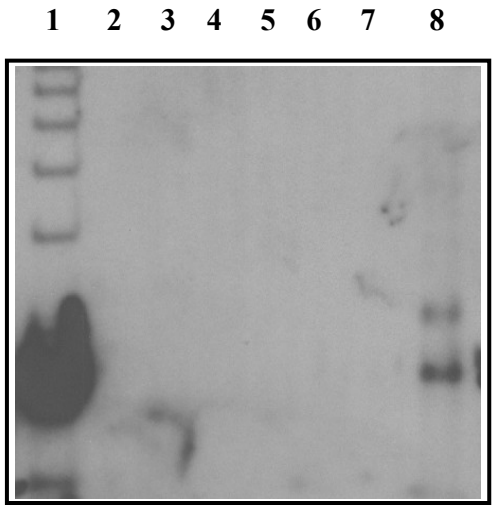


Fig.4 Southern blot of genomic DNA from several founder (Fo) animals.

The genomic DNAs were digested with Eco RI and hybridized with the CAT probe. Out of 20 founder animals, 2 were found to be positive for the construct. In this panel, founder animal #6 (lane 8) was identified as a positive founder animal whereas the other 5 animals (lane 2-7) were negative. 1 Kb ladder was run to estimate the length of the hybridization signal (lane 1).

3.1.2.3 The testis-specific expression of the transgene

To determine the tissue-specific expression and the transcriptional activity of the transgene, total RNA was prepared from various adult tissues from a homozygous transgenic mouse, and hybridized with a CAT probe. Only in testis, the transcript of expected size of about 1 kb could be detected. However, no transcript was detected with the RNA samples from brain, heart, lung, kidney and skeletal muscle tissues.

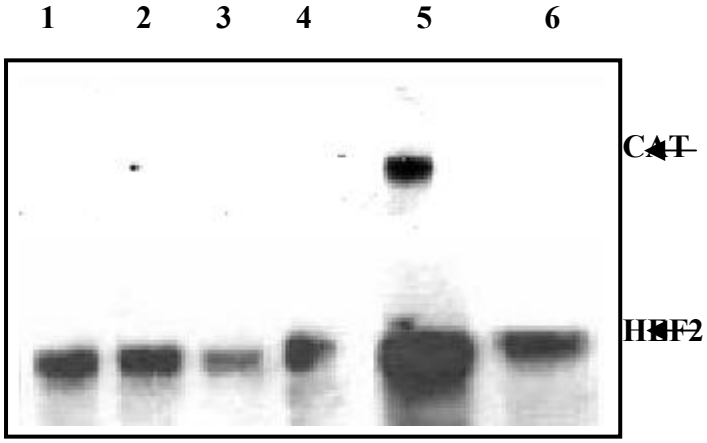


Fig.5 Northern blot analysis of various tissues from Tnp2-147 homozygous transgenic mouse.

Total RNA (15 ug per lane) was prepared from brain (1), heart (2), lung (3), kidney (4), testis (5), and skeletal muscle (6) tissues of homozygous adult mice and separated on an RNA-agarose gel. The blot was hybridized with the 650 bp CAT fragment and rehybridized with HEF to show the integrity of the RNA samples.

3.1.2.4. The testis-specific expression of transgenic CAT protein

In order to investigate the CAT enzymatic activity in Tnp2-147 transgenic line, different tissues were analyzed by non-radioactive CAT assay. The CAT activity can be measured by following the conversion of chloramphenicol to its 1-acetyl and 3-acetyl derivates. After the separation of reactions by thin layer chromatography, the presence of CAT enzyme activity was detected only in testis but not in brain, heart, lung, liver, spleen, kidney, ovary, uterus, skeletal muscle.

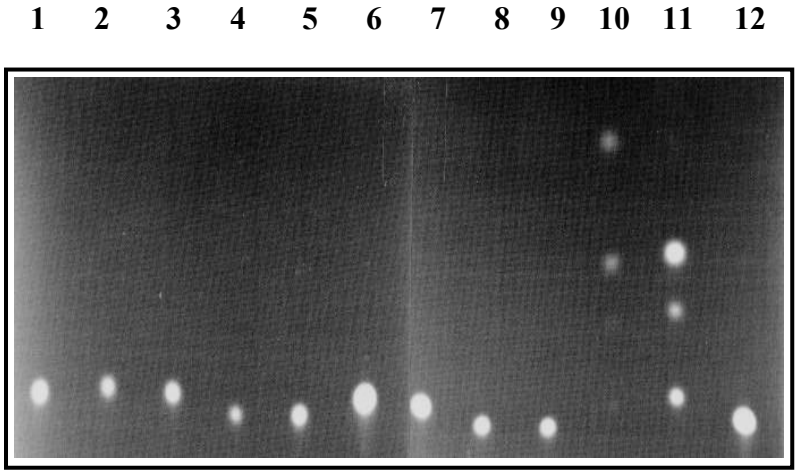


Fig.6 Non-radioctive CAT assay was employed to show the CAT enzymatic activity in Tnp2-147 homozygous mice.

50 µg total protein extract from a number of tissues, such as brain (1), heart (2), lung (3), liver (4), spleen (5), kidney (6), ovary (7), uterus (8), skeletal muscle (9), and testis (10) were used to perform the CAT assay. Only in testis, CAT enzyme activity could be detected but not in other tissues analyzed. CAT enzyme (11) was included as positive control and testicular protein extract from a non-transgenic mouse (12) as negative control.

3.1.2.5 The expression of the transgene during testis development

The exact timing of the expression of the transgene in testis was further investigated by developmental northern blot analysis. For this purpose, testis RNA was isolated from various postnatal stages of homozygous mice, and hybridized with the CAT probe. The first CAT transcript was detected at day 25 which corresponds to the spermatid-stage.

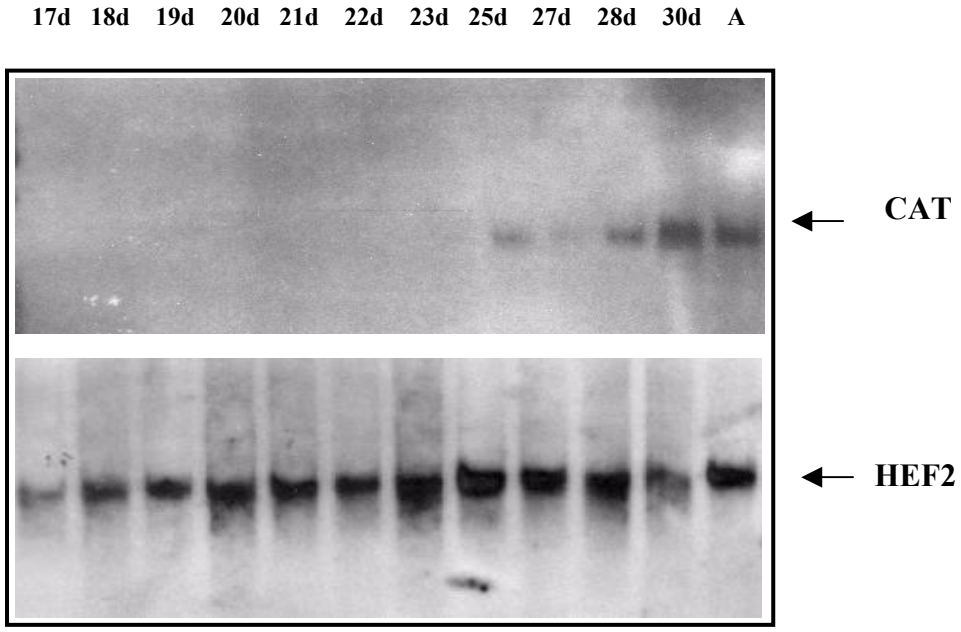


Fig.7 Northern blot analysis by using RNA from various postnatal stages obtained from homozygous mice to show the expression of CAT reporter gene.

15 μg total testis RNA isolated from various postnatal stages of homozygous Tnp2-147 transgenic mice was separated on an RNA-agarose gel, blotted and probed with the 650 bp CAT probe. To show the integrity of the RNA samples, the blot was rehybridized with HEF probe.

In order to compare the expression of the transgene mRNA with the expression of endogenous Tnp2 mRNA in the transgenic line Tnp2-147, the developmental northern blot from homozygous mice was hybridized with the Tnp2 cDNA probe. The first endogenous Tnp2 RNA in the transgenic line was detected at day 23.

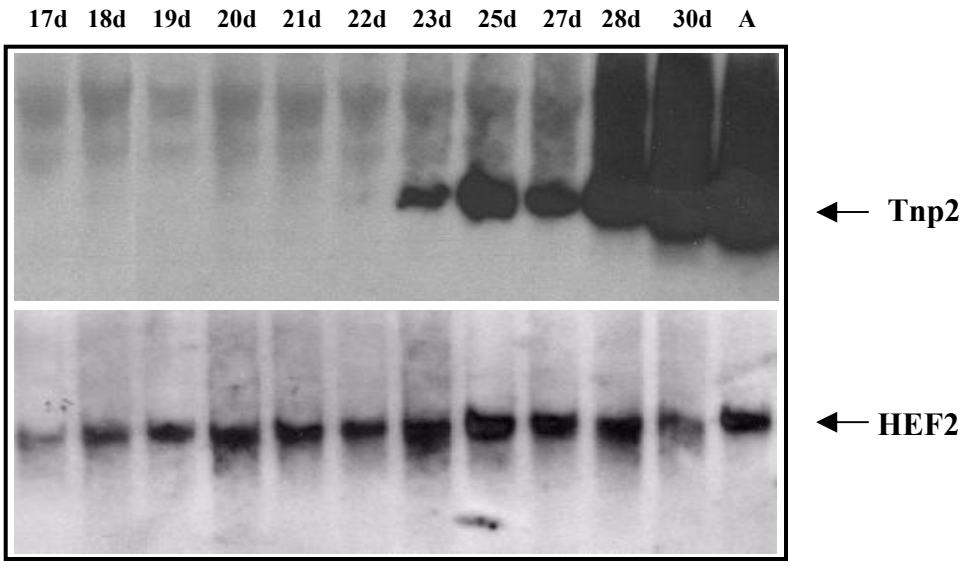


Fig.8 Northern blot analysis by using RNA from various postnatal stages obtained from homozygous mice to show the endogenous Tnp2 mRNA.

15 μg total testis RNA isolated from various postnatal stages of homozygous Tnp2-147 transgenic mice was separated on an RNA-agarose gel, blotted and hybridized with the Tnp2 cDNA probe.

3.1.2.6 The expression of transgenic CAT protein during testis development

The expression profile of transgenic CAT protein during testis development in RTnp2-147 transgenic mice was analyzed by CAT assay. For each reaction, 50 μg of total testis protein extract of various postnatal stages from homozygous transgenic mice were used. Reactions were separated by thin layer chromatography and visualized by UV-light. The presence of CAT protein was observed at day 25 which reflects the translation of trangenic CAT mRNA at haploid stage.

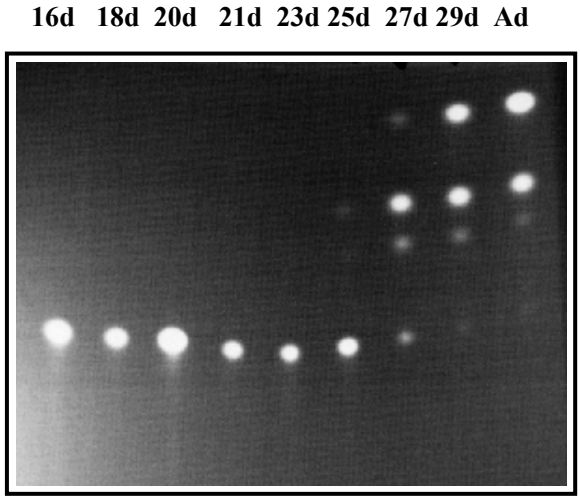


Fig.9 Non-radioactive CAT assay by using total testicular protein extracts from different postnatal stages of Tnp2-147 homozygous mice.

50 ug of total testis protein extract from various development stages of homozygous transgenic mice were used to perform CAT assay. The presence of CAT protein could be detected first at day 25 which corresponds to the translation of the reporter gene at haploid stage.

3.2 Translational regulation of rat Tnp2 mRNA

To investigate the role of 3' UTR in translational control of Tnp2 gene, we generated transgenic mice harboring a construct of 5' UTR of Tnp2 gene, CAT reporter gene and SV40 splicing and polyadenylation signal sequences. Secondly, the role of 3' UTR in translational repression of Tnp2 mRNA and the importance of this process in spermiogenesis were aimed to be examined by the transgenic mice line that carry the construct where the 3' UTR of Tnp2 gene was exchanged with 3' UTR of human growth hormone (hGH) gene.

3.2.1 In vivo studies

3.2.1.1 Generation of Tnp2-SV40 transgenic mouse line

3.2.1.1.1 Plasmid construction for the transgenic mice

For the generation of transgenic mice, a fusion construct of 2.1 kb which contained 525 nts genomic fragment of 5‘ untranslated region of rat Tnp2 gene, 650 nts of CAT reporter gene, and 950 nts of SV40 splice and polyadenylation sequences was used.
The 525 nt long Sst I/Bam HI 5' genomic fragment of rat Tnp2 gene was cloned in pBLCAT3 vector which readily contained CAT and SV40 sequences. For the microinjection, 2.1 kb Sst I/Sst I fragment was used.

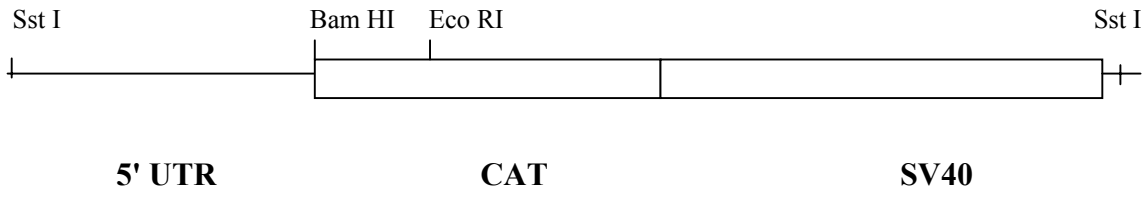


Fig.10 Structure and restriction map of the microinjected Tnp2-SV40 fusion construct.
The 2.1 kb SstI/Sst I fragment which was used for the generation of trangenic mice contained 525 bp 5' UTR of rat Tnp2 gene, 650 bp of CAT reporter gene and 950 bp of SV40 splicing and polyadenylation sequence.

3.2.1.1.2 Tissue-specific expression of the transgene

To determine the transcriptional activity and the tissue-specific expression of the transgene, total RNA was prepared from several tissues of a heterozygous transgenic mouse, and hybridized with the 1.6 kb CAT-SV40 probe. In none of the analyzed tissues, such as brain, heart, lung, kidney, spleen, testis, and skeletal muscle, the presence of the expression of the transgene could be detected.

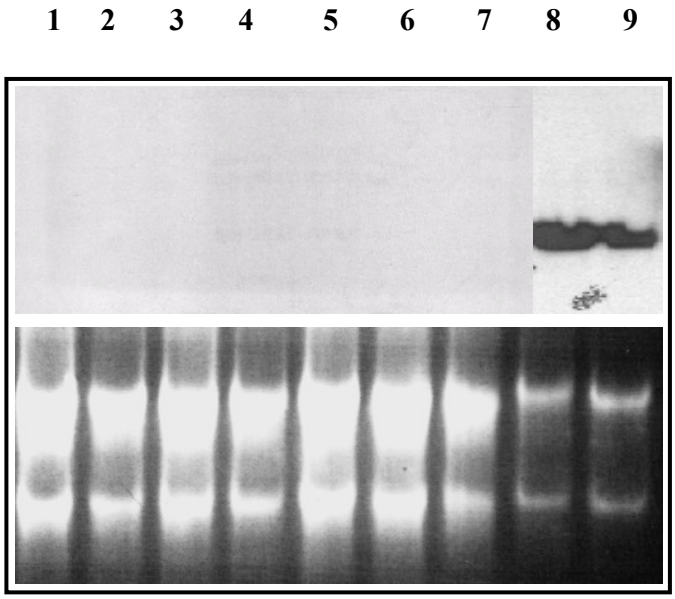


Fig.11 Northern blot analysis of various tissues from Tnp2-SV40 transgenic mouse.
15 µg of total RNA from brain (1), heart (2), lung (3), kidney (4), spleen (5), testis (6), and skeletal muscle(7) tissues of a heterozygous transgenic mouse were used to perform the northern blot analysis. RNA samples from two different transgenic mice lines which carry CAT reporter gene were included as positive controls (8,9). RNA samples were separated on a 1% agarose gel, blotted and hybridized with the CAT-SV40 probe. The integrity of RNA samples are shown by EtBr staining of the RNA gel.

3.2.1.1.3 Tissue-specific expression of the transgenic CAT protein

To be able to determine the CAT enzyme activity in Tnp2-SV40 transgenic line, total protein was isolated from various tissues of a heterozygous transgenic mouse, such as brain, heart, lung, liver, kidney, and testis. 50 µg total protein extract was used to perform CAT assay, then

reaction samples were separated by thin layer chromatography, finally visualized by UV-light. However, in none of the tissues analyzed, CAT enzyme activity could be detected.

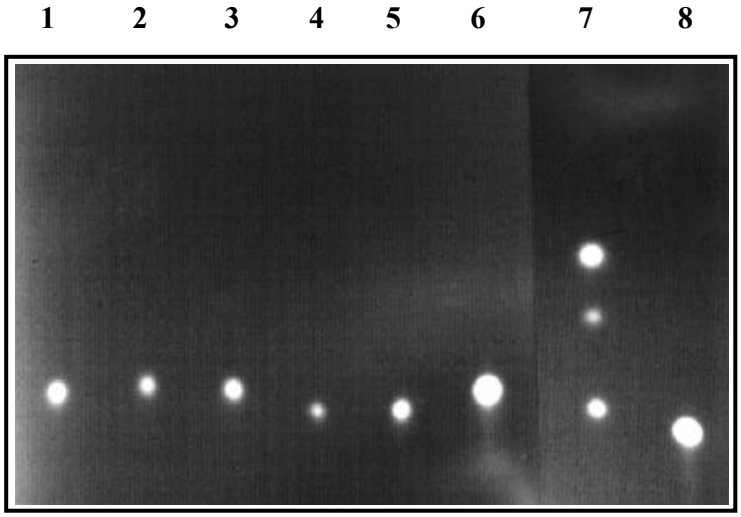


Fig.12 Non-radioactive CAT assay for Tnp2-SV40 transgenic mice line.
50 µg of total protein extract from brain (1), heart (2), lung (4), liver (5), kidney (6), and testis(8) of a heterozygous Tnp2-SV40 transgenic mouse were used to carry out CAT assay. No CAT enzymatic activity could be detected in any of the tissues used. CAT enzyme supplied by the manufacturer was included as positive control (7).

3.2.1.2 Generation of Tnp2-hGH transgenic mouse line

3.2.1.2.1 Plasmid construction for the transgenic mice

The 1.2 kb transgene contained 525 nts 5 'UTR of rat Tnp2, the complete coding sequences, exon 1 and exon 2 which are 325 nts and 18 nts, respectively, intronic sequence of 192 nts, and the 160 nts of 3' noncoding region of human growth hormone (hGH) including polyadenylation signal.
The 897 nts long Bgl I/Kpn I fragment which contained the 550 nts 5' UTR and exon 1 of Tnp2 was isolated from a rat cosmid clone which was kindly provided by Dr. G. Schlüter, Institute of Human Genetics, Goettingen. Intronic sequence and exon 2 were amplified by PCR by using modified primers having Kpn I and Eco RI cleavage sites. 3' UTR of hGH was amplified by PCR on human genomic DNA by again using modified primers having the

recognition sites for Eco RI and Hind III. These three fragments were successively cloned into pRSetA vector which was digested by Bgl I and Hind III. For the microinjection of the fertilizated mouse oocytes, the 1.2 kb Sst I/Hind III fragment was used.

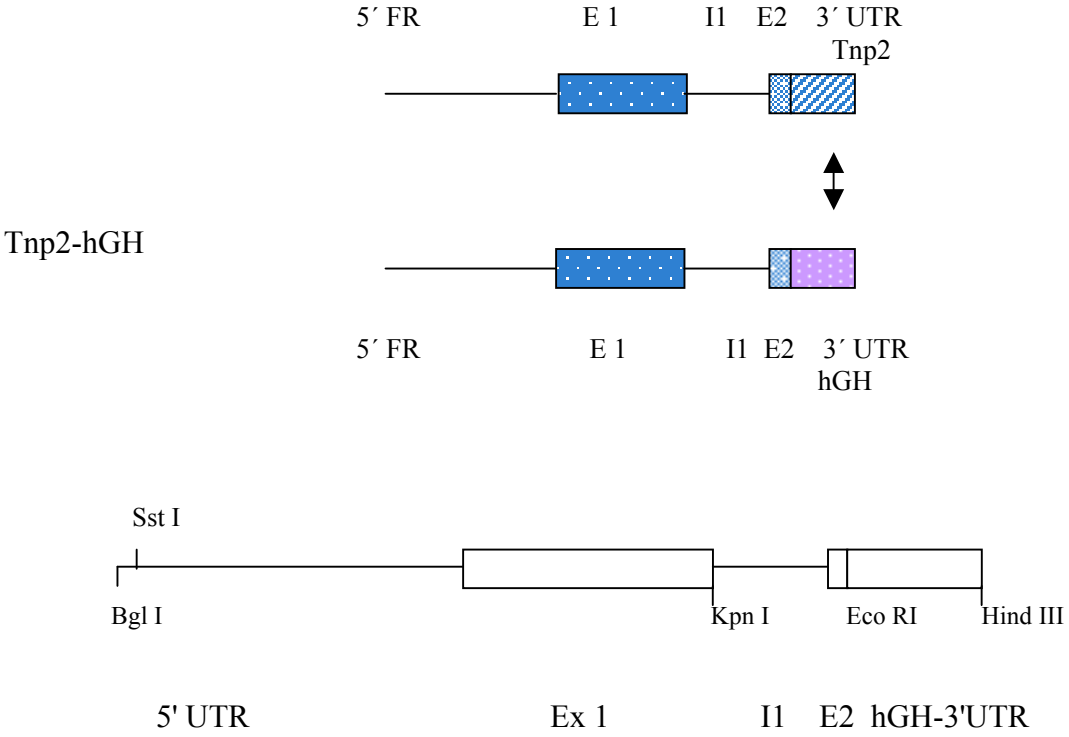


Fig.13 The structure and the restriction map of the fusion construct used in the generation of Tnp2-hGH transgenic mice.

The fusion construct contains 525 nts 5' UTR, coding and noncoding regions of rat Tnp2 gene, and the complete 160 nts of human growth hormone (hGH) 3' UTR.

CTGCCCGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGT

hGH-3'UTR-F

TGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAATTAAGTTGCATCATTTTGC

Poly-A signal

TGACTAGGTGTCCTTCTATAATATTATGGGGTGGAGGGGGGTGGTATGGAGCA

hGH-3'UTR-R

Fig.14 Nucleotide sequence of 3' UTR of human growth hormone (hGH) (Accession no. M13438).

160 bp 3' UTR of hGH was amplified by PCR using modified primers having Eco RI and Hind III recognition sites on human genomic DNA.

3.2.1.2.2 Genomic integration of the transgene

For the identification of the positive founder animals which carry the transgenic construct, the genomic DNAs that were isolated from mouse tails were analyzed by dot-blotting and the membrane was hybridized with 160 nts 3' UTR of hGH. Then, the transgenity of the positive founder animals was confirmed by southern blotting and hybridization. The genomic DNAs from F_0 animals were double digested with Sst I and Hind III overnight, separated on 1% vertical agarose gel, blotted and finally hybridized with 3' UTR of hGH probe which was labeled with ^{32}P. Out of 31 animals, one of them was identified as a positive founder animal by dot-blot analysis, and the integration of the construct into the genome was verified by southern blotting as well.

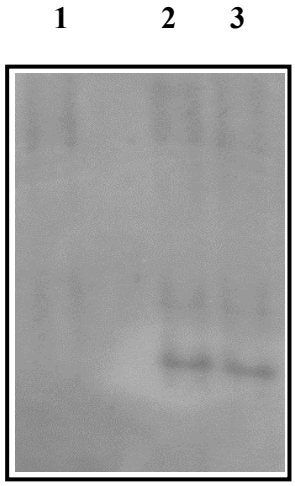


Fig.15 Southern blot analysis of genomic DNA from founder and F1 transgenic mice.
10 µg genomic DNA from non-trangenic (lane 1), transgenic founder (F_0) animal (lane2) and transgenic F1 animal (lane 3) were digested with Sst I and Hind III, and hybridized with radioactive labeled 3' UTR of hGH. The presence of 1.3 kb hybridization signals confirmed the result obtained from dot-blot analysis.

For the establishment of the transgenic line, the positive male founder mouse was bred with wild type NMRI female mice to obtain heterozygous mice. Out of 43 offsprings comprising 18 female and 25 male mice, none of the female progeny was found to be positive for the

construct whereas all of the male progenies were identified as positive. This result indicated the Y-chromosomal integration of the transgene for Tnp2-hGH construct.

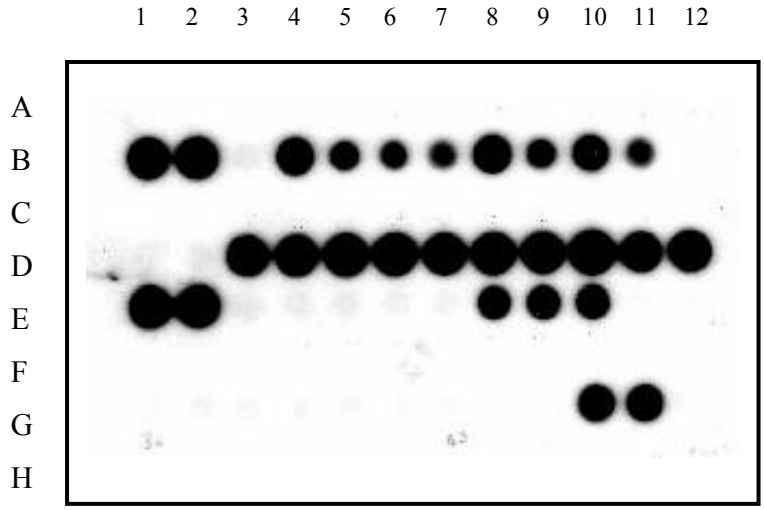


Fig.16 Dot-blot analysis of genomic DNA from F1 generation animals of Tnp2-hGH transgenic line.

43 offsprings of F1 generation from breeding of positive founder mouse with wild type female mouse were analyzed by dot blotting. 10 μg of genomic DNA was transferred on a membrane by dot-blotting, and hybrized with ^{32}P labeled hGH-3' UTR probe. None of the female F1 offsprings, such as B3, B12, D1-2, E3-7, E11-12, G1-7, was found to be positive for the construct whereas all of the F1 male offsprings were carrying the construct.

3.2.1.2.3 Tissue-specific expression of the transgene

In order to investigate the transgene expression in different tissues, northern blot analysis was carried out. RNA from brain, heart, lung, liver, kidney, spleen, testis and muscle tissues from a transgenic male mouse was isolated, separated on vertical agarose gel, blotted and hybridized with 160 nts 3' UTR of hGH probe which only detects the Tnp2 transcript originating from the transgenic construct.

The presence of about 1 kb transcript in testis showed the expression of the transgene only in testis but not in other tissues examined such as brain, heart, lung, liver, kidney, spleen and

skeletal muscle. The filter was then rehybridized with the human elongation factor (HEF) probe to show the integrity of the RNA samples.

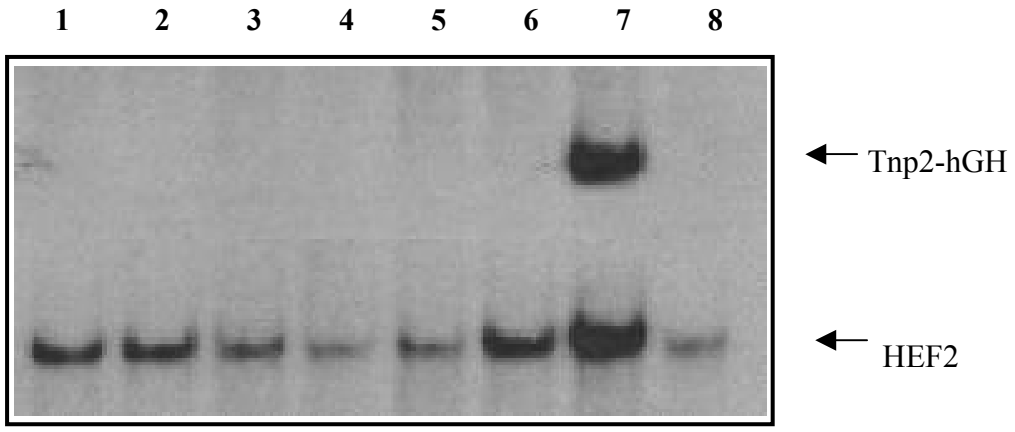


Fig.17 Northern blot analysis to show the testis-specific expression of the transgene.
RNA from various tissues of a transgenic Tnp2-hGH male mouse was isolated, separated on 1% vertical RNA-agarose gel, blotted and hybridized with radioactively labeled 3'UTR hGH probe. No transcript could be detected in brain (1), heart (2), lung (3), liver (4), kidney (5), spleen (6) and skeletal muscle (8), but only in testis (7). The filter was later rehybridized with HEF probe. The RNA-ladder from GIBCO/BRL was used to estimate the size of the transcript.

3.2.1.2.4 The expression of the transgene during testis development

During postnatal development in the male mouse, the first spermatocytes in meiotic prophase would be observed between days 10 and 12 after birth. At 15 to 17 days, 30 % of tubules contain cells in late pachytene, and at 20 to 22 days, spermatids can be observed for the first time in about 35 % of tubules (Austin and Short, 1982). Thus, by analyzing the testis at 3 different stages during the first 3 weeks of life, an indication can be obtained of developmental stage at which a transcript is first formed.We analyzed the expression profile for Tnp2-hGH mRNA in transgenic mice by northern blot in the range of 17 to 31 days. The first transcript could be detected at day 24 which corresponds to the spermatid-specific expression.

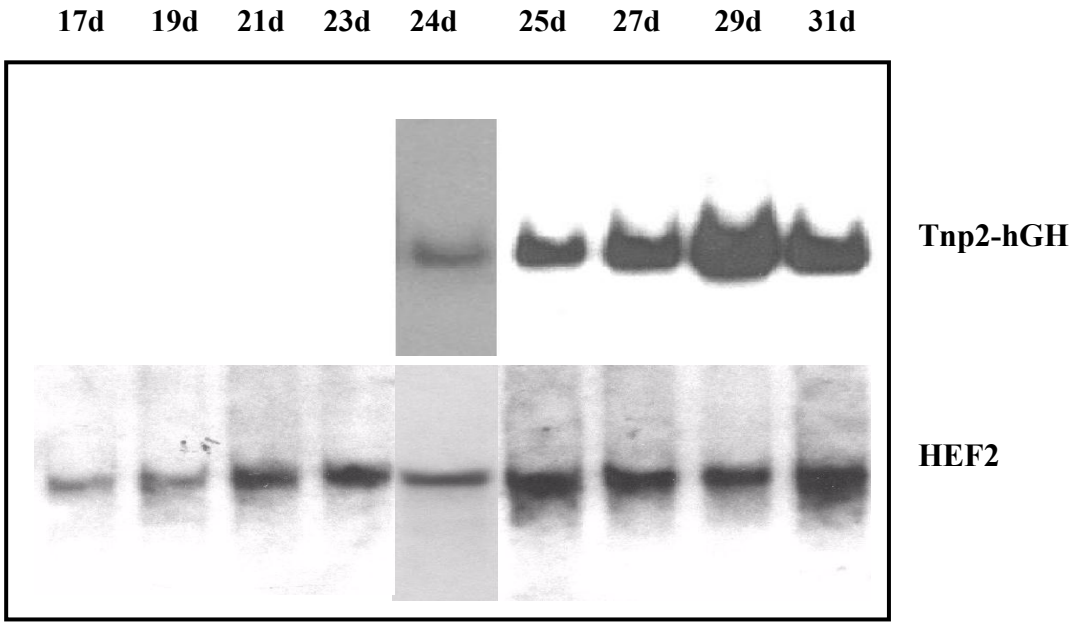


Fig.18 Developmental northern blot analysis to show the spermatid-specific expression of the transgene.

20 μg testis RNA isolated from various postnatal stages was separated on 1% RNA-agarose gel, blotted, and hybridized with 3' UTR hGH probe. The first hybridization signal was detected at day 24. The blot was rehybridized with 1.6 kb HEF probe to demonstrate the RNA integrity.

The expression pattern of endogenous Tnp2 gene in Tnp2-hGH transgenic mice was analyzed by RT-PCR. For this purpose, 5 μg of total RNA isolated from various stages of Tnp2-hGH transgenic mice were used to carry out reverse transcription reaction with an oligo$(dT)_{17}$ primer. Second PCR amplification was performed by two Tnp2 gene-specific primers and yielded a 343 bp PCR product. The first endogenous Tnp2 transcript could be detected at day 23. Contamination of genomic DNA produced a 533 bp product if no or few Tnp2 mRNA was present (21d, 23d). At higher Tnp2 transcript levels, amplification of the smaller product from RNA templates outcompeted reaction from the genomic templates.

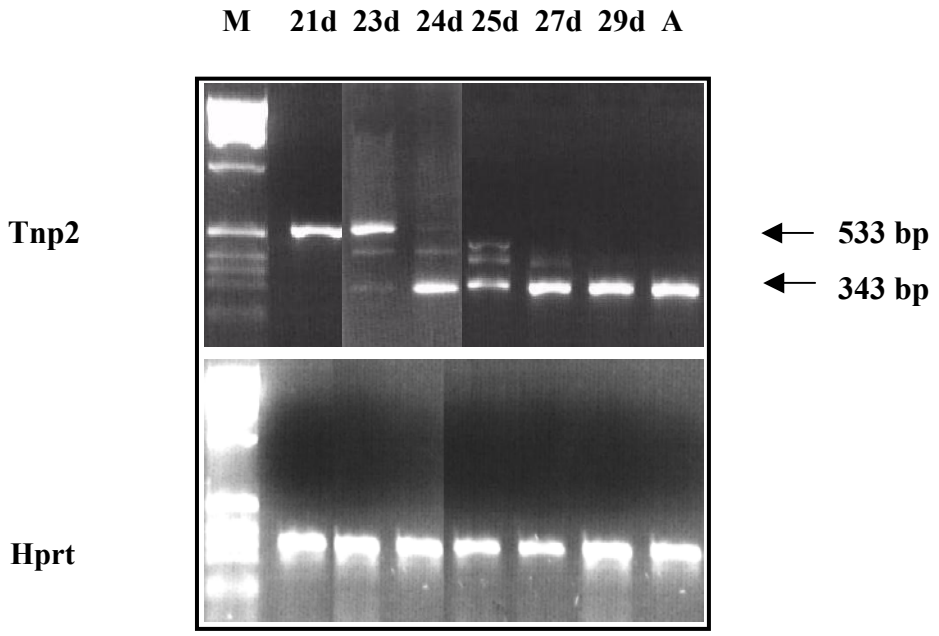


Fig.19 Developmental RT-PCR with total testicular RNA from Tnp2-hGH transgenic mice to show the expression of endogenous Tnp2 mRNA.

The first reaction of RT-PCR was carried out with an oligo $(dT)_{17}$ primer with using total RNA from various postnatal stages of Tnp2-hGH transgenic mice. Second reaction was performed with using Tnp2 gene-specific primers, the amplification from RNA yielded 343 bp PCR product whereas the amplification from DNA resulted in 533 bp PCR product. Integrity of RNA samples was analyzed by subsequent PCR from the same reverse transcription reaction with primers specific for hypoxanthine phosphoribosyltransferase (Hprt) transcripts. (Lower panel)

3.2.1.2.5 The expression of endogenous Tnp2 mRNA during testis development

To profile the expression of endogenous Tnp2 mRNA during the spermatogenic cell differentiation, and to compare it with the expression pattern of transgenic Tnp2 mRNA, we carried out developmental northern blot analysis. Total testicular RNA from various stages of NMRI mice were isolated and separated on an RNA-agarose gel, and the filter was hybridized with the Tnp2 probe. The first endogenous RNA could be detected at day 23 which corresponds to haploid stage and coincides with the appearance of endogenous Tnp2 mRNA in Tnp2-hGH transgenic mice line as well.

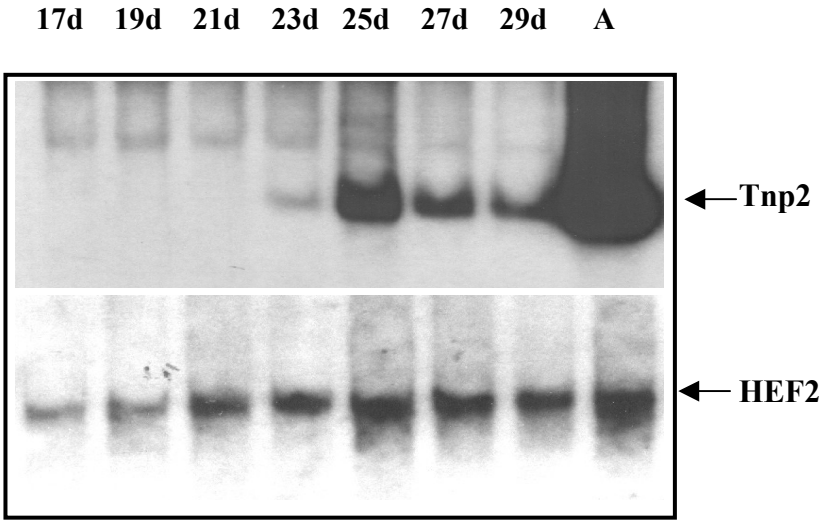


Fig.20 Developmental northern blot analysis to show the spermatid-specific expression of endogenous Tnp2 mRNA in NMRI wild type mice.

20 µg testis RNA from different stages from NMRI wild type mice were separated on RNA-agarose gel, blotted, and hybridized with the Tnp2 cDNA probe. The Tnp2 transcript was first detected at day 23. The blot was rehybridized with 1.6 kb HEF probe to demonstrate the RNA integrity and amount.

Likewise for Tnp2-hGH transgenic mice line, the expression pattern of endogenous Tnp2 mRNA was further analyzed by RT-PCR which is a more sensitive method than northern blot analysis. The detection of Tnp2 mRNA at day 23 by RT-PCR confirmed the result we obtained by developmental northern blot analysis.

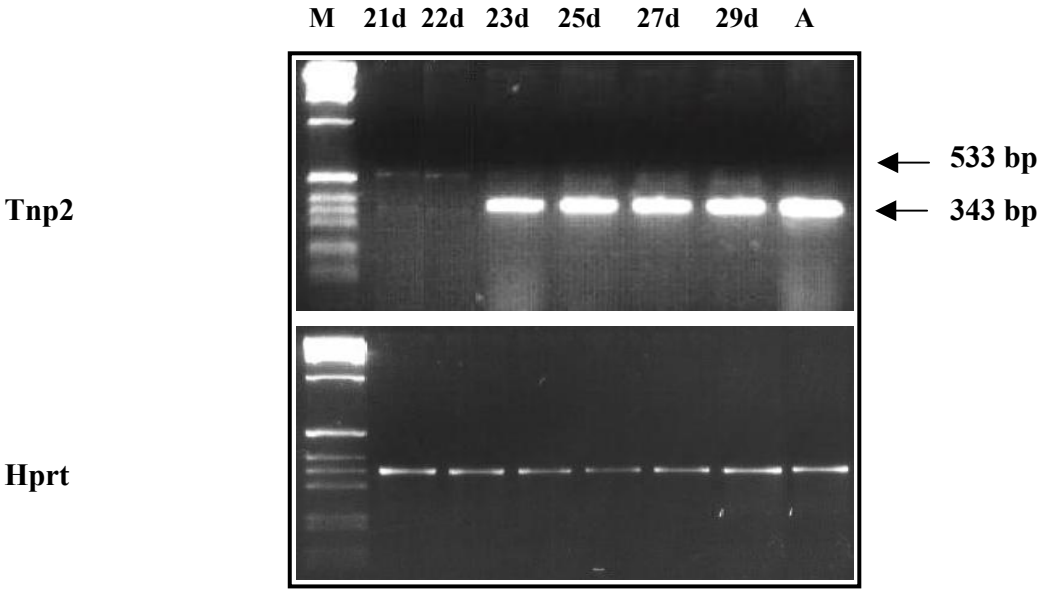


Fig.21 Developmental RT-PCR with total testicular RNA from NMRI wild type mice to show the expression of endogenous Tnp2 mRNA.

5 µg testis RNA from different stages of wild type mouse were used for RT-PCR. 1st strand synthesis was carried out with an oligo (dT) 17 primer, and the second PCR reaction was performed with Tnp2 gene-specific primers. The first endogenous RNA was detected at day 23, reflecting the expression of the Tnp2 mRNA in haploid stage and confirming the result obtained by northern blot analysis.

3.2.1.2.6 The expression of transgenic Tnp2 protein during testis development

The expression of transgenic Tnp2 protein throughout the developmental stages were examined by carrying out immunoblot analyses with nuclear basic protein extracts from various developmental stages isolated from Tnp2-hGH transgenic mice. After extraction of basic nuclear proteins, they were separated on 10% SDS-PAGE, electroblotted, and stained with Ponceau S to demonstrate the integrity and the amount of protein loaded to each lane. Later, the presence of protein was detected by using anti-Tnp2 antibody which was kindly provided by S.W. Kistler, University of South California, USA. The transgenic Tnp2 protein was first detected at day 24 in the Tnp2-hGH transgenic mouse line.

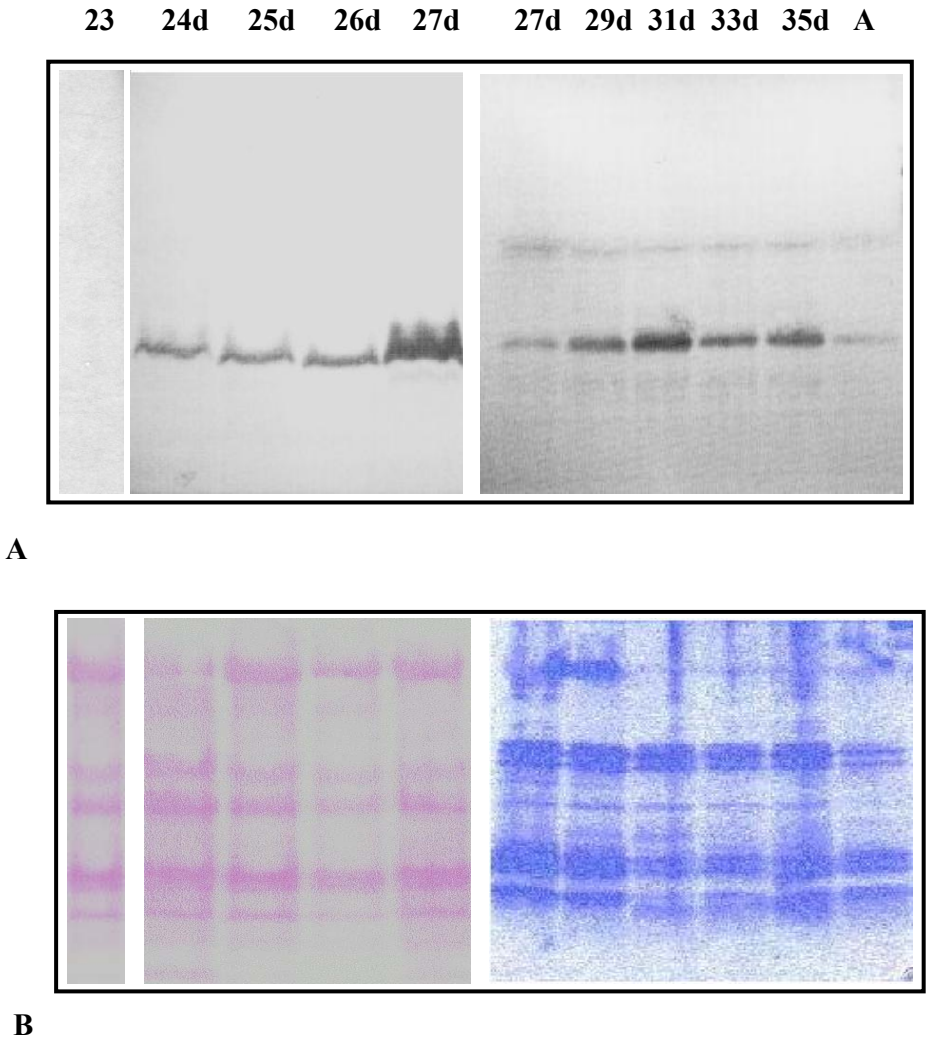


Fig.22 Developmental western blot of transgenic Tnp2 protein with anti-Tnp2 antibody. Basic nuclear proteins were extracted from various postnatal stages of the Tnp2-hGH transgenic mice, separated by 10% SDS-PAGE, and the transgenic protein was first detected on day 24 by using anti-Tnp2 antibody. Before going on with immunodetection, western blots were stained with Ponceau S to demonstrate the integrity and the amount of protein loaded to each lane (lower panel).

3.2.1.2.7 The expression of endogenous Tnp2 protein during testis development

In order to compare the transgenic Tnp2 protein expression in Tnp2-hGH transgenic mice with the endogenous Tnp2 protein expression in wild type mice, the same experiment which was done for the transgenic line was carried out for the wild type NMRI mice. The basic nuclear protein extracts of testes from 20 to 35 days old mice were examined by western

blotting with anti-Tnp2 antibody. The endogenous Tnp2 protein could be first detected at day 26 in NMRI mouse strain.

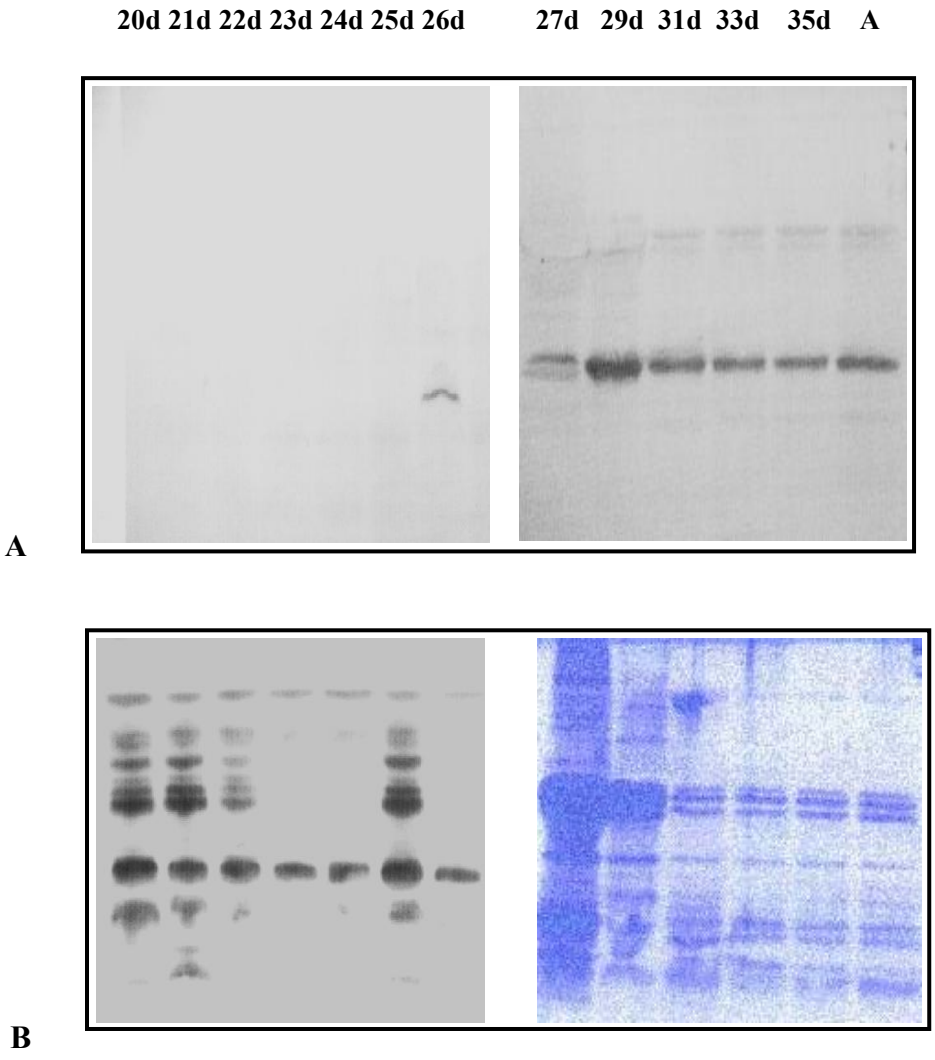


Fig.23 Developmental western blot of endogenous Tnp2 in NMRI mouse strain.
Aliquots of basic nuclear protein extracts from wild type NMRI mice of several postnatal stages were separated by SDS-10 % PAGE, blotted, stained with Ponceau S (lower panel, B), and finally immunodetection was carried out with anti-Tnp2 antibody (A).

3.2.1.2.8 Immunohistochemical localization of transgene Tnp2 protein

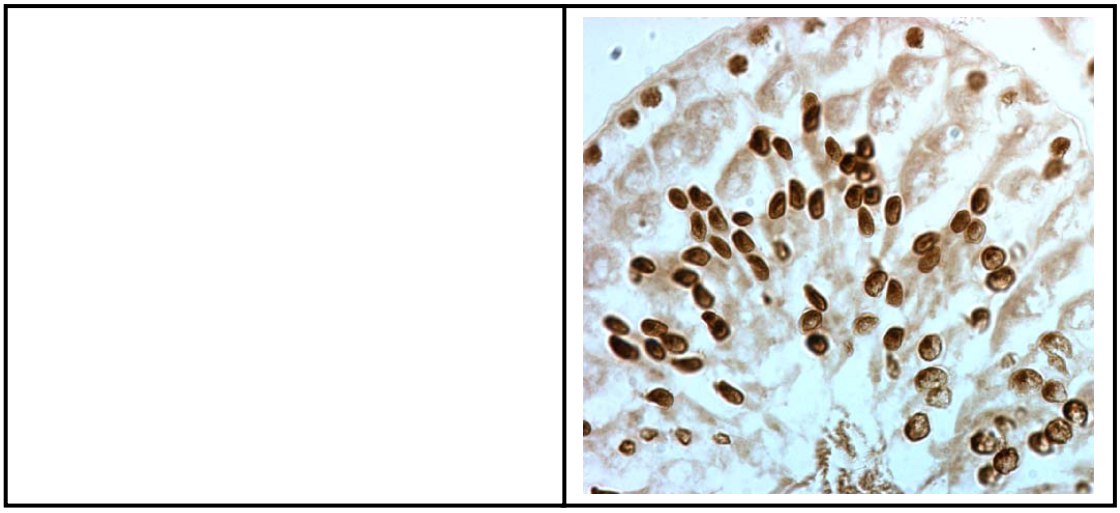
In order to examine the cell types in testis which contain Tnp2 protein, immunoperoxidase staining with the anti-Tnp2 antibody was employed. Paraffin-embedded tissue sections were

prepared from a heterozygous Tnp2-hGH adult transgenic mouse and NMRI wild type adult mouse. To increase the sensitivity of the protein detection on paraffin-embedded tissue sections, peroxidase anti-peroxidase method was employed (2.2.17.3).

The presence of Tnp2 protein could be detected starting from elongated spermatids in NMRI wild type mouse whereas in Tnp2-hGH transgenic mouse, the transgenic Tnp2 protein was observed in elongating spermatids as well.

This result confirmed the premature translation of the transgenic Tnp2 protein in Tnp-hGH mouse line showed by developmental western blots and immunodetection by anti-Tnp2 antibody.

A



B

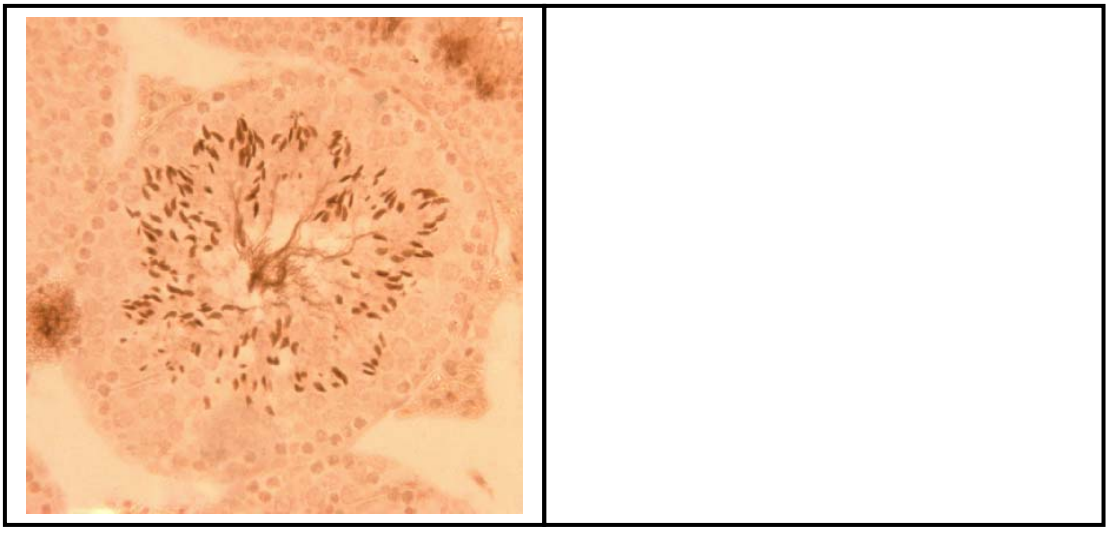


Fig.24 Immunohistochemical localization of Tnp2 protein in adult Tnp2-hGH transgenic mouse testis, and wild type adult mouse testis.

The presence of Tnp2 protein in elongating spermatids in Tnp2-hGH transgenic mouse testis could be detected by anti-Tnp2 antibody whereas in wild type mouse testis, it could detected in elongated spermatids.

3.2.1.2.9 Electron microscopy of adult testis from Tnp2-hGH transgenic mouse

In order to investigate the effect of premature expression of transgenic Tnp2 protein on spermatid morphology, the testis from adult Tnp2-hGH transgenic mouse was examined by electron microscopy. Tnp2 protein is known to play role in nuclear reorganization in haploid spermatids by involving the change of nucleosomal chromatin to the highly condensed chromatin. Therefore, we investigated the spermatid and sperm morphology in Tnp2-hGH transgenic mice by electron microscopy whether there are any abnormalities in spermatid head morphology. However, we could not observe any abnormal head structure in spermatids which might have resulted from an earlier nuclear condensation due to the premature appearance of Tnp2 protein in spermatid nucleus.

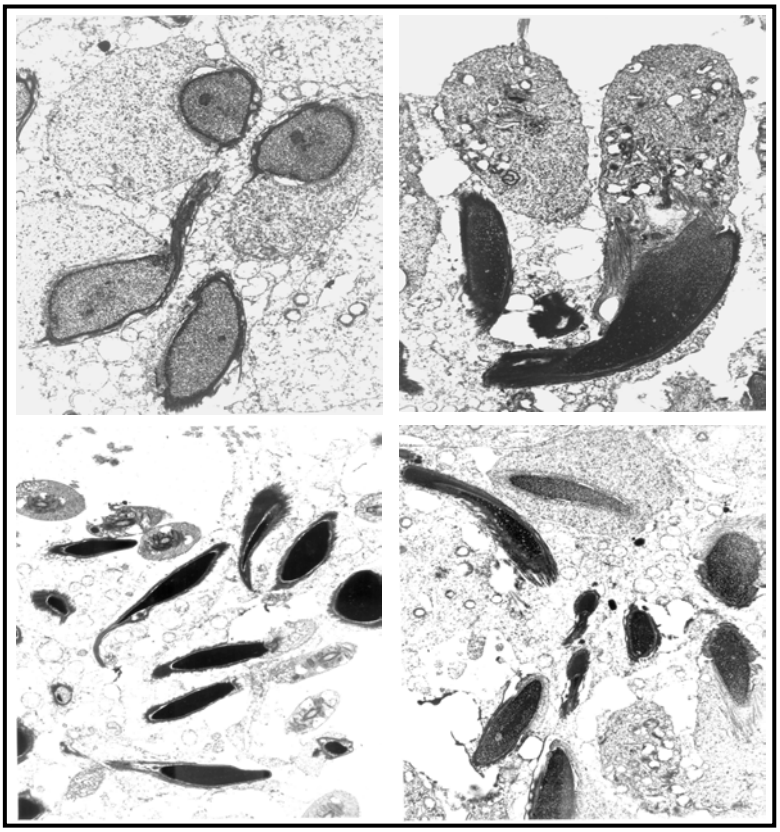


Fig.25 Electron micrograph of testis from Tnp2-hGH transgenic mice.
Due to the translation of Tnp2 protein prematurely in Tnp2-hGH transgenic mouse line, we examined testis from an adult transgenic mouse by electron microscopy. However, there could be no evidence of an earlier nuclear condensation in round, elongating and elongated spermatid resulting in an abnormal head morphology was observed.

3.2.2 In vitro studies

Both in vivo and in vitro studies indicate that the 3‘ untranslated region (UTR) is sufficient and necessary to achieve the translational regulation of haploid-stage specific mRNAs, and additionally, it is supposed that it is often regulated by the interaction between specific cytoplasmic proteins, so called RNA-binding proteins and the regulatory elements within the 3‘ untranslated regions.

3.2.2.1 Secondary structure prediction for 3' UTR of Tnp2 mRNA

It is known that 3' untranslated region of many genes contain binding sites for regulatory proteins to achieve translational control. To date, almost all RNA regulatory proteins bind loops, loops and stems, bulges or combinations thereof. Therefore, we analyzed the rat Tnp2 3' UTR for regions of putative secondary structures by applying the computer algorithm of Zuker (Zuker, 1989). Analysis of secondary structure of 3' UTR of Tnp2 mRNA revealed some stem and loop structures. However, any of these regions can be protein binding sites, therefore functional studies should be carried out to determine if any of them are actual sites for protein binding.

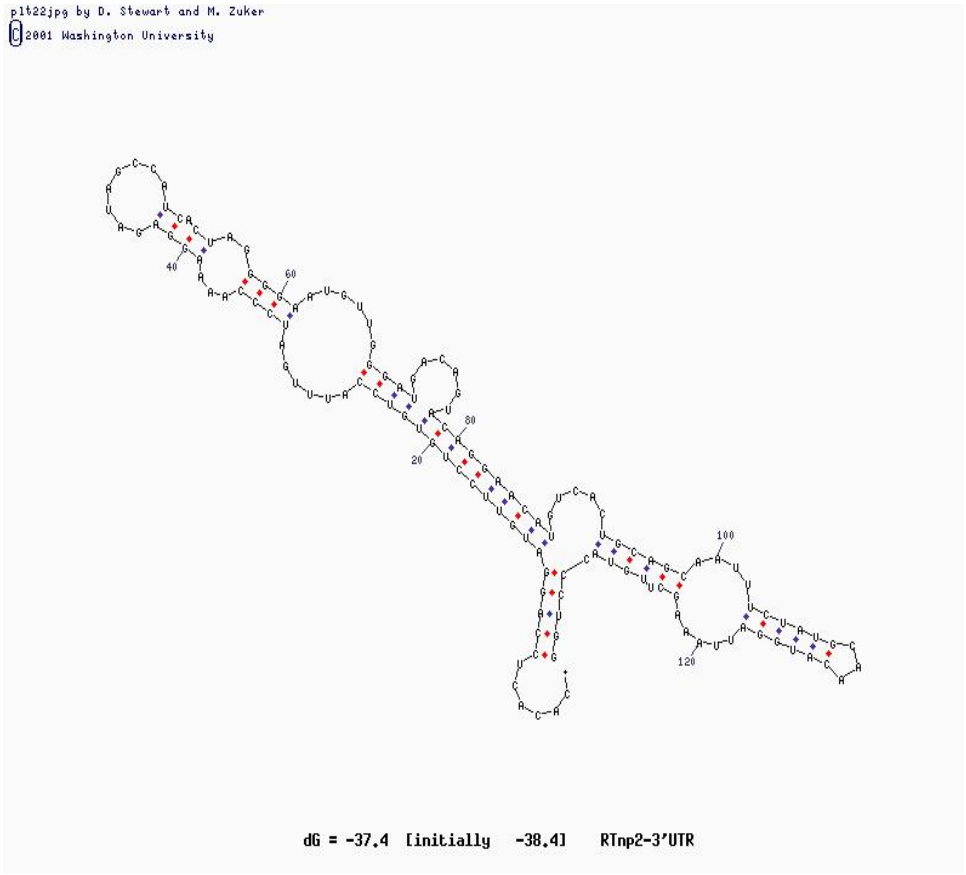


Fig.26 Possible secondary structure of 3' UTR of rat Tnp2 mRNA.

The prediction is accomplished according to computer algorithm of Zucker, "mfold" (http://bioinfo.math.rpi.edu/~mfold/rna/form1.cgi), to yield a structure of minimum free energy of -38.4 kcal/mole.

3.2.2.2 RNA-affinity chromatography

In order to show the binding of cytoplasmic testis proteins to the 3‘ UTR of rat Tnp2 mRNA, we performed RNA-affinity chromatography. For this purpose, the 3‘ UTR of Tnp2 gene was amplified by a modified reverse primer which had 30 nts T nucleotides at the 3‘end, and cloned in pBluescript II SK +/- vector which made it possible to generate in vitro transcripts of the cloned fragment. The poly (A) tailed 3‘ UTR transcript of Tnp2 gene was then immobilized in the column by poly(U) agarose beads. The cytoplasmic testicular extract was loaded onto the column and incubated at room temperature for an hour in the presence of heparin and tRNA to minimize the nonspecific bindings. Then, the column was extensively washed and bound proteins were step-eluted by increasing salt concentration.

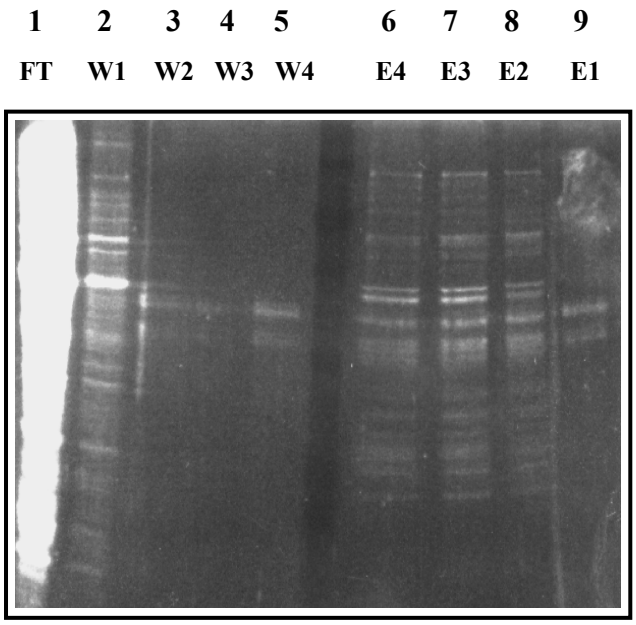


Fig.27 SDS-PAGE analysis of the protein fractions of the RNA affinity column.
Aliquots of protein from each purification step were resolved by SDS-10 % PAGE and stained with SyproOrange. Lane 1 represents the flowthrough fraction; lanes 2-5 contain the four washing steps, W1 to W4, lanes 6-9 contain elution steps E1 to E4 with 1 M KCl, 2 M KCl, 3 M KCl, and 5 M NaCl step elution fractions, respectively.

3.2.2.3 Northwestern blot analysis

To demonstrate the specific binding and enrichment of testicular cytoplasmic protein(s), we analyzed the fractions from RNA-affinity column by northwestern blot analysis. For this purpose, 1/10 of each purification step was separated by 10 % SDS-PAGE, blotted and probed with radioactively labeled in vitro transcribed 3‘ UTR of Tnp2 gene. This method enables to detect the binding of RNA molecules to proteins. We could show the binding of two proteins, about 114 kDa and 55 kDa, to 3‘UTR of Tnp2. It is noteworthy that elution of 55 kDa protein at 3 M KCl but not at 1 M or 2 M KCl salt concentration indicate that the binding of 55 kDa protein to 3’ UTR is stronger than the binding of 114 kDa protein which could be eluted with 1 M KCl. It is even remarkable that elution of 55 kDA protein was first possible with 3 M KCl which is a stringent condition considering the previous results with this method where the elution fractions were from 0.5 M to 2 M KCl.

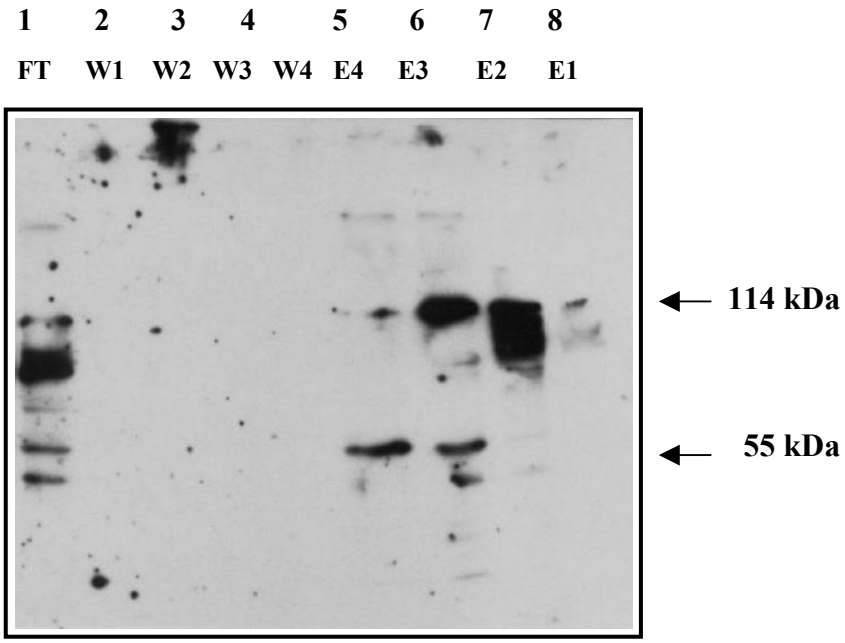


Fig.28 Northwestern blot analysis of the protein fractions of the RNA affinity column. Aliquots (30 µl) of proteins from each purification step were resolved by SDS-10 % PAGE and transferred to nitrocellulose filter. The membrane was renatured and probed with 32P-labeled transcript of the 3‘ UTR of rat Tnp2. W1 to W4, washing-step fractions; E1 to E4, step elution fractions. The pre-stained protein marker from Sigma was used to estimate the size of the proteins.

4. DISCUSSION

Spermiogenesis is the process of differentiation of round spermatids into mature spermatozoa by undergoing complex morphological, biochemical and physiological modifications. While in round spermatids histones and non-histone proteins are replaced by transition proteins, in elongating spermatids, transition proteins are removed from the condensing chromatin and replaced by protamines. On the tightly packed DNA-protamine complexes transcription ceases several days before the completion of spermiogenesis. Thus, as major modifications in both nuclear and cytoplasmic structures continue throughout spermiogenesis, stringent temporal and stage-specific gene expression is a prerequisite for the correct differentiation of round spermatids into mature spermatozoa. The genes for transition proteins and protamines are transcribed in round spermatids and elongating spermatids. Transcription is supposed to be regulated via methylation and trans-acting factors which bind to cis-elements in the promoter region. The transcripts are stored as ribonucleoprotein particles in a translationally repressed state for several days and are translated in elongated spermatids. It has been demonstrated, in haploid spermatids, essentially every mRNA exhibits evidence for translational repression. Translational regulation involves protein repressors that bind to the poly-A tail or specific RNA sequences located mostly in 3' UTR.

4.1 Transcriptional regulation of rat Tnp2 gene

In order to investigate the promoter sequence responsible for testis- and spermatid-specific expression of rat Tnp2 gene, we started analyzing the 5' regulatory region of the gene. By primer extension analysis, we demonstrated that the transcription start site for rat Tnp2 gene is located ~70 nts upstream from the translational start site of Tnp2 mRNA. Finding of a consensus TATA box sequence (TATATAA) with an expected distance which is 24 nts upstream from transcription start site confirmed the putative transcription site that was determined experimentally.

An earlier study from our institute by D. Böhm showed that a 525 nts 5‘ untranslated region of rat Tnp2 gene could direct the testis- and haploid-specific expression of the Tnp2 gene in transgenic mice. For this study, a construct consisting of 525 bp 5’ UTR, CAT gene as reporter gene, 3’ UTR and 3’ genomic flanking region of Tnp2 gene was used for generating transgenic mouse. Northern blot analysis and CAT assays demonstrated that 525 bp 5’ untranslated region is sufficient for the testis- and spermatid-specific expression of CAT reporter gene under Tnp2 promoter sequence.

In order to analyze this region further, we shortened the 5‘ regulatory region to 147 nts and generated transgenic mouse carrying 147 nt 5‘ UTR of rat Tnp2, the coding region of CAT gene as reporter gene, followed by 3‘ UTR and 3‘ genomic flanking region of rat Tnp2. The region of 147 nts corresponds to the region –74 to +73 relative to the transcription start site. We examined the tissue-specific expression of the transgene in Tnp2-147 transgenic mouse line by northern blot analysis and showed that the transgene is expressed only in testis but not in any other tissue. Similarly, haploid-stage specific expression of the transgene was demonstrated by developmental northern blot analysis. The first transcript could be detected at day 25 which corresponds to the haploid stage of spermatogenesis. These expression studies revealed that a 147 bp region of –74 to +73 of the rat Tnp2 is sufficient to direct testis- and spermatid-specific transcription in transgenic mice.

Furthermore, the expression of fusion protein in Tnp2-147 transgenic mouse line was also investigated. For this purpose, a non-radioactive CAT assay was employed which is based on the use of a fluorescent chloramphenicol (CAM) substrate. The detection of CAT protein only in testis and at day 25 confirmed our expression results in the direction of the testis- and spermatid-specific expression of transgene in Tnp2-147 transgenic mouse.

Other transgenic mice studies have also shown that relatively short 5‘ upstream sequences can direct tissue- and stage-specific expression of haploid-stage specific expressed genes in testis. Howard et al. (1993) showed that the promoter region of –91 to +17 was sufficient for the proper tissue- and stage-specific expression of angiotensin-converting enzyme (ACE) in transgenic mice. Zambrowicz et al. (1993) demonstrated that a 113 bp region from –150 to –37 of the mouse protamine 1 can successfully direct spermatid-specific transcription in transgenic mice. For rat histone H1t gene, studies in transgenic mice revealed that the region from –141 to +70 was sufficient to confer spermatocytes-specific expression of the gene (Clare et al., 1997).

Few essential transcriptional regulatory motifs have been identified in genes with similar temporal expression in differentiating male germ cells. Although promoter analyses have detected conserved sequence motifs in the promoters of temporally expressed genes, minimal promoter sequences of 100-200 nucleotides lacking many of these elements can direct germ-cell specific expression of reporter genes in transgenic mice.

```
   bovine  .......... .....CATAC CCCCCTGTGG CCAGTGCCAT CACAATCGGG
     pig   ....AGGCCA CATCTGTTAC ACCCCTGAGG CCTGTGCCAT CACAATCGGT
   human   .....AGCCA CATCTCTCAC ACCCCTGTGG CCTGGGCCAT CATAATCAGC
     rat   .....TGCCA TACCTGTCAC .CCTCTGTGG CCTGTGCCAT CATAATTGG.
   mouse   GGCTTTGTCA TACCTGTCAC .CCTCTGTGG CCTATGCCAT CATAATCGG.
Consensus  ......gcca .a.ctgtcAC .CCcCTGtGG CCtgtGCCAT CAtAATcgG.
                          Half CRE             Prot 1 C
           51                                              100
   bovine  CCCAACTATA TAACCAGG.. GGCTGCCAGG GCCTCTGTGA AGCTGGGTCT
     pig   CCCGACTATA TAACCAGG.. GGCTGCCAGG GCCTCTGTGA AGCTGGGTGT
   human   CCCAACTATA TAACCAGGTG GGCTGCCAGG GCCTCTGTAA AGCTAGGCCT
     rat   CCCAGCTATA TAACTAGG.. GGCT.CAGAG GACTCCCCAA AGCGAGGCCT
   mouse   CCCAGCTATA TAACTAGG.. GGCT.GTGGG GACTCCCCAA AGCCGGGCCT
Consensus  CCCaaCTATA TAACcAGG.. GGCTgccagG GcCTCtgtaA AGCtgGGccT
                TATA box                      Tsp

           101                                             150
   bovine  GCCAGAAGAG GAGGAGGAGG CGGCGGCCCC TGCCCCTCTA AGCGAGGCCG
     pig   GCC.GAAGAG .AGGAAGAGA GAGAG..CTC TGCCCCTCCA AGCGCGGCCT
   human   GCT.GG.GAG .AGGATGAGG AGGAG..CCC TGCCCCTCCA AACGTGGCCT
     rat   GCTGGGAGAA GAGGAGGAGG AGGAAGTCTC TGCCCCTCCG AGTGTGGCCT
   mouse   GCTGGGAG.. GAGGAGGAGG AGGAAGTCTC TGCCCC...G AGTGTGGCCT
Consensus  GCt.GgaGag gAGGAgGAGg agGagg.CtC TGCCCCtcca AgcGtGGCCt

           151        163
   bovine  CCCCCCACCC ATG
     pig   CCCATG.... ...
   human   CCTATG.... ...
     rat   CCCATG.... ...
   mouse   CCCATG.... ...
Consensus  CCcatg.... ...
```

Table 1 Alignment of the 5‘ proximal regions of the mouse, rat, bovine, pig and human Tnp2 genes. (Accession Nos.: Bovine: X56400, pig: M80677, human: U15422, rat: Z46939, mouse: Z46939)

The 5’ untranslated region chosen for the Tnp2-147 transgenic mouse study was compared for different species to identify the conserved sequences within this region. The region of 147 nts upstream of the translational site was used which corresponds to the region of –74 to +73 for rat Tnp2 5’ regulatory sequence.

To investigate conserved DNA elements in the region that we used for generating transgenic mice, we compared 147 bp relative to the translation start codon of Tnp2 genes from different species. Multiple alignment of these sequences by CLUSTAL program (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) revealed a high sequence homology within this region. There are several similar or identical sequences in the 5' flanking region. One of the conserved sequence is the TATA box at position –24 which is necessary for proper transcription initiation by RNA polymerase II. However, some testis-specific genes, such as Bax inhibitor-1 and SOGGY do not contain a TATA box. (Jean et al., 1999, Kaneko et al., 2000). Another conserved sequence which is located upstream of TATA box is a modified CAAT box at position –43. A similar modified box is found in the promoter region of testis specific gene for rat and mouse proacrosin. The CAAT box has been found in a variety of promoters and plays an essential role in their activity. A number of different proteins bind to CAAT box which are expressed in all tissues while others in tissue-specific manner. Another sequence element that is conserved between transition protein genes is the 11 bp motif TGCCATCATAA at position –46 which is identical in 9 of 11 positions to sequence TGACTTCATAA. This sequence was found in promoter of mouse protamine 1 gene as the binding site for testis-specific transacting factor Tet-1. In vitro transcriptional and binding assays demonstrated that the Tet-1 site is responsible for testis-specific transcriptional activation of mouse protamine 1 gene. In a transgenic mouse study, it was shown that a 113 bp region which contains the Tet-1 sequence can direct the spermatid-specific transcription. The same sequence was also reported as protamine 1 consensus (Prot1 C) by Queralt et al. (1993) who sequenced protamine 1 gene of several species, such as guinea pig, gorilla, orangutan, anubis baboon, and red monkey to identify the conserved elements in 5' flanking regions of protamine 1 genes by comparison. Another conserved element is TGTCACC which is similar to the TGTGAGG motif reported for protamine 1 gene. It is noteworthy that this motif was found to be located 20 upstream of Prot1 C sequence in protamine 1 gene from several species. In Tnp2 5' regulatory sequence, it was also identified at 19 upstream of Prot1 C sequence.

One motif, the cyclic cAMP response element (CRE), present in the promoters of many testis-specific genes, does seem to be functionally important. The expression of cAMP response element modulator (CREMτ) was shown to be restricted to testis. Unlike other CREM spliced forms such as CREMα, -β, -γ which act as repressors, CREMτ retains its transactivation domain and functions as an activator. During spermatogenesis, CREMα, -β, -γ transcripts are

present at low levels in post-meiotic germ cells. As meiosis proceeds, splicing produces high levels of CREMτ transcripts in post-meiotic round spermatids. In recent studies, the specific role of CREMτ in spermiogenesis was addressed by generating CREMτ-mutant mice that showed postmeiotic arrest at the first stages of spermiogenesis. Absence of the CREMτ gene resulted in the lack of expression of CREMτ-dependent genes such as protamine 1 and 2, and calspermin. There is direct evidence that CREMτ is one of the transcription factors responsible for the postmeotic expression of the calspermin and transition protein 1 gene. Although Tnp1 gene contains perfect CRE sequence, TGACGTCA at position –97, in Tnp2 5' regulatory gene, only half CRE sequence, GTCA, could be identified at position –63. Sequence analysis identifies the same half CRE sequence in mouse protamine 2 as well. The affinities for binding vary among the different CREs, but wide range of binding suggests that CREMτ recognizes a number of different promoter targets in spermatids which share at least the conserved half site CRE, GTCA, in either strand.

4.2 Translational regulation of rat Tnp2 mRNA

4.2.1 Tnp2-SV40 transgenic mouse line

Translational control is especially important towards the end of spermatogenesis since the global transcription from the haploid genome ceases several days before the completion of spermiogenesis and the synthesis of the transition proteins and protamines. Both in vitro and in vivo studies have shown that the 3' untranslated region is necessary and sufficient to mediate translational repression of spermatid-specific expressed genes.

Braun et al. (1995) demonstrated that the 3' UTR of mouse protamine 1 (mP1) gene is necessary and sufficient for the proper temporal expression of mP1 mRNA in transgenic mice. They established two different transgenic mice lines; in both lines the human growth hormone (hGH) gene was expressed under the control of mP1 promoter, but as 3' UTR, in one line 3' UTR of hGH was used whereas in the other 3' UTR of mP1. In both lines, hGH mRNA was expressed in round spermatids which coincides with the expression of endogenous protamine 1 mRNA. However, the translation of hGH mRNA was repressed until the time of the endogenous protamine 1 mRNA in transgenic line carrying the construct with 3' UTR of mP1 while an immediate translation of the hGH mRNA in round spermatids was

observed followed by its transcription in transgenic line carrying the construct with hGH 3' UTR. This study provided evidence that the replacement of the 3' UTR of mP1 with the 3' UTR of hGH abolished its delay of translation, thus indicating that 3' UTR of mP1 mRNA determines the fate of protamine transcripts**.**

To investigate whether the same translational repression through 3' UTR occurs for Tnp2 mRNA, we generated transgenic mice, namely Tnp2-SV40, where we replaced the 3' UTR of Tnp2 gene with SV40 splice and polyadenylation sequence, and included 525 nts of 5' UTR and CAT gene coding sequence as reporter gene. With this construct, we expected to abolish the translational delay for the CAT mRNA. In other words, due to the absence of 3' UTR of Tnp2 gene, we expected to observe an immediate translation of CAT mRNA followed by its transcription in round spermatids.

The expression of CAT reporter gene was analyzed by northern blot analysis of various RNA samples from a heterozygous trangenic mouse. However, we couldn't detect the presence of CAT transcript in any of the tissues. As expected, CAT assay performed on total protein extract from several tissues revealed no CAT enyzmatic activity. This unexpected result can be explained in two ways: It might be possible that SV40 splice and polyadenylation sequences were not capable of conferring mRNA stability for the transgenic transcript. Concerning this point, we can give two contradictory examples. First, Nayernia et al. (1994) successfully used the same SV40 sequence to show the testis-specific expression of the CAT reporter gene under proacrosin promoter in transgenic mice. On the other hand, Stewart et al. (1988) failed to detect SV40 early gene expression stabilized with SV40 sequences under the control of protamine 2 promoter region in one of their transgenic lines. Second reason might be found in the transcriptional regulation, i.e that some regulatory sequences for transcription lie in 3' UTR and/or 5' flanking region of rat Tnp2 gene.

4.2.3 Tnp2-hGH transgenic mouse line

In order to demonstrate the role of 3'UTR of rat Tnp2 gene in the translational repression of Tnp2 mRNA, we generated transgenic mice which harboured 525 nts 5'UTR of rat Tnp2 gene, coding and non-coding regions of Tnp2, and 3'UTR of hGH. In our transgenic mice, we could show the testis- and spermatid-specific expression of the transgene by northern blot

analysis. The trangene expression was shown to be only in testis but not in any of the tissues analyzed tissues, such as brain, heart, lung, liver, kidney, spleen or skeletal muscle. The haploid-stage specific expression of the transgene was examined by developmental northern blot. The first transcript was detected at day 24 which corresponds to the haploid-stage of spermatogenesis. In order to be able to compare the expression of transgenic Tnp2 mRNA with the endogenous Tnp2 mRNA, transcription of endogenous Tnp2 gene was extensively analyzed as well. The expression of endogenous Tnp2 in Tnp2-hGH transgenic mouse during testis development was investigated by RT-PCR and the presence of endogenous Tnp2 mRNA could be shown at day 23. Besides, the expression of endogenous Tnp2 mRNA was further analyzed in NMRI wild type mouse by both northern blot analysis and RT-PCR.For RT-PCR, two Tnp2 gene-specific primers were used. The forward primer lies in exon 1 while the reverse primer in exon 2, thus the amplification of the complete coding sequence of Tnp2 gene was possible. Due to the presence of 190 nts intron, amplification from the DNA template and RNA template could be easily detected. Amplification from DNA template yielded a 533 bp PCR product whereas amplification from RNA template resulted in a 343 bp PCR product. By both methods, the endogenous Tnp2 transcript could be shown first at day 23. This one day delay in the expression of transgenic mRNA might partly be explained by locus specific influences, interfering with faithful regulation of the transgene with the 525 bp promoter region. Furthermore, even if there is no evidence of further regulative elements in the 5' direction, it cannot be ruled out that the 525 bp promoter lacks certain elements which are required for proper timing of the Tnp2 transcription. Finally, as the 3' UTR is involved in translational control, at the same time it may influence (i.e lower) transcript stability, resulting in an altered transcript turnover. Since accumulation of the transcript would be delayed, it may be possible that we detected the transgenic Tnp2 transcript in later germ cell stages.

Furthermore, the expression of transgenic Tnp2 protein during testis development was analyzed by western blotting and immunodetection with an anti-Tnp2 antibody. At day 24 it was possible to detect the presence of transgene Tnp2 protein. Recalling the transcription of trangenic Tnp2-hGH mRNA at day 24, it was obviously shown that the transcription and the translation of the transgenic Tnp2-hGH gene and mRNA occur at the same day. In other words, in our Tnp2-hGH transgenic line where the 3‘ UTR of Tnp2 was exchanged with 3‘ UTR of hGH, we didn't observe the translational delay but rather could show the immediate translation of the transgene following its transcription. We analyzed the expression of endogenous Tnp2 protein during testis development in NMRI wild type mouse to compare

our finding in Tnp2-hGH transgenic line. The expression of endogenous Tnp2 protein in NMRI wild type mouse was extensively examined by immunodetection in stages of 20 day old to 35 day old mice. The expression of endogenous Tnp2 protein could be first detected at day 26. Expression studies of endogenous mRNA and protein of Tnp2 in wild type mouse clearly showed that there is a translational delay of 2-days for Tnp2 mRNA in wild type mouse. However, we could show the immediate translation of transgenic Tnp2-hGH mRNA in the transgenic line. This shows that 3‘ UTR of Tnp2 mRNA is necessary and sufficient for the translational repression of Tnp2 mRNA.

An interesting question in sperm morphogenesis is whether the temporal expression of testis nuclear proteins is actually necessary for normal spermatid differentiation. This question was answered for protamine 1 by Braun et al. in 1995. They demonstrated the need for translational repression for protamine 1 mRNA in transgenic mice where they showed premature translation of protamine 1 mRNA by exchanging 3' UTR of protamine 1 with 3' UTR of hGH. As a result of premature accumulation of Prm 1 mRNA, they observed dominant male sterility accompanied by a complete arrest in spermatid differentiation, early condensation of spermatid nuclear DNA, abnormal head morphogenesis, and incomplete processing of Prm2 protein in transgenic mice. Although we could show an earlier translation of Tnp2 protein in our Tnp2-hGH transgenic line, we could observe neither infertility in male mice nor precocious nuclear condensation nor arrest in spermatid differentiation. The reason for this difference in these two studies can be that we could generate only one transgenic line whereas Braun et al. (1995) analyzed 7 transgenic lines and found out 4 lines to show sterility in male mouse whereas in 3 transgenic lines, male mice were found to be fertile. Besides, since the construct was integrated in Y-chromosome in Tnp2-hGH transgenic line, it was not possible to establish a homozygous line. This results in the production of half amount of Tnp2 protein which may cause a dosage-effect of the protein in our observations.

Spermatid nuclear condensation normally begins in the step 12 elongating spermatids and is thought to be mediated by transition proteins. On the basis of relative timing of events, it has been proposed that the transition proteins initiate nuclear condensation and that the protamines complete the process. For this reason, we analyzed the spermatid morphology in Tnp2-hGH transgenic line by electron microscopy to see if the earlier translation of the transgenic Tnp2 protein has any effect on especially spermatid head morphology. However, we could observe neither any abnormalities in spermatid head structure nor abnormal nuclear

shaping nor a general failure to complete the latter stages of spermiogenesis. Although the premature translation of protamine 1 mRNA in transgenic mice had such drastic effects on spermatid morphology, we didn't observe similar results in our Tnp2-hGH transgenic mice. This might be due to the fact that Tnp2 protein just initiate the nuclear condensation in spermatids whereas protamines complete the process. Therefore, the premature translation of protamine mRNA might have more profound effect on early onset of nuclear condensation.

4.2.3 RNA-affinity chromatography

Translation of mRNAs including testis and haploid-specific mRNAs is often regulated by specific cytoplasmic proteins which interact with regulatory elements within their 3' untranslated regions. Braun et al. (1993) have demonstrated that the 3' UTR of mouse protamine 1 gene contains all the elements required for proper translational regulation of protamine 1 mRNA in transgenic mice. Further studies revealed several cis-acting elements in the 3' UTRs of protamine 1 and 2 mRNAs and trans-acting factors that recognize them. All of the cis-acting sequences needed for the translational regulation of protamine 1 has been mapped to 37 nucleotides 5'-most region and 62 nucleotides in the 3' most region of the protamine 1 3' UTR. Several RNA-binding proteins that influence the translation through binding to the 3' UTR of mouse protamine 1 and 2 mRNAs have been identified with the help of gel mobility shift assays and UV-crosslinking experiments. Screening of male germ cell cDNA expression libraries with 3' UTR has also yielded several clones encoding RNA-binding proteins such as protamine 1 RNA-binding protein (Prbp), spermatid perinuclear RNA binding protein(Spnr) and testis nuclear RNA-binding protein (Tenr). Kwon and Hecht (1991, 1993) have described two regions of the Prm-1 and Prm-2 of 3'UTRs, Y and H boxes, that interact with testis proteins. These proteins are believed to be sequence-specific DNA-binding proteins and nonspecific RNA binding proteins. Tafuri et al. (1993) cloned the gene for one of these proteins and named as Mouse Y box Protein MSY1. Recently, Davies et al. (2000) have cloned 2 new members of the Y box member, namely MSY3 and MSY4, by yeast three hybrid assay. They showed that MSY4 binds to a conserved 7-nt sequence in the Prm1 3'UTR and MSY2 to the 3'-most 37 nts of Prm1 3'UTR.

In order to show the presence and subsequently to isolate the RNA-binding protein(s) which is responsible for the translational regulation of rat Tnp2 mRNA, we employed RNA-affinity

chromatography. The in vitro transcribed 3' UTR of Tnp 2 gene was immobilized by poly (U) agarose beads through the interaction of its poly (A) tail with the poly (U) agarose beads. The cytoplasmic proteins were isolated from testis and allowed to bind to immobilized 3‘ UTR in vitro transcribed RNA in column. Finally, bound proteins were successively eluted with increasing salt concentration. The elution fractions were analyzed by northwestern analysis which allows the binding of radioactively labelled 3' UTR of Tnp2 gene with the immobilized testicular cytoplasmic proteins on nitrocellulose filters. By northwestern analysis, we could show the specific binding of two proteins which are about 114 and 55 kDa to the 3' UTR of Tnp2 mRNA. The 114 kDa protein could be eluted with 2 M KCl whereas 55 kDa protein was detected first in the third elution step which was 3 M KCl. Elution of 55 kDa protein with a higher salt concentration may indicate that the binding of this protein to the Tnp2 3’ UTR is stronger than that of 114 kDa protein. It is even noteworthy that elution conditions for these 2 proteins are more stringent than that of the other studies where the elution fractions were 0.5 M to 2 M KCl. This result suggests a specific interaction between 55/114 kDa proteins with 3’ UTR of Tnp2 mRNA. However, it was not possible to make a preparative column and a protein gel to purify these proteins, as there were many other proteins present in the last 2 elution fractions.

The RNA-affinity chromatography method which was employed in this study was used by Gu et al. (1995) to purify a 65 kDa testicular protein which binds to the 5' unstranslated region of testis-specific Cu/Zn superoxide dismutase (SOD-1) mRNA, thus represses its translation *in vitro*. Secondly, Wu et al. (1997) performed the same method to purify and characterize the mouse testis-brain RNA-binding protein (TB-RBP). They showed that TB-RBP specifically binds to highly conserved cis-acting sequences, Y and H sequence elements of the 3' UTR of mouse protamine 2, in the 3' UTR of a number of testicular and brain mRNAs, including protamines 1 and 2. It was also shown that TB-RBP represses the in vitro translation mRNA constructs containing specific conserved sequences of protamine 2.

5. SUMMARY

Stringent temporal and stage-specific gene expression is a prerequisite for the correct differentiation of male germ cells during spermatogenesis. Post-transcriptional control is especially important towards the end of spermatogenesis since global transcription ceases several days before the completion of spermiogenesis. Thus, mRNA storage and translational activation play prominent roles in the expression of many spermatid and spermatozoan proteins that are synthesized in late stages of germ cell maturation. Indeed, in early spermatids, many RNAs, such as protamine and transition protein transcripts, are translationally repressed with long poly(A) tracts and stored as ribonucleoprotein particles for up to a week.

To investigate the 5' regulatory region of rat Tnp2 gene, the transcription start point was experimentally determined. Primer extension analysis revealed that the transcription start site is about –70 bp upstream of the translational start codon. A consensus TATA box was identified at an expected distance of 24 bp upstream of the transcription start point. Moreover, a modified CAAT sequence was found at position –37 relative to the transcription start site. Transgenic mice were generated which harbored a construct that consisted of 147 bp 5' UTR of Tnp2 gene, CAT gene as reporter gene, 3' UTR and 3' genomic flanking region of Tnp2 gene. The CAT mRNA was detected only in testis and at day 25 post-natal by nothern blot analysis. These results demonstrate that a promoter region of -74 to 70, relative to transcription site, of rat Tnp2 gene can confer the testis- and spermatid-specific expression of the CAT reporter gene in transgenic mice.

It has been shown that the 3' UTR of testis-specific mRNAs contains all the elements required for the proper translational regulation. To investigate the relevance of the 3' UTR in translational control of Tnp2 mRNA, the Tnp2-SV40 transgenic mouse line was generated. Tnp2-SV40 construct contained 525 bp 5' UTR of Tnp2 gene, CAT gene and SV40 3' UTR including polyadenylation signal. With this construct, we expected to abolish the translational

delay for the CAT mRNA. However, neither transgenic transcript nor transgenic protein were detected by northern blot analysis and CAT assays.

The role of 3' UTR of Tnp2 mRNA and the importance of translational repression were further investigated by Tnp2-hGH transgenic mouse line where the 3' UTR of Tnp2 was replaced with 3' UTR of hGH. The presence of the transgenic transcript was detected at day 24 by northern blot analysis. Developmental western blot analysis demonstrated that the transgenic Tnp2 mRNA was first translated at day 24. These results showed the immediate translation of transgenic Tnp2 mRNA followed by its transcription due the absence of 3' UTR of Tnp2. Although, the endogenous Tnp2 mRNA was transcribed on the same day as the transgenic Tnp2 expression, at day 24, the translation of endogenous Tnp2 mRNA was accomplished at day 26 which is a 2 days of delay compared with transgenic Tnp2 protein. In other words, due to the absence of 3' UTR of Tnp2, transgenic Tnp2 mRNA was translated prematurely. These results clearly demonstrated that 3' UTR of rat Tnp2 mRNA is necessary and sufficient for the translational repression of Tnp2 mRNA during spermiogenesis. The testis from Tnp-hGH transgenic mouse was examined by electron microscopy to investigate whether the premature expression of transgenic Tnp2 protein had any effect on spermatid morphology. However, no abnormal head structure due to the possible earlier condensation of chromatin in spermatids could be observed.

To show the presence and subsequently enrich and isolate the RNA-binding proteins which play a role in the translational control of Tnp2 mRNA, we employed RNA-affinity chromatography. Analyzing the elution fractions obtained from the column revealed the specific binding of 2 cytoplasmic proteins to the 3' UTR of Tnp2 mRNA. Northwestern blot analysis demonstrated that a 114 kDa protein which was eluted with 1 M KCl binds to the 3' UTR. Except from114 kDa protein, 55 kDa protein was also shown to interact with 3' UTR of Tnp2. Elution of this protein with a higher salt concentration of 3 M KCl indicates a stronger binding of this protein to the 3' UTR of rat Tnp2 mRNA. However, attempts for the further purification of these proteins were not successful.

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PUBLICATIONS

1. **Analysis of the CFTR gene in Turkish cystic fibrosis patients: Identification of three novel mutations (3172delAC, P1013L, and M10281).**
Onay, T., Topaloglu, O., Zielenski, J., Gokgoz, N., Kayserili, H., Camcioglu, Y., Cokugras, H., Akcakaya, N., Apak, M., Tsui, L-C., Kirdar, B. (1998)
Human Genetics 102: 224-230.

2. **Rat transition nuclear protein 2 regulatory region directs haploid expression of reporter gene in male germ cells of transgenic mice.**
Nayernia, K., Böhm, D., Topaloglu, O., Schlueter, G., Engel, W. (2001)
Molecular Reproduction and Development, 58(4):368-375.

3. **Teratozoospermia in mice lacking the transition protein 2 (Tnp2).**
Adham, M. I., Nayernia, K., Goettges-Burkhardt, E., Topaloglu, O., Dixkens, C., Holstein, A. F., Engel, W. (2001)
Molecular Human Reproduction, in press.

4. **Cystic fibrosis mutations and associated haplotypes in Turkish CF patients.**
Onay, T., Topaloglu, O., Zielenski, J., Gokgoz, N., Kayserili, H., Camcioglu, Y., Cokugras, H., Akcakaya, N., Apak, M., Tsui, L-C., Kirdar, B. (2001)
Human Biology, in press.

CURRICULUM VITAE

Özlem Topaloglu, born on 23 November 1971 in Istanbul, Turkey

1989-1993	Department of Biology, Middle East Technical University, Ankara, Turkey
1993	B.Sc in Biology
1993-1995	Department of Molecular Biology, Bogazici University, Istanbul, Turkey
1994	M. Sc in Biology
1995-2001-05-17.1.1.1	Ph. D study at Institute of Human Genetics, Georg-August-Universitaet, Goettingen, Germany