GENE EXPRESSION OF THE TESTIS-SPECIFIC HISTONE (H1t) IN THE SPERMATOGENESIS OF THE STALLION

MÁRCIA CRISTINA
OLIVEIRA CAVALCANTI

INAUGURAL DISSERTATION
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From the Institute for Veterinary Anatomy -Embryology and -Histology
of the Justus-Liebig-University Giessen,

Supervisor: Prof. Dr. Martin Bergmann

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submitted by

MÁRCIA CRISTINA OLIVEIRA CAVALCANTI

Veterinarian from Recife (Brazil)

Giessen 2008

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Dekan: Prof. Dr. Dr. Habil. G. Baljer

1. Referee: Prof. Dr. Bergmann
2. Referee: Prof. Dr. Schuler

Day of the Disputation: 04.February 2008

For my Family

(Deus é fiel)

„I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistence acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation."

Márcia Cristina Oliveira Cavalcanti

1 Literature survey

1.1 Anatomy and histology of the testis

The testis is surrounded by a dense connective-tissue capsule, called the tunica albuginea, which is covered anterior and lateral with the remnants of the processus vaginalis. The partial septum of the testis is called the mediastinum. This area consists of a connective tissue in which an anastomotic network of ducts can be identified: the rete testis. The tunica albuginea is formed by a connective tissue in which smooth-muscle fibers can be found, the latter being responsible for the capacity of the capsule to contract in response to pharmacological stimuli. The inner surface of the tunica albuginea is a highly vascular connective tissue termed the tunica vasculosa. The spermatogenic tubules extend as loops from the mediastinum testis, both ends of each loop communicating via single straight tubules, the tubuli recti. The organization of the intertubular tissue varies dramatically between species, but contains the blood vessels, lymphatics, and nerve fibres. The Leydig cells are scattered in groups in the intertubular tissue in relation to the vasculature and the lamina propria of the seminiferous tubules, the outer layers of which consist of modified smooth-muscle cells termed myoid cells (Neil and Knobil, 1988; Cerveny et al., 2005; Wrobel and Bergmann, 2006).

1.1.1 Spermatogenesis

Spermatogenesis is the process of germ cell development. Spermatogonia undergo successive mitotic and meiotic divisions (spermatocytogenesis) and a metamorphic change (spermiogenesis) to produce spermatozoa. The sperm cell development is a cyclic and highly coordinated process in which diploid spermatogonia differentiate into mature haploid spermatozoa (Fig. 1) (Pickett et al., 1989). This highly organized process encompasses different cell associations of the seminiferous epithelium called stages of spermatogenesis. The sequence of events that occurs from the disappearance of a given cellular association to its reappearance constitutes the cycle of seminiferous epithelium (Bergmann, 2006). One of the most productive self-renewing systems in the body is spermatogenesis, lasting between 30 and 75 days depending on species (Russell et al., 1990). Although it is not yet established which genes regulate the duration of spermatogenesis, recent work has demonstrated that the spermatogenic cycle length is under the control of germ cell genotype (Leal and Franca, 2006). The general organization of spermatogenesis is in all mammals a very important mechanism (Sharpe, 1994). However, there are some specific characteristics concerning the

types and the number of spermatogonial generations. The major criteria for the identification of the stage lie in the morphological characteristics of spermatids, in particular, in the nucleus and acrosomic system (Russell et al., 1990; Hess, 1990). With this method, the number of stages and the features used for the classification scheme will vary between species and even among different investigators studing the same species (Hess, 1990). Another method, the tubular morphology system, is based on the shape and location of spermatid nuclei, presence of meiotic divisions, and overall seminiferous epithelium composition. Although the basic structure of the testis is highly conserved among vertebrates (Capel, 2000), specific characteristics of the testis structure might be found for a particular species. Quantitative data can be used to answer important questions about the testis function and to provide a more complete understanding of spermatogenesis (Russell et al., 1990; França et al., 2002).

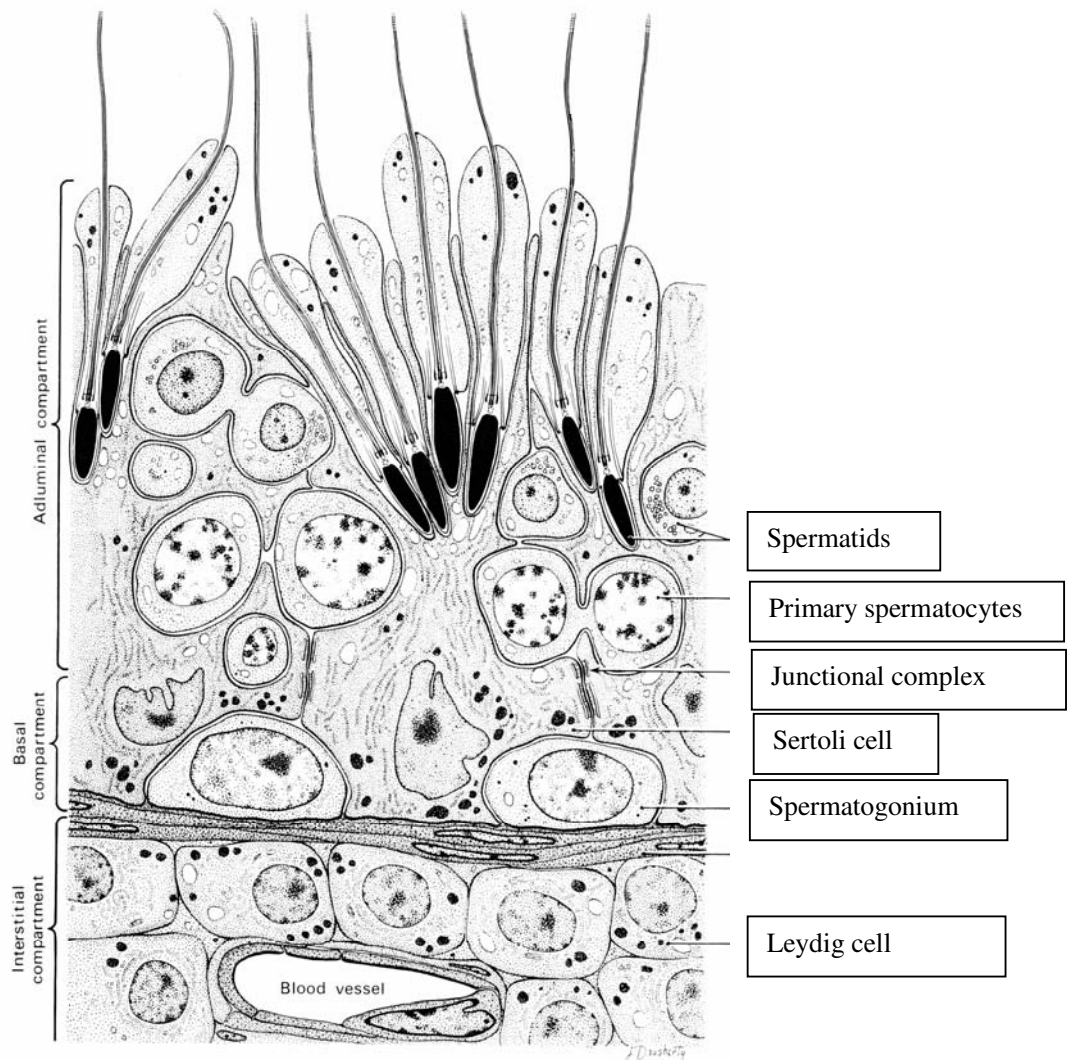


Fig. 1: Sector of the germinal epithelium in the stallion seminiferous tubule showing the relationship of germinal cells and adjacent Sertoli cells in seminiferous epithelium. Spermatogonia, primary spermatocytes, secondary spermatocytes, and spherical spermatids all develop in the space between two or more Sertoli cells and are in contact with them. Primary spermatocytes are moved by the Sertoli cells from the basal compartment through the junctional complexes and into the adluminal compartment (Pickett et al., 1989).

1.1.2 Mitosis and meiosis

Mitosis is the process of cell duplication - two daughter cells are formed with exactly the same DNA and chromosomal content of the original diploid (2n) mother cell. Human cells contain 46 chromosomes - 22 pairs of homologous autosomes and one pair of sex chromosomes. Mitosis (M) encompasses just one step in the eukaryotic cell cycle: first gap (G_1) > synthesis (S) > second gap (G_2) > mitosis (M) > cytokinesis (C). Cells grow during the dominant G_1 phase. Replication of chromosomes occurs in the S phase. Preparation for mitosis takes place during G_2 - replication of organelles and synthesis of microtubules. Interphase includes the combined stages G_1, S, and G_2. During mitosis chromosomes condense, the nuclear envelope disappears, spindle fibers begin to form microtubules (prophase), centromeres of duplicate sister chromatids align along the spindle equator (metaphase), chromatids separate and migrate toward opposite poles (anaphase), the mitotic apparatus is disassembled, autonomous nuclear envelopes are established, and the chromosomes uncoil (telophase) (Table 1) (for review Blow and Tanaka, 2005). The final stage of the cell cycle, when cell division actually occurs, is called cytokinesis (C). Meiosis is a special process of reductional cell division; it results in the formation of four gametes containing half (1n) the number of chromosomes found in somatic cells. Haploid gametes unite at fertilization to create a diploid zygote. In mammals the heterogametic male (XY) determines the sex of the embryo. Approximately one-half of spermatozoa contain either an X or Y chromosome (the sex chromosomal complement of mammalian females is XX, and therefore, the ovar can only contribute an X chromosome to the offspring). Genes carried on the X chromosome that inhibit spermatogenesis are inactivated in XY somatic cells (for review Campbell and Reece, 2001). Steps of meiosis are outlined in Table 1. Meiosis differs from mitosis in two critical respects. During prophase of meiosis I, chromosomes pair along their length and come in contact in discrete areas of synapsis (chiasmata). Chromatids can exchange base pairs by crossing-over. The recombination of segments of chromosomes allows for continual generation of genetic variability (i.e., rapid evolutionary progress) and provides a mechanism for correcting damage in the DNA helix. Secondly, nonidentical sister chromatids do not replicate between serial nuclear divisions. Meiosis II is essentially mitotic (Russell et al., 1990).

Table 1: Meiosis

INTERPHASE: replication of DNA (2n 4c)
PROPHASE I
Leptotene: condensation of chromatin
Zygotene: conjugation of homologues
Pachytene: crossing-over and recombination
Diplotene: synaptonemal complexes dissociate
Diakinesis: chiasmata disappear and homologues begin to repel
METAPHASE I: one face of each homologue centromere binds to a spindle fiber
ANAPHASE I: homologous pairs separate and begin to move
TELOPHASE I: chromosomes migrate to each pole, cell division (1n 2c)
PROPHASE II: spindle fibers rearrange and chromosomes recondense
METAPHASE II: chromosomal pairs align along spindle equator
ANAPHASE II: sister chromatids separate and move to opposite poles
TELOPHASE II: each daughter cell nucleus has one set of chromosomes (1n 1c)

1.1.3 Spermatocytogenesis

During spermatocytogenesis, stem cells called spermatogonia proliferate by mitosis. Spermatogonia are diploid and the stem cells along the route of spermatogenesis. They are always situated in contact with the basal membrane of the seminiferous tubule. The spermatogenesis starts when a single A1-spermatogonium divides to form a pair of A1-spermatogonia, this is the commitment of these germinal cells to divide futher and differentiate into A2-spermatogonia and ultimately give rise to spermatids and spermatozoa (Pickett et al., 1989).

Type A spermatogonia have a rounded to oval nucleus with fine chromatin grains and one or two nucleoli, the type B spermatogonia have a more rounded nucleus with chromatin granules of variable sizes that are often attached to the nuclear membrane and contains one nucleolus. At the end of the differentiation phase, the most mature spermatogonia divide to form the young primary spermatocytes.

Specifically, type B cells divide to form preleptotene spermatocytes. Preleptotene are the last cells of the spermatogenic sequence to go through the S-(synthetic) phase of the cell cycle. In addition, the germ cell number is quadrupled after the completion of the first and second meiotic divisions (Fig. 2) (Bergmann, 2006). Characteristically, a long meiotic prophase in which recombination occurs is followed by two rapid divisions, the end result being the production of haploid spermatids. The presence of leptotene cells signals the initiation of meiotic prophase. In the transition from preleptotene to leptotene, nuclei gradually loose their peripheral chromatin and form fine chromatin threads that can be seen by light microscopy. In zygotene cells, the homologous chromosomes have become paired. In pachytene cells, the chromosomes have become fully paired. In virtually all mammalian species, the pachytene phase of meiosis occupies over a week and typically lasts 1.5-2 weeks, but has a fixed duration for each particular species. Genetic recombination, known as crossing over, occurs during this period (Russel et al., 1990).

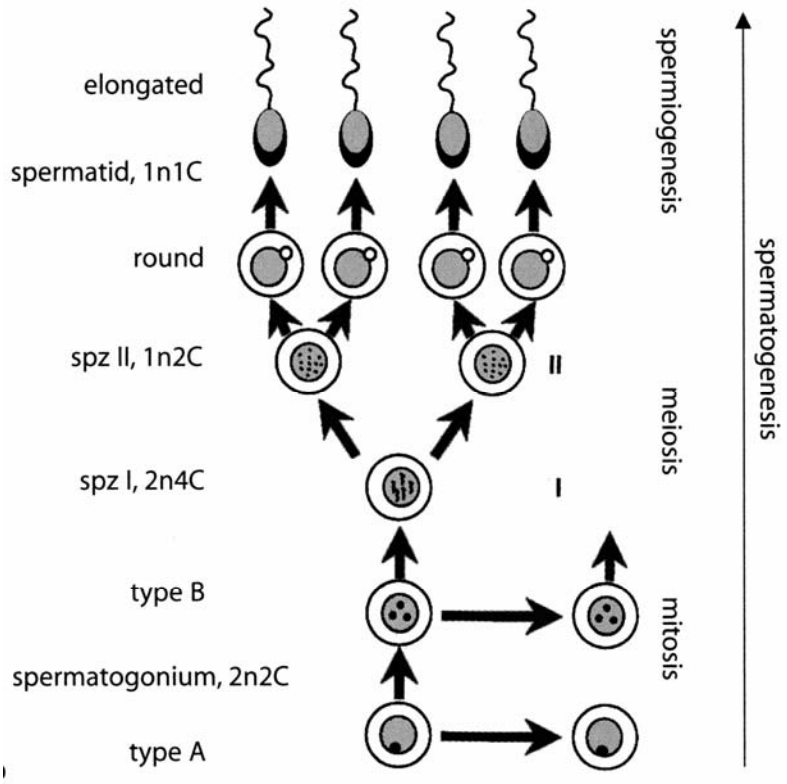


Fig. 2: Process of spermatogenesis (Bergmann, 2006).

1.1.4 Spermiogenesis

Throughout spermatocytogenesis, cells retain a rounded configuration. Spermatids undergo a dramatic change in form during spermiogenesis - into the streamline spermatozoa adapted for fertilization (Fig. 3). Spermiogenesis involves nuclear condensation, formation of the acrosomal cap and development of the tail. The acrosome is derived from the Golgi apparatus. Centrioles (points of organization of spindle fibers) migrate to a postnuclear region after the completion of meiosis. The distal centriole provides a template for accretion of cytoskeletal elements comprising the contractile lattice of the tail. Mitochondria become concentrated into the sheath of the middle piece. Cells do not divide during spermiogenesis, which is one of the most phenomenal cell transformations in the body (Russel et al., 1990; Holstein et al., 2003).

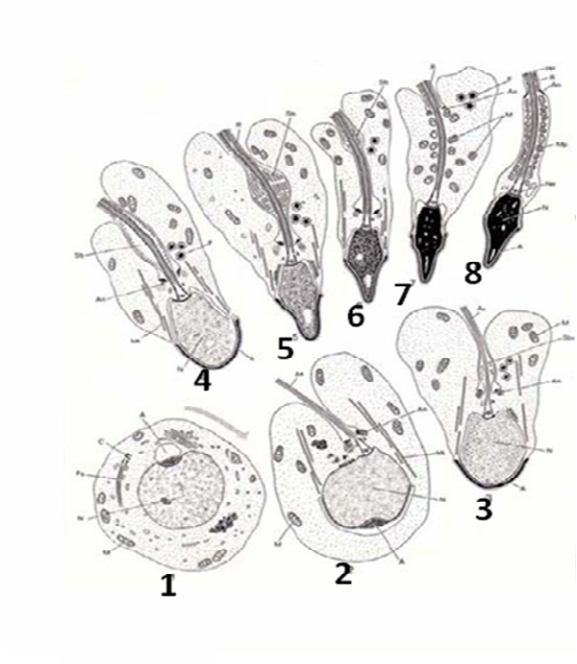


Fig. 3: Steps of spermatid differentiation: (1) Immature spermatid with round shaped nucleus. The acrossome vesicle is attached to the nucleus; the tail anlage fails contact to the nucleus. (2) The acrossome vesicle is increased and flattened over the nucleus. The tail contacted the nucleus. (3-8) Acrosome formation, nuclear condensation and development of tail structures take place. The mature spermatid (8) is delivered from the germinal epithelium. Semi-schematic drawing on the basis of electron micrographs (Holstein et al., 2003).

1.1.5 Sertoli cells

The Sertoli cells, named after the Italian histologist Enrico Sertoli (1842-1910), are easily identifiable by their nuclei. They are not part of the sperm cell line, but constitute „nurse cells" that sustain the proper environment for spermatozoa to develop (Griswold, 1998). Although a Sertoli cell has a very extensive and branching cytoplasmic structure, little of the Sertoli cell´s true extent can be seen in the light microscope (Russel, 1993a, b).

The nucleus of Sertoli cells is ovoid or angular, large and lightly stained, slightly separated from the tube border and often contains one or two prominent nucleoli. The long axis of the nucleus is oriented perpendicular to wall of the tubule. A fold in the nuclear membrane is characteristic for Sertoli cell but not always visible in the light microscope (Russel, 1993a, b; de Kretser and Kerr, 1994) (Fig. 2).

Sertoli cell functions are involved in maintenance of the integrity of the seminiferous epithelium, compartmentalization of the seminiferous epithelium, secretion of fluid to form a tubular lumen, participation in spermiation, phagocytosis, delivery of nutrients to germ cells, steroidogenesis and steroid metabolism, movement of cells within the epithelium, secretion of proteins, regulation of the spermatogenic cycle, target for hormones in the testis and mediator of hormone effects (Russel et al., 1990).

1.1.6 Hormonal regulation of spermatogenesis

The hormonal regulation of spermatogenesis is organized as a control circuit with a negative feed-back mechanism involving the hypothalamus, pituitary gland, and testis. Specific neurons of the hypothalamus synthesize gonadotropin-releasing hormone (GnRH), which induces the production of two hormones within the pituitary, luteinizing hormone (LH) and follicle stimulating hormone (FSH). While a high pulse rate of GnRH release results in the production of LH, a low pulse rate of GnRH results in the production of FSH. Within the testis, LH causes synthesis of testosterone by intertubular Leydig cells, which negatively influences hormone release in the hypothalamus and pituitary. The FSH acts on intratubular Sertoli cells and also induces the production of androgen-binding protein (ABP) by means of which testosterone can pass the Sertoli-Sertoli junctional complexes, and also induces the production of activin and inhibin by Sertoli cells which both influence hormone release in the hypothalamus and pituitary (for review Pickett, 1989, Holdcraft and Braum, 2004; Brehm and Steger, 2005) (Fig. 4).

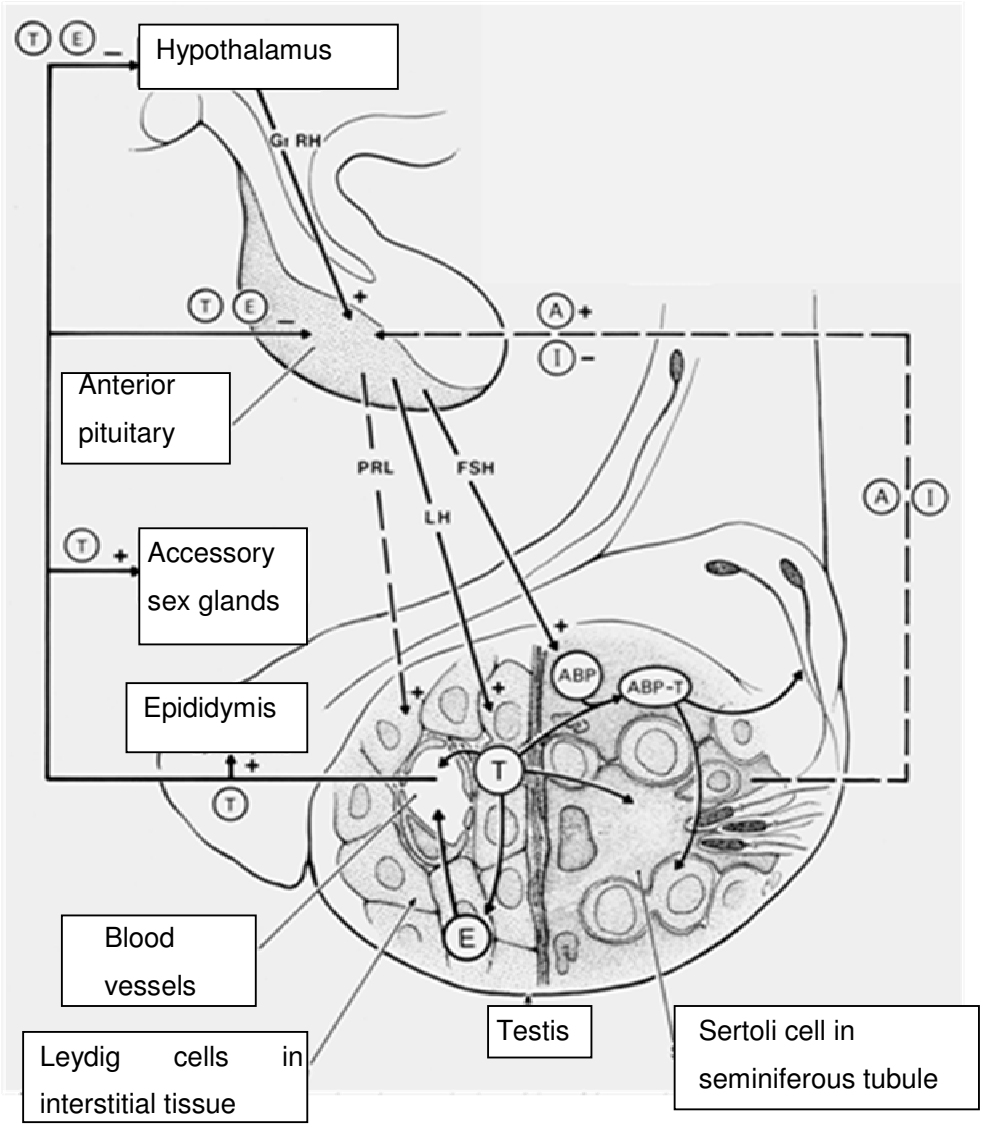


Fig. 4: Hormonal regulation during the spermatogenesis of the stallion. Interrrelationship of hypophyseal hormones acting on Leydig cells and Sertoli cells of the seminiferous tubules and feedback control of gonadal hormones on the hypothalamus and adenohypophysis. An increased level of testosterone in pheripheral blood as a result of increased production by the testes, feeds back on the hypothalamus and adenohypophysis to suppress discharge of GnRH and LH respectively. Circulating FSH acts directly on Sertoli cells, which secrete two protein hormones: inhibin and activin. Adequate concentrations of testosterone and FSH must be present to stimulate Sertoli cells to produce an environment appropriate for normal spermatogenesis. (A) activin, (ABP) androgen-binding protein, (E) estradiol or other estrogens, (GnRH) gonadotropin-release hormone, (I) inhibin, (LH) luteinizing hormone, (FSH) follicle-stimulating hormone, (PRL) prolactin, (T) testosterone (Pickett, 1989).

1.1.7 Mammalian spermatogenic cycle, stage and wave

The sperm cells are differentiating in distinctive associations. A spermatogenic cycle is defined as the time until the reappearance of the same stage within a given segment of the tubule (Parvinen et al., 1986). Each stage of the cycle follows in an orderly sequence along the length of the tubule and the distance between the same stages is called the spermatogenic wave (Bergmann, 2006). One tubule can contain numerous complete waves. Adjacent segments of the tubule communicate in some unknown manner. The number of stages in a particular species is thus defined as the number of morphologically recognizable germ cell associations within the testis. The number of stages within a spermatogenic cycle and the number of cycles required for the completion of spermatogenesis varies between species (Hess, 1990; Onyango et al., 2000) (Table 2).

Table 2: Stage of the seminiferous epithelium cycle (numbers) and duration of spermatogenesis (days) in various species (Russell et al., 1990)

Species	Number	Cycle
Boar	8	8.6
Bull	8	13.5
Dog	8	13.6
Human	6	16
Mouse	12	4.5
Rat	14	4.5
Stallion	8	12.2

1.1.8 Spermatogenesis of the stallion (*Equus caballus*)

In the adult stallion, billions of spermatozoa are produced daily in the convoluted seminiferous tubules. The testes of an adult stallion produce about 70.000 spermatozoa each second during the breeding season, production of each individual spermatozoon requires about 57 days (Johnson, 1985, 1990). When spermatozoa are liberated from seminiferous epithelium, fluid carries them from the convoluted seminiferous tubules into straight seminiferous tubules and the tubules of the rete testis where additional fluid may be added. The suspension of spermatozoa is moved rapidly through ductuli efferentes testis into the proximal epididymis. From histologic examination of stallion testes, detailed analyses resulted in recognition of eight different cellular associations, or stages based on four or five specific types of germinal cells grouped together (Fig. 5a, b) (Johnson et al., 1990; Amann, 1993).

The exact number of cellular association depends on the criteria used for identification of each grouping of germinal cells. In each stage or cellular association, the four or five types of germinal cells are associated in a specific layered pattern. Each layer is one generation of germinal cells, which is 12.2 days more developed than the layer below. The youngest generation is located along the wall or lamina propria of the seminiferous tubule. Older generations are found closer to the tubular lumen. In a normal testis, germinal cells are always found in these specific cellular associations or stages. The cells present in each cellular association can be determined by reading upward in a column from the lamina propria toward the tubular lumen (generations five to one) (Amann, 1993). The width of each column depicts the relative duration of each cellular association. If a fixed point within a seminiferous tubule would be viewed over time, germinal cells developing at that point would sequentially acquire the appearance of each of the eight cellular associations characteristic of stallions (Johnson et al., 1990).

Description of the individual stages of the equine spermatogenic cycle according to Johnson et al. (1990):

Stage I. From complete disappeareance of mature spermatids lining the tubular lumen to onset of elongation of spermatid nuclei

Stage II. From onset of elongation to end of elongation of spermatid nuclei

Stage III. From end of elongation of spermatid nuclei to start of the first meiotic division

Stage IV. From start of the first to end of the second meiotic division

Stage V. From end of the second meiotic division to initial appearance of type B2 spermatogonia

Stage VI. From initial appearance of type B2 spermatogonia to when all bundles of elongated spermatids begin to migrate towards the lumen of the seminiferous tubule

Stage VII. From the time all bundles of elongated spermatids have begun to migrate toward the tubular lumen until they reach the lumen and B2 spermatogonia are no longer present

Stage VIII. From appearance of preleptotene primary spermatocytes and when elongated spermatids line the tubular lumen until complete disappearance of mature spermatids lining the tubular lumen

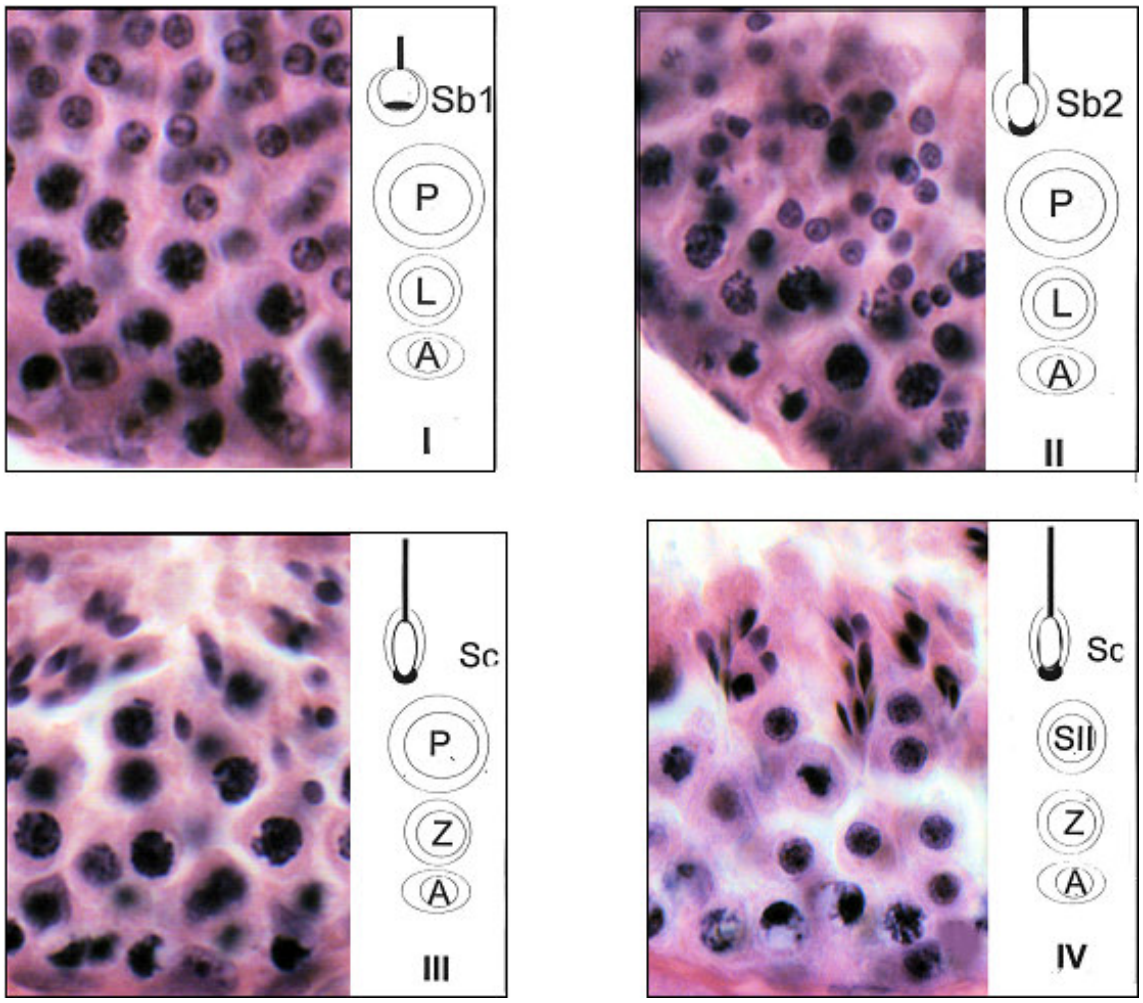


Fig. 5a: Stage I, II, III and IV of the cycle of the seminiferous epithelium during the spermatogenesis of the stallion according to Johnson et al., 1990. (A) spermatogonium type A, (L) leptotene spermatocyte, (Z) zygotene spermatocyte, (P) pachytene spermatocyte, (SII) second spermatocyte, (Sd1, Sd2) spermatids. Paraffin section with haematoxylin-eosin (H&E) staining. Primary magnification: x 40.

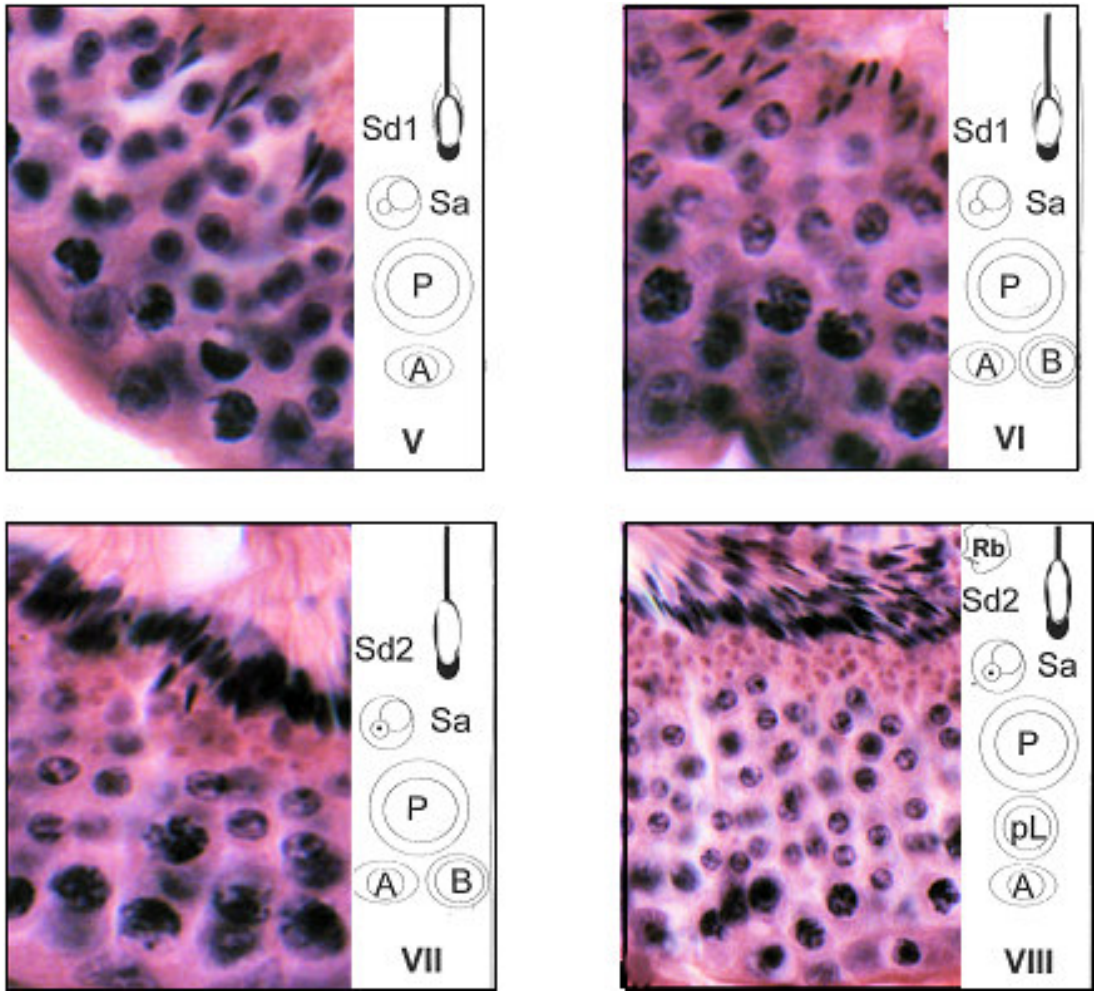


Fig. 5b: Stage V, VI, VII and VIII of the cycle of the seminiferous epithelium during the spermatogenesis of the stallion according to Johnson et al.,1990. (A) spermatogonium type A, (B) spermatogonium type B, (pL) preleptotene spermatocyte, (P) pachytene spermatocyte, (Sa) round spermatid, (Sd1, Sd2) spermatids, (Rb) residual body. Paraffin section with haematoxylin-eosin (H&E) staining. Primary magnification: x 40.

1.1.9 Cryptorchism in the stallion

The cryptorchism occurs spontaneously in many mammals and is relatively common in pigs, horses and humans. When the testis does not descend properly, spermatogenesis does not proceed and although androgens are produced, the secretion rate is usually lower than normal, particularly if the condition is unilateral (Cox, 1993), because then there is no compensatory stimulation by the increased levels of luteinizing hormone (LH) (Risbridger et al., 1981). Spermatogenesis can be initiated in an abdominal testis by cooling it artificially, so it appears that the temperature is the key (Frankenhuis and Wensing, 1979). The reason for the testis making this remarkable journey is not clear. Although the scrotal testis is sensitive to warming the body temperature, movement to a cooler environment cannot be the prime motive, since testicular migration within the abdominal cavity occurs in many mammals without any change in temperature (Carrick and Setchell, 1977). In stallions, cryptorchism is an anomaly and cryptorchid stallions have the distinct tendency to get spermatogenenic defects (spermatogenic arrest of spermatogonia, spermatogenic arrest of primary spermatocytes). Histologically, the seminiferous tubules of stallion cryptorchid testes are smaller than tubules of normal testes and contain many layers of epithelial cells at different stages of embryological differentiation, with scattered primordial germ cells (Al-Bagdadi et al., 1991).

There are four types of cryptorchidism according to Van der Velden (1990) and Mottershead (2000): (1) the hardest type is a complete abdominal retention. The testicle is fully retained in the abdomen and is mobile within the abdominal cavity. Typically this testicle will be small and flabby and the testicle is not externally palpable, (2) incomplete abdominal retention is the testicle retained and is not mobile within the abdominal cavity, but is usually located close to the deep inguinal ring, with portions of the testicles attached tissue passed through the vaginal ring, where they can sometimes be palpated externally with the horse standing and often when the horse is laid down under anaesthesia, (3) the permanent inguinal retention the testicle has descended through the deep inguinal ring, but is trapped within the inguinal canal, (4) temporary inguinal retention is the condition that holds the most hope for the horse's owner. The retained testicle may be palpable within the inguinal canal with the horse standing, but will certainly be palpable with the horse laid down under anaesthesia. The right testicle is the one most commonly affected (in more than three-quarters of cases). With this type of retention the testicle will usually descend spontaneously by the time the colt is three years of age.

1.2 Histone - Protamine replacement during the spermatogenesis

During spermiogenesis, haploid spermatids undergo complex morphological, biochemical, and physiological modifications that result in the formation of mature spermatozoa. The replacement of histones, the major protein constituents of eukaryotic chromatin, by protamines, the principal basic nucleoproteins of mature spermatozoa, causes the compaction state of the chromatin, resulting in profound changes in both nuclear shape and size (Steger et al., 1999). Until now the histone-protamine replacement has been investigated more closely in humans than in other mammalian species (Seyedin and Kistler, 1980; Meistrich et al., 1985; Koppel et al., 1994; Drabent et al., 1996, 1998; Steger et al., 1998). In the stallion spermatogenesis, histone to protamine exchange has not yet been examined so far. In mammals, at least six somatic subtypes (H1.1 - H1.5 and H1°), one oocyte-specific and two testis-specific linker histones H1t (Lennox et al., 1983) and HILS1 are expressed (Khochbin, 2001; Drabent et al., 1996; Iguchi et al., 2003).

During meiosis, part of the somatic histone variants (Franke et al., 1998) are gradually replaced by testis-specific subtypes, such as H1t (Doenecke et al., 1994, Bartell et al., 1996). Both histones and nonhistone proteins are replaced by transition protein and are then eliminated from the cell (Steger et al., 1999). In men with normal spermatogenesis, the testis-specific histone (H1t) mRNA can be observed in mid- and late pachytene spermatocytes (stage III - V), concomitant with the onset of H1t transcription. H1t protein appears in pachytene spermatocytes (stage III) and remains present as a nuclear protein constituent up to step 5 spermatids (stage V) (Steger et al., 1998).

In humans, histones are partially retained in nuclei of mature spermatids and spermatozoa and occur together with protamines, which first appear in nuclei of step 4 spermatids and persist in all sperm nuclei (Gatewood et al., 1990; Lelannic et al., 1993; Lescoat et al., 1993; Prigent et al., 1996). The nuclear status of sperm is an important parameter in assessment of male fertility. Therefore, it is evident that stringent temporal and stage-specific gene expression is a prerequisite for the correct differentiation of round spermatids into mature spermatozoa (Steger et al., 1999).

1.2.1 The linker histone

The basic unit of chromatin is the nucleosome, which consists of 146 base pairs of DNA wrapped around an octamer of core histones, including two molecules of H2A, H2B, H3 and H4 (Luger et al., 1997; Wolfe, 1998) (Fig. 6). In mammals, at least six somatic subtypes (H1.1 - H1.5 and H1°), one oocyte-specific and two testis-specific linker histones H1t (Lennox et al., 1983) and HILS1 are expressed (Khochbin, 2001; Drabent et al., 1996; Iguchi et al., 2003). Linker histones are essential for maintaining chromatin structure and regulating gene transcription in germinal cells. Testis-specific linker histone H1t binds more weakly to linker DNA than other H1 variants and may be important for DNA repair, although such a function has not been demonstrated in knockout mice. The H1t had also by far the least condensing activity (Khadake and Satyanarayana Rao, 1995). The testis-specific histone H1t is abundant in pachytene spermatocytes so far in rats, mice (Seyedin et al., 1981; Kremer and Kistler, 1991), and humans (Steger et al., 1999). Therefore it is critical for maintenance of chromatin structure and regulation of gene transcription in these cell types (Wilkerson et al., 2002a). The H1t promoter contains several elements found in the promoters of other H1 genes, but transcriptional regulation of the gene differs markedly from the other H1 family members. For example, H1a and H1c genes are also transcribed in primary spermatocytes but they are not tissue-specific like H1t. Several promoter elements are involved in the specific activation of the H1t promoter in spermatocytes, several additional elements are involved in repressing activity of the gene in non-expressing cell type (Wilkerson et al., 2002b). This may be important to treat some types of male infertility, therefore is necessary a better understanding of the transcriptional regulation by the H1t promoter or H1t promoter elements. (Wilkerson et al., 2002b).

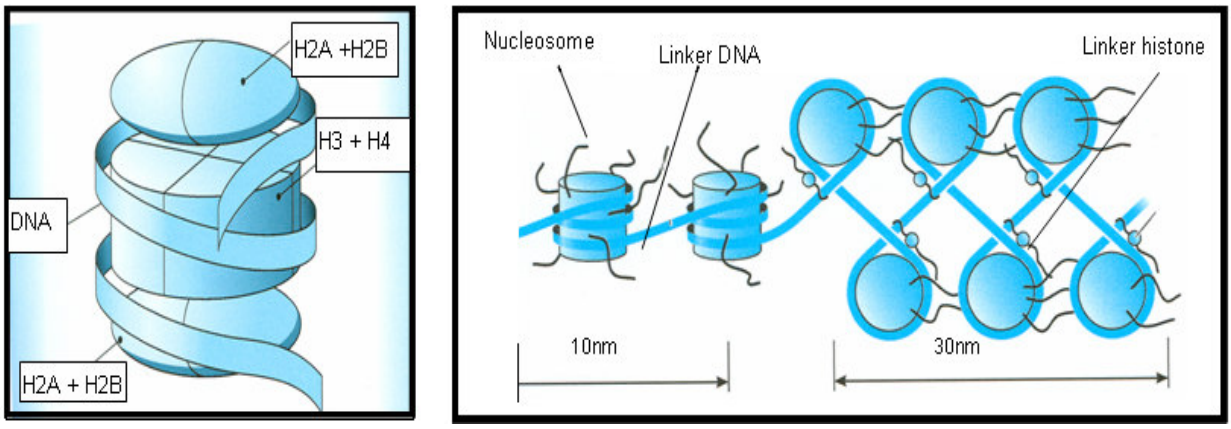


Fig. 6: H1 histones bind to the linker DNA between nucleosome core particles and facilitate the folding of nucleosomes into the 30 nm chromatin fiber and higher order chromatin structures (Wolfe, 1998).

1.2.2 Transcriptional regulation of the histone H1t gene

There is a high degree of similarity among histone H1 promoters. The proximal promoter of most H1 genes contain a GC- rich region within the leader region, a TATA box, a CCAAT box, a GC-box and an AC box (Osley, 1991). The steady state level of H1 mRNA appears to play an important role in rate of synthesis of the histones (Dominski and Marzluff, 1999). The most H1 histone genes including H1t are clustered on chromosome 6 in humans (Albig et al., 1993; Koppel et al., 1994). The highly conserved TATA binding protein (TBP) binds to the TATA box to start the formation of a transcription initiation complex (Nakajima et al., 1988). Factors important for enhanced transcription of the cell cycle-regulated H1 genes during S-phase bind to the CCAAT box. There are two proteins that have been described that bind to the CCAAT box. One of these is HiNF-B or H1TF-2 (van Wijnen et al., 1988a, b; Gallinari et al., 1989; Martinelli and Heintz, 1994), which is likely identical to NF-Y, the canonical heteromeric CCAAT box binding protein (Mantovani, 1999). HiNF-B and H1TF-2 are biochemically indistinguishable heteromeric DNA binding activities. The second protein, which recognizes both the histone H1 CCAAT box and AC box, is HiNF-D. It contains the homeodomain protein CDP-cut as its DNA binding subunit (van den Ent et al., 1994; van Wijnen et al., 1996; Nepveu, 2001).

Sp transcription family members, that either activate or repress transcription depending upon the specific factor, bind the GC-box. Sp1 is a potent transcriptional activator (Courey and Tjian, 1988), but Sp3 may activate or repress transcription, depending upon the gene and the

cell type involved (Hagen et al., 1994; Birnbaum et al., 1995). A transcriptional factor responsible for activating the H1 gene during S-phase of the cell cycle binds to the AC box, similar to the S-phase binding of factors to the CCAAT box (Coles and Wells, 1985).

1.2.3 Transcriptional activation of the H1t gene

Several regions within the testis-specific histone H1t promoter serve to activate transcription (Fig.7). The 40 bp TE element, important for transcription, contains three subelements. The TE1 and TE2 subelements are imperfect inverted repeats (Wolfe and Grimes, 1993; van Wert et al., 1998; Wilkerson et al., 2003), but only TE1 serves as a transcriptional activator (Wilkerson et al., 2002a; Wilkerson et al., 2003). The GC-box 1 located between these two subelements contributes to transcriptional activator (Wilkerson et al., 2002a, b). TE1 binds specifically to nuclear protein from primary spermatocytes to give a low mobility complex in electrophoretic mobility shift assays (Grimes et al., 1992a, b; Wolfe et al., 1995; van Wert et al., 1998).

No other tissue or cell type produces the low mobility TE complex that is seen with this probe. TE1 and TE2 have similar sequences. By the EMSA competition assays it has been shown that the TE1 probe competes in the binding of spermatocyte nuclear proteins with the TE2 probe and the TE2 probe competes in the binding with the TE1 probe (Wolfe et al., 1995). However, there are important functional differences in these two sites. TE2 appears to serve as a repressor binding site in some cell types (Wilkerson et al., 2003). The H1t promoter provides spermatocytes-specific transcription in transgenic mice (van Wert et al., 1995, 1998; Bartell et al., 1996). Mutagenesis of the TE site accomplished by replacing the entire TE element with a heterologous DNA fragment, leads to inactivation of the rat H1t promoter in transgenic mice (van Wert et al., 1998).

Sequences with homology to TE1 are found in several other testis genes. For example, the sequence is found in the LDHc promoter (van der Hoorn et al., 1991; Wilkerson et al., 2003), the D element of RT7 promoter (van der Hoorn and Tarnasky, 1992) and the D element of protamine 1 promoter (Johnson et al., 1988). However, it is not present in other testis-specific histones or core histone genes. Transgenic animal studies show an inactivation of the H1t promoter when the TE element is mutated. The biochemical protein-DNA binding studies and the sequence homologies with promoter elements in other testis genes suggests that nuclear transcription factors in primary spermatocytes bind to the TE1 and TE2 subelements (Wilkerson et al., 2002a). More recently has been demonstrated that both the TE1 subelement and the GC-box 1 are necessary for full activity of the H1t promoter in transient expression

assays (Wilkerson et al., 2002a). Mutagenesis of either site caused a decrease in reporter gene expression in testis Leydig cells, in the germinal derived GC-2spd cell line as well as in C127I and NIH3T3 cells but caused an increase in promoter activity in GC-2spd, Leydig, and C127 cells (Wilkerson et al., 2003).

Therefore, the TE1 and TE2 sites are functionally different and it is likely that different proteins bind to each site in vivo (Wilkerson et al., 2003). EMSA supershift assays show that Sp1 and Sp3 from total testis, from an enriched population of primary spermatocytes, and from cultured cell lines bind to the GC-box 1 (Wilkerson et al., 2003).

Western blots reveal the presence of both Sp1 and Sp3 in testis primary spermatocytes (Wilkerson et al., 2003), but the relative abundance of Sp1 and Sp3 variants change dramatically as germinal cells mature. The 95 and 105 KDa forms of Sp1 are abundant in several rat tissue and cell lines but a previously unidentified 60 KDa form is more abundant in spermatocytes and early spermatids (Wilkerson et al., 2002a, b). The levels of the typical Sp1 and Sp3 variants were highest in testis cells from sexually immature 9-day old rats, the levels dropped in spermatocytes and early spermatids and the levels were lowest in late spermatids (Wilkerson et al., 2002a).

Coexpression of Sp1 in transient transfection assays along with an H1t-promoter luciferase expression vector in GC-2spd germinal cell line led to a 6-fold increase in H1t promoter activity. Coexpression of Sp3 also unregulated the H1t promoter but only by 2-fold in GC-2spd cells. Expression of Sp1 and Sp3 unregulated the h1t promoter in other cell lines but to a lower degree. Upregulation was mediated primarily by GC-box 1, because an H1t promoter construct containing a mutant GC-box 1 had little change in activity with coexpression of Sp1 or Sp3 (Wilkerson et al., 2002b).

Finally, a region designated the H1t/Tg box, located in the distal promoter about 500 bp upstream from the H1t mRNA start site has been identified (Drabent and Doenecke, 1997). This element is reported to function cooperatively with the AC box to activate transcription in some cell lines (Drabent and Doenecke, 1997). Thus, several sites within the H1t proximal and distal promoter have been identified that can serve as binding sites for transcriptional activators in transient expression.

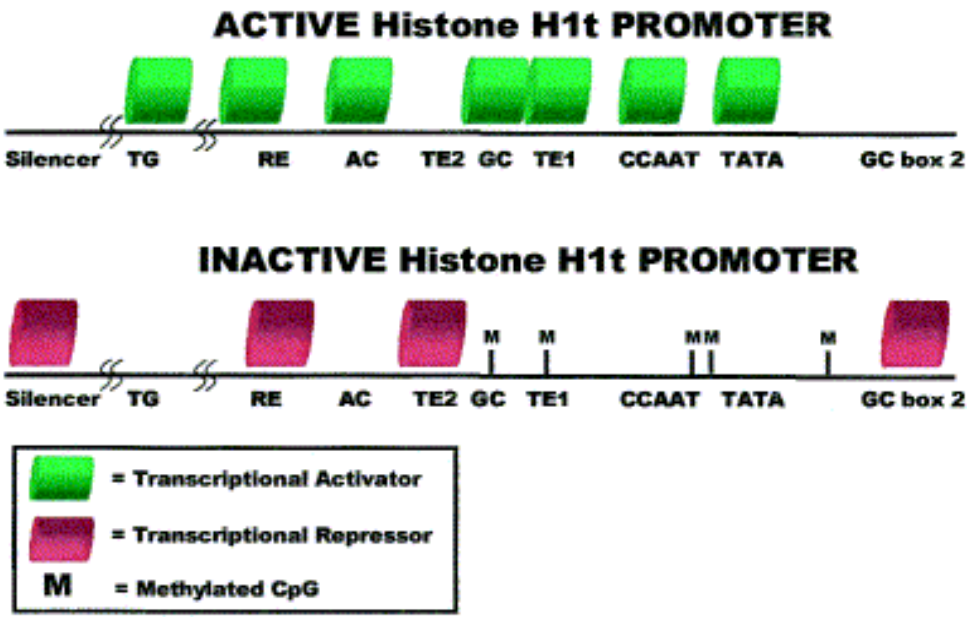


Fig. 7: Model of the histone H1t promoter. In the top panel the promoter is shown in the transcriptionally active state where transcriptional activators are shown bound to the TATA-box, CCAAT-box, the TE1 element, the GC-box 1, the AC-box and the 5′end of the RE element. In the lower panel the promoter is shown in the transcriptionally inactive state with transcriptional repressors shown bound to the GC-box 2, the TE2 element, the 3′ end of the RE element and to the distal silencer element. Methylated CpG dinucleotides are marked with an M (Grimes et al., 2003).

1.2.4 Transcriptional repression of the H1t gene

To fully understand the mechanisms of transcriptional regulation of the testis-specific histone gene, it is important to examine not only sequence elements that lead to transcriptional activation but also elements that lead to transcriptional repression (Fig. 7). Transcription of this gene is repressed in all cells types except primary spermatocytes and several proximal and distal promoter regions that contribute to repression have been identified (Wilkerson et al., 2002a). A GC-rich repressor region that is located downstream from the H1t TATA box (GC-box 2) is reported to be involved in silencing of the H1t gene in some non-germinal cells (Clare et al., 1997). Although Sp1 and Sp3 are reported to be able to bind to this element, other factors appear to repress H1t expression at this site. The TE2 subelement within the H1t/TE element serves as a repressor binding site in some cell lines (Wilkerson et al., 2003).

TE1 and TE2 are imperfect inverted repeats, but these two elements appear to have different functions depending upon the cell line being examined. Mutagenesis of TE1 leads to downregulation of H1t promoter activity in GC-2spd cells, C127 cells and NIH3T3 cells, while mutagenesis of TE2 leads to upregulation of promoter activity in GC-2spd cells, C127 cell and Leydig cells (Wilkerson et al., 2002a, 2003). This finding was surprising because both elements bind nuclear proteins from primary spermatocytes and both elements compete with each other when binding nuclear proteins in EMSAs (Wilkerson et al., 2002b). A sequence element, designated RE, is located in the proximal promoter between 130 and 106 bp upstream from the transcription start site (Wolfe and Grimes, 2003). The element spans approximately 24 base pairs and is centered 15 base pairs upstream from the 5´end of the AC box. The 3´end of the bipartite element serves as a binding site for a transcriptional repressor in several cell lines, but the 5´end of the element serves as a binding site for a transcriptional activator in primary spermatocytes. The repressor binding site functions in several cell lines such as NIH3T3 and GC-2spd cells, but the repressor binding proteins are clearly different from those that bind to the GC-box 2 element that is located downstream from the TATA box (Wilkerson et al., 2002b).

Deletion of the RE region leads to a 4-fold increase in H1t promoted reporter gene activity in NIH3T3 cells (Wolfe and Grimes, 1993). In comparison to the activity of other linker histone promoters, the activity of the H1t RE mutant rises to greater than 60 % of the activity of the wild type cell cycle-regulated H1d promoter. Although nuclear proteins from NIH3T3 cells bind the RE element, nuclear proteins that form the RE complex are not present in rat primary spermatocytes where the promoter is active. A factor that represses transcription binds to the 3´end of the bipartite RE element in cells where the H1t promoter is inactive, but a different factor, based in part upon mobility in EMSAs that activates transcription binds to the 5´end of the element in primary spermatocytes where the promoter is active (Wolfe and Grimes, 1993). A strong DNase I footprint forms over the RE element when nuclear proteins from primary spermatocytes are used for binding assays, but a footprint is not present over this region when using nuclear extract from rat liver.

This element is contained in an H1 promoter domain that also contains the H1/AC-box and that is involved in the binding of HiNF-A, a nuclear factor also found to bind to similar regions in promoter of human H4 and H3 histone genes (van Wijnen et al., 1988b). The 3´end of the RE element is conserved in many H1 genes and therefore may play a role in cell cycle regulation of the linker histones. Thus, the 3´end of the RE element, the TE2 subelement located within the H1t/TE element and the GC-box 2 downstream from the TATA box all

serve as binding sites for transcriptional repressors in the proximal promoter region of the histone H1t gene (Wilkerson et al., 2002b).

1.3 Aims of the study

It is known, that peripubertal stallions around two years of age show low fertility compared to older animals (Melo et al., 1998) whichs may be due to incomplete histone-protamine exchange during spermiogenesis. This premature subfertility has been associated with a prolonged H1 expression (Rizgalla, 2002). To our knowledge, this is the first study on H1t-gene and cell specific expression during normal and well-known premature subfertility during the peripubertal development in stallions' spermatogenesis.
The aim of the study was:

1. Clone the equine H1t-mRNA sequence.

2. To evaluate the stage- and cell-specific expression of testicular H1t on the mRNA and protein level during equine spermatogenesis.

3. To examine the possible relationship between the level of H1t gene expression and well-known premature subfertility in the stallion.

2 Materials and Methods

2.1 General histological methods

2.1.1 Tissue collection

Testicular samples for this study were collected from 24 castrated stallions at the Department of Equine Surgery, Justus-Liebig-University in Giessen. Equine organs from the Depatment of Veterinary Pathology, Justus-Liebig-University Giessen were used for this study. Tissue collection and conservation aimed at preserving protein and mRNA in order to study their expression patterns.

2.1.2 Paraffin material

A. Fixation

After castration, the samples were fixed in Bouin´s solution. For (in-vitro) RT-PCR, testicular samples were frozen immediately in liquid nitrogen and stored at minus 80° C until RNA extraction. The samples were fixed with Bouin´s for 24 hours. All testes samples were cut in small pieces for a better penetration of Bouin´s solution. After fixation, samples were washed daily during 7 days with fresh 70 % ethanol until complet elimination of the yellow colour. Subsequently, samples were put in plastic biopsy punnets and drained with the dehydration equipment.

Bouin´s solution:

- Picrin acid solution 15 ml
- Formalin 35% 5 ml
- Glacial acetic acid 1 ml

B. Dehydration

Thereafter, samples were immersed in series of graded ethanol for 24 hours to dehydrate the tissue, followed by xylene as a clearing agent and finally hot molten paraffin wax (impregnation).

Running program:

- Ethanol 80 % for 2 hours
- Ethanol 96 % for 2 hours
- Absolute ethanol for 3 hours
- Absolute ethanol for 3 hours
- Absolute ethanol for 3 hours
- Xylene for 1 hour
- Xylene for 45 minutes
- Xylene for 45 minutes
- Paraffin 59 ºC for 40 minutes
- Paraffin 59 ºC for 40 minutes
- Paraffin 59 ºC for 40 minutes

C. Embedding

In this process, the paraffin wax at 60 ºC will replace the water and allowed to cool and harden.

D. Surface coating of the slides

To obtain sufficient adhesion of the tissue sections, the slides have to be pretreated with APTEX:

- Sort the slides in a cuvette
- Wash 1 x in distillet water, dry off
- Wash 1 x in acetone, dry off
- Put the slides for 5 minutes in 2 % APTEX (4 ml APTEX in 196 ml acetone)
- Put the slides for 2 minutes in fresh tap water
- Wash the slides for 2 minutes with flowing tap water
- Put the slides for 2 minutes in di
- Slides overnight at 37 ºC into the dryer

E. Sectioning

After embedding in paraffin wax, the tissue was sectioned into 6 µm sections using a microtome. All the slides were put overnight into the dryer at 37 ºC.

F. Haematoxylin and eosin staining (H&E)

To facilitat evaluation of the tissue under a microscope, the slices have to be stained with one or more dyes. Haematoxylin and eosin (abbreviated H&E) are the most commonly used stains in histology and histopathology. Haematoxylin stains nuclei blue and eosin stains the cytoplasm pink.

H&E protocol:

- 3 x 10 minutes xylene
- 2 x 5 minutes absolute ethanol
- 1 x 5 minutes 96 % ethanol
- 1 x 5 minutes 80 % ethanol
- 1 x 5 minutes 70 % ethanol
- 1 x 5 minutes 50 % ethanol
- 1 x 5 minutes deionized water
- 7 minutes haematoxylin (Mayer)
- 15 minutes in running water
- 7 minutes in 1 % eosin
- Wash 1 x shortly in water
- Wash shortly in 70 % ethanol
- Wash shortly in 80 % ethanol
- 1 x 2 minutes 80 % ethanol
- 1 x 2 minutes 96 % ethanol
- 3 x 2 minutes absolute ethanol
- 3 x 10 minutes xylene
- cover the slides with glycerol gelatine

2.1.3 Histology of the testicular samples

In order to obtain testes with different spermatogenesis development were used: cryptorchid testes (spermatogonia arrest), testis from 6 month old animal (prespermatogonia/spermatogonia), 1 year old testes (spermatogonia/primary spermatocytes), 1. 5 years old testes (spermatogenic development until elongated spermatids) and adult testes (complete spermatogenesis) (Table 3) (Fig. 8-10).

After histological analyses, these testes were selected for RT-PCR, IHC, in situ hybridisation, qReal-Time PCR and Western blot analyses.

Table 3: Histological analysis of all the samples (le – left testis, ri – right testis, m – month, yr – year).

Sample Nr.		Age	Testis	Histology
1	295.04le	1.5 yrs	Inguinal	Spermatogonia arrest
2	01.05 ri	2 yrs	Inguinal	Spermatogonia arrest
3	319.04	6 m	Scrotal	Spermatogonia arrest
4	33.00 le	-	Inguinal	Spermatogonia arrest
5	34.05 ri	2 yrs	Abdominal	Spermatogonia arrest
6	72.05 le	2 yrs	Abdominal	Spermatogonia arrest
7	72.05 ri	2 yrs	Abdominal	Spermatogonia arrest
8	104.05ri	2.5 yrs	Inguinal	Spermatogonia arrest
9	29.02 le	1 yr	Scrotal	Primary spermatocyte arrest
10	29.02 ri	1 yr	Scrotal	Primary spermatocyte arrest
11	294.04ri	1.5 yrs	Scrotal	Elongated spermatids
12	295.04ri	1.5 yrs	Scrotal	Elongated spermatids
13	14.05 ri	3 yrs	Scrotal	Complete spermatogenesis
14	20.05le	3 yrs	Scrotal	Complete spermatogenesis
15	20.05ri	3 yrs	Scrotal	Complete spermatogenesis
16	40.05 le	2 yrs	Scrotal	Complete spermatogenesis
17	41.05 le	2 yrs	Scrotal	Complete spermatogenesis
18	41.05 re	2 yrs	Scrotal	Complete spermatogenesis
19	45.05 le	8 yrs	Scrotal	Complete spermatogenesis
20	45.05 ri	8 yrs	Scrotal	Complete spermatogenesis
21	293.04	4 yrs	Scrotal	Complete spermatogenesis
22	313.04	11 yrs	Scrotal	Complete spermatogenesis
23	317.04le	2 yrs	Scrotal	Complete spermatogenesis
24	317.04ri	2 yrs	Scrotal	Complete spermatogenesis

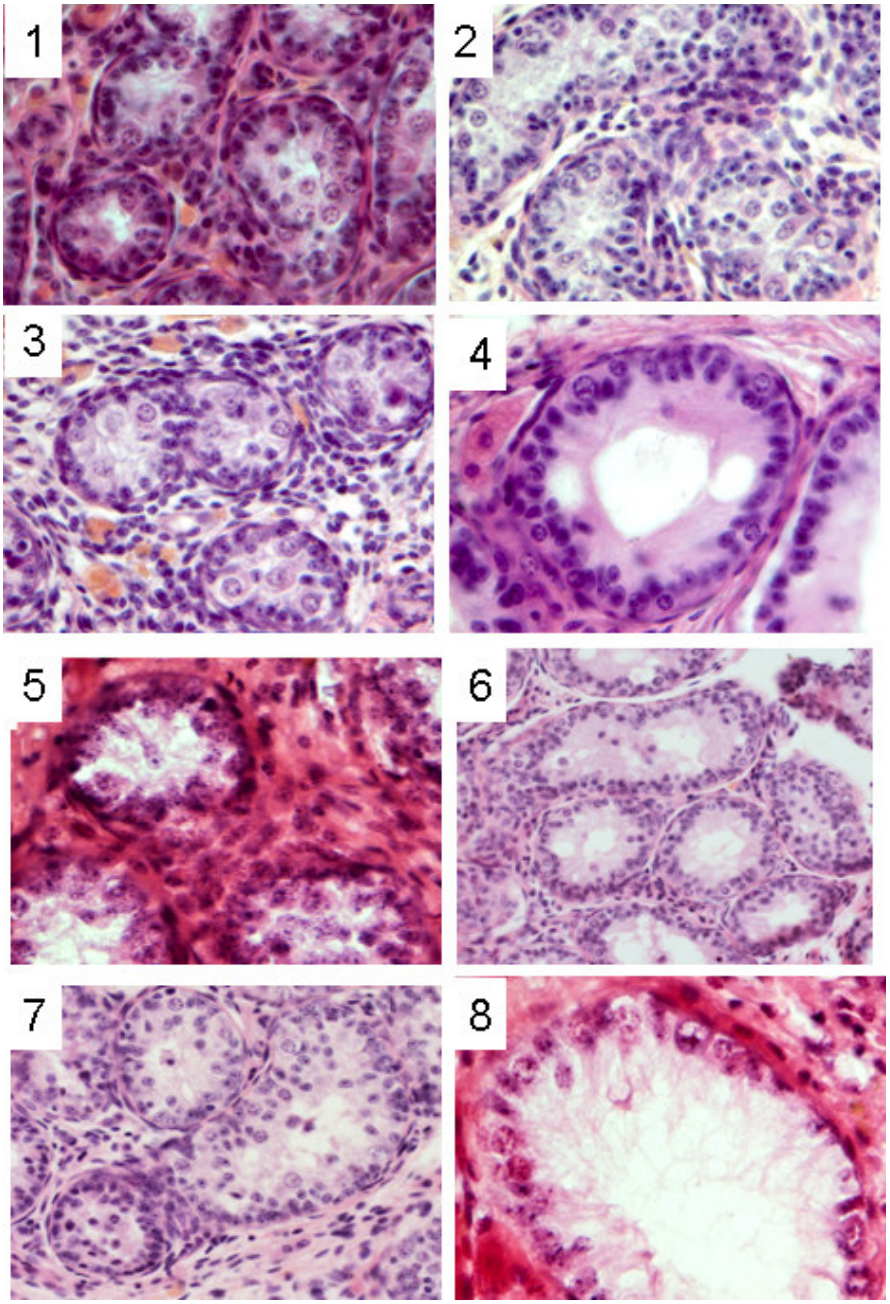


Fig. 8: (1-8) Seminiferous tubules of testes showing arrest of spermatogenesis at the level of spermatogonia. Paraffin section with haematoxylin-eosin (H&E) staining. Primary magnification x 40.

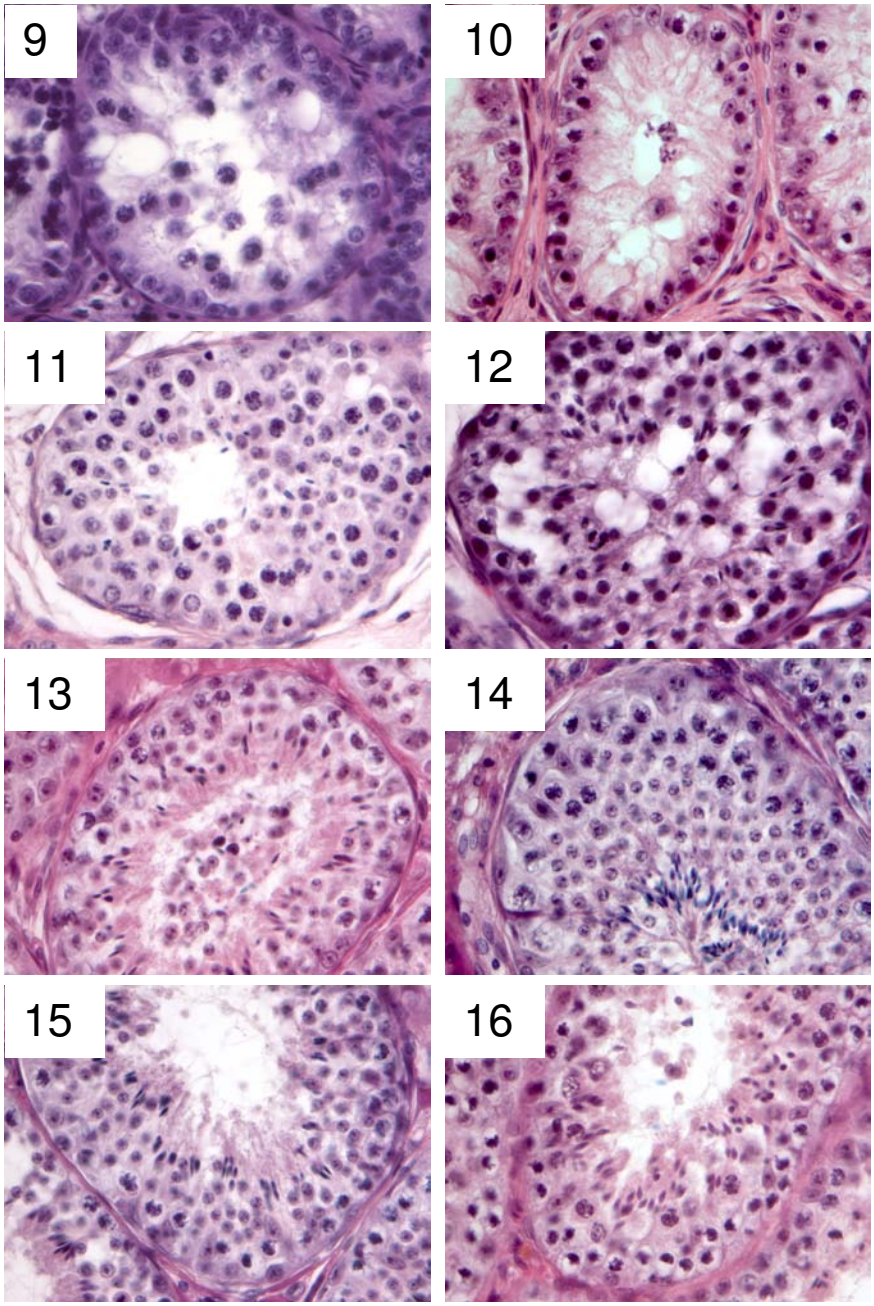


Fig. 9: (9-10) Seminiferous tubules of testes showing arrest of spermatogenesis at the level of primary spermatocytes, (11-12) Seminiferous tubule showing qualitative intact spermatogenesis, (13-16) Seminiferous tubules of testes showing normal stallion spermatogenesis. Paraffin section with haematoxylin-eosin (H&E) staining. Primary magnification x 40.

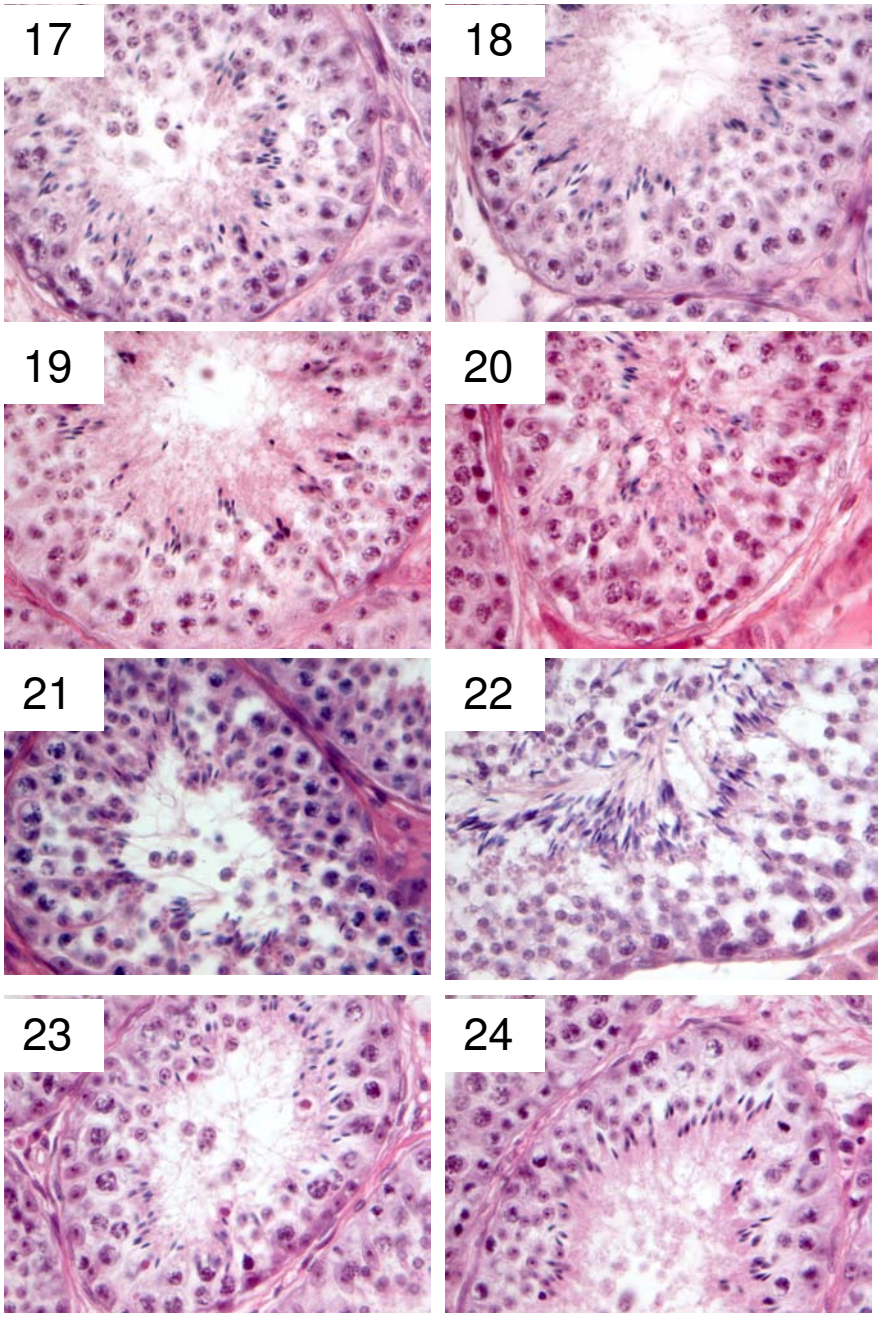


Fig. 10: (17-24) Seminiferous tubules showing complete spermatogenesis. Paraffin section with haematoxylin-eosin (H&E) staining. Primary magnification x 40.

2.2 General molecular biology methods

2.2.1 RNA isolation with TRIzol® Reagent

TRIZOL Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. Preparation of mRNA by means of Trizol reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is a sophisticated modification of the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). During sample homogenization or lysis, TRIZOL reagent maintains the integrity of the RNA. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol.

A. DEPC water preparation:

- 2 l distillet water plus 2 ml dietylpyrocarbonate (DEPC). Shake.
- Put for 1 hour at 37 °C into the dryer; autoclave overnight.

B. RNA-extraction:

- 0.2 N HCl: 5.2 ml 25 % HCl plus 200 ml DEPC water
- Weigh 100 mg of frozen tissue
- Homogenize the frozen tissue and add 1 ml TRIzol® Reagent
- Leave 5 minutes at room temperature
- Add 200 µl chloroform, homogenize well
- Leave 5 minutes at room temperature
- Centrifuge 15 minutes at 4 °C (18.000g)
- After the centrifugation, see 3 phases in the tube: colorless (RNA/DNA), the white (interphase) and red contains proteine (Fig. 11)

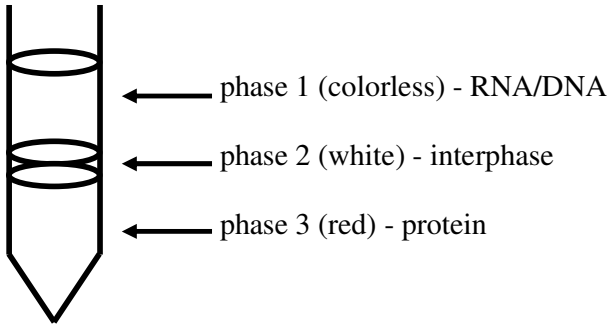


Fig.11: Separation of three phases after centrifugation.

C. Determination of the RNA concentration

For the determination of the RNA concentration the UVette® cuvette was be used. Place 69 µl of the buffer (0,1M Tris-HCL-buffer) and blank the cuvette. Then add 1 µl of the sample and mix well (but gently without making bubbles!) pipetting up and down.

D. DNase treatment

For the removal of contaminating DNA from RNA samples especially prior to RT-PCR experiments.

For 20 µl estimated:

- x µl RNA (circa 15 µg)
- 3 µl DNase I
- 2 µl 10x DNase I buffer
- x µl DEPC water
- Incubate 30 minutes in water bath at 37 °C
- Inactivate 10 minutes in water bath at 72 °C

2.2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

In molecular biology, reverse transcription polymerase chain reaction (RT-PCR) is a laboratory technique for amplifying a defined piece of a DNA molecule. The RNA strand is first reverse transcribed into its DNA complement or complementary DNA, followed by amplification of the resulting DNA using polymerase chain reaction. Polymerase chain reaction itself is the process used to amplify specific parts of a DNA molecule, via the enzyme DNA polymerase. In the first step of RT-PCR, called the "first strand reaction," complementary DNA is made from a messenger RNA template using dNTPs and a RNA-dependent DNA polymerase, reverse transcriptase, through the process of reverse transcription.

The above components are combined with a DNA primer in a reverse transcriptase buffer for one hour at 37 °C. After the reverse transcriptase reaction is complete and complementary, DNA has been generated from the original single-stranded mRNA, standard polymerase chain reaction, termed the "second strand reaction," is initiated (Bartlett and Stirling, 2003).

- A thermostable DNA polymerase and the upstream and downstream DNA primers are added
- The reaction is heated to temperatures above 37 °C to facilitate sequence specific binding of DNA primers to the cDNA
- Further heating allows the thermostable DNA polymerase (transcriptase) to make double-stranded DNA from the primer bound cDNA
- The reaction is heated to approximately 95 °C to separate the two DNA strands
- The reaction is cooled down for the annealing of the primers. The cycle will be repeated for several times.

After approximately 30 cycles, millions of copies of the sequence of interest are generated.

A. First strand cDNA synthesis

For first strand cDNA synthesis Superscript II RT polymerase (Qiagen, Hilden) was used.

Master Mix (Superscript II RT polymerase) for 20 µl estimated:

- x µl DEPC water
- 1 µl oligo dT-15 primer

- x µl RNA (circa 8 µg)
- 10 minutes 70 °C and 3 minutes cold water
- 4 µl 5x first strand buffer
- 2 µl 0,1 M DTT
- 1 µl 10 mM dNTPs mix
- 1 minute 37
- 1 µl Superscript II reverse transcriptase
- 1 h 37 °C

B. RT-PCR

RT-PCR master mix for 50 µl estimated:

- 36.5 µl DEPC water
- 5 µl 10x PCR puffer
- 4 µl 25 mM MgCL2
- 1 µl 10mM dNTPs mix
- 1 µl 5´primer (10 pmol)
- 1 µl 3´primer (10 pmol)
- 1 µl cDNA
- 0.5 µl Taq DNA polymerase

Subsequently, PCR was performed using the equine specific H1t oligonucleotide primers (Table 4) with the following cycling conditions:

- 1x 95 °C for 2 minutes
- 10x [45 °C for 1 minute, 65 °C for 1 minute, 72 °C for 2 minute]
- 20x [95 °C for 45 seconds, 60 °C for 30 seconds, 72 °C for 45 seconds]
- 72 °C for 8 minutes

ß-actin was used as positive control for the same cDNA preparations according to Buff et al., (2002).

Table 4: Oligonucleotide primers used for PCR.

Name	Orientation	Sequence (5'→3')	Fragment
H1t_cloning	Forward	GCG CGC CCT ACC CTA TAT AAG	334 bp
	Reverse	GGC TGT TAT TCT TCT CTA CGT CG	
H1t_stallion	Forward	GCC AGC AGC CCC AGC CGA AC	201 bp
	Reverse	CTG CCA GCG CCT TCT TGA GAG	
H1t_pPCR	Forward	AAG CCT CCA GCC AAG AAG CG	98 bp
	Reverse	CAA CTT GGA CAC AGA CGA ACC	
β-actin	Forward	ACA GGT CCT TAC GGA TGT GG	255 bp
	Reverse	TGG GTG ACA TCA AGG AGA AG	

2.2.3 Analysis of PCR products using agarose gel electrophoresis

Agarose gel electrophoresis is a method used in biochemistry and molecular biology to separate DNA strands by size and to estimate the size of the separated strands by comparison to known fragments (DNA ladder). This is achieved by pulling negatively charged DNA molecules through an agarose matrix with an electric field. Shorter molecules move faster than longer ones (Sambrook and Russel, 2001).

A. Making a 2 % agarose gel

- 1 g agarose powder
- 50 mL 1 x TAE buffer
- Heat 1 - 2 minutes

Allow to cool before putting 10 µl EtBr into the gel. Caution when working with the EtBr, the EtBr is a dangerous carcinogen. After pouring the liquid into the gel plates you have to be sure that the gel plates have been taped strongly.

B. Loading the gel

- Load a total of 15 µl of gel mixed with DNA sample
- 2 µl loading dye
- 10 µl DNA sample
- 3 µl RNA free water
- Let the gels run for 30 minutes from negative to positive.

C. Examining the gels

Place the gel on the UV transilluminator and look for orange and pink bands of DNA. There should be no bands visible in the negative control lane.

2.2.4 Measurement of nucleic acid samples

The NanoDrop® ND-1000 Spectrophotometer allows an accurately and reproducible measure of nucleic acid samples without dilution. To do this, the instrument automatically detects the high concentration to calculate the absorbance. A sample measurement output is shown below (Fig. 12). All the cDNA samples were calculated in 90 ng/µl.

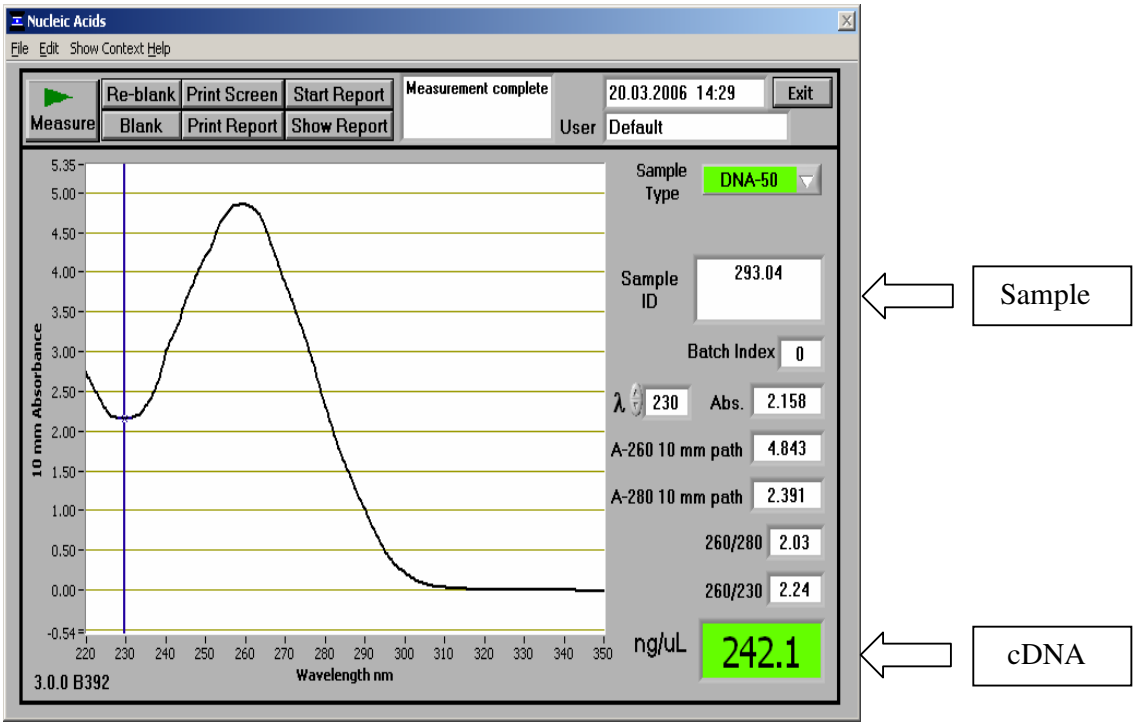


Fig. 12: Measurement of the cDNA samples for quantitative Real-time RT-PCR.

2.2.5 Quantitative Real-Time RT-PCR

The use of the real-time polymerase chain reaction (PCR) to amplify cDNA products reverse transcribed from mRNA is on the way to becoming a routine tool in molecular biology to study low abundance gene expression and provide the necessary accuracy and produces reliable as well as rapid quantification results. Real-time RT-PCR is highly sensitive and allows quantification of rate transcripts and small changes in gene expression (Pfaffl, 2001).
Real-time RT-PCR amplification was performed with the equine specific oligonucleotide primers H1t_pPCR_F/R using the qPCR Master Mix for SYBR Green I detection according to the manufacturer's protocol (EUROGENTEC, Seraing). All Real-Time reactions were run in the ICyclerIQ (Biorad) with the following thermal profile: a step 2 minutes 50° C, HotGoldstar activation /UNG inactivation 6 minutes 95 °C, followed by 40 amplification cycles each consisting of denaturation for 1 minutes at 95 °C, annealing for 30 seconds at 55 °C, and elongation for 30 seconds at 72 °C. As negative control we used kidney tissue and water as template. In this experiment we didn't use endogenous control because was very difficult to find a housekeeping-gene with a stabile expression in stallion testes. The relative H1t expression was calculated in the statistical analysis by the signal threshold cycle (Ct).

- Each 25 µl reaction contained:
- 12.5 µl of 2 x reaction buffer
- 0.75 µl diluted- SYBR Green I
- 2.5 µl of each H1t_ qPCR primers
- 4.25 µl of RNAse free water
- 5 µl of template cDNA

2.2.6 Statistical analysis

The statistical analysis was done with the statistical program package BMDP (Dixon, 1993). The repeated measurements of the quantitative real-time RT-PCR were summarized to arithmetic mean within a case. These data were analysed by two different ways of definition of the grouping.

In the First case, the data were grouped by the stage of cell development in the spermatogenesis: prespermatogonia/spermatogonia; primary spermatocytes; complete spermatogenesis. Secondly, the grouping was done by age: ≤ 2.5 years; ≥ 3 years.

In the first case, the groups were compared by a one-way analysis of variance (program BMDP70). In the case of global differences between the groups, a pair wise group comparison with the Tukey-test was done (BMDP7D).

In the second case, a non-linear regression analysis was performed to describe the relationship between mean Ct-value and age of the stallion (program BMDP3R). The non-linear regression model was: $Y = P1 \exp (P2.X) + P3$ with the following meanings: Y = measured mean Ct-value = dependent variable; X = age in years = independent variable; P1, P2 and P3 = model coefficients with P1 = total decrease of Ct-value; P2 = exponential decrease and P3 = final Ct-value for high age.

2.3 Partial cloning of the equine H1t-mRNA

In biology, it collectively refers to processes used to create copies of DNA fragments (Molecular Cloning), cells (Genetic Cloning), or organisms (Sambrook and Russel, 2001). BLAST search of the GenBank/EBI/DDBJ database with the human H1t sequence (GenBank Accession No.60094) revealed that no nucleotide sequence existed for the equine H1t gene. In order to get this sequence, we derived several oligonucleotid primers from the human H1t sequence and used it for RT-PCR amplification from 100 mg stallion testes RNA. A PCR fragment of 334 bp was obtained with the H1t_ cloning_ F/R primers (see Table 4) using the following thermocycling conditions:

- 1 cycle of 94 °C x 2 minutes
- 12 cycles of 94 °C x 15 seconds, 66°C minus 0.5 °C each cycle x 30 seconds
- 72 °C x 30 seconds
- 25 cycles of 94 °C x 15 seconds
- 60 °C x 30 seconds
- 72 ° C x 30 s plus 10 seconds each cycle

The amplicon was gel purified with Qiaex II (Quiagen, Hilden, Germany) and controlled by electrophoresis. Subsequently, the aplicons were cloned into the pT-Adv vector (Fig. 13).
Three different clones were sequenced on both strands using ABI 373 DNA sequencer. Consensus sequences of these clones were used to design oligonucleotid primers specific for equine H1t for further RT-PCR analyses. These primers are referred to as H1t_ stallion_ F/R (see Table 4).

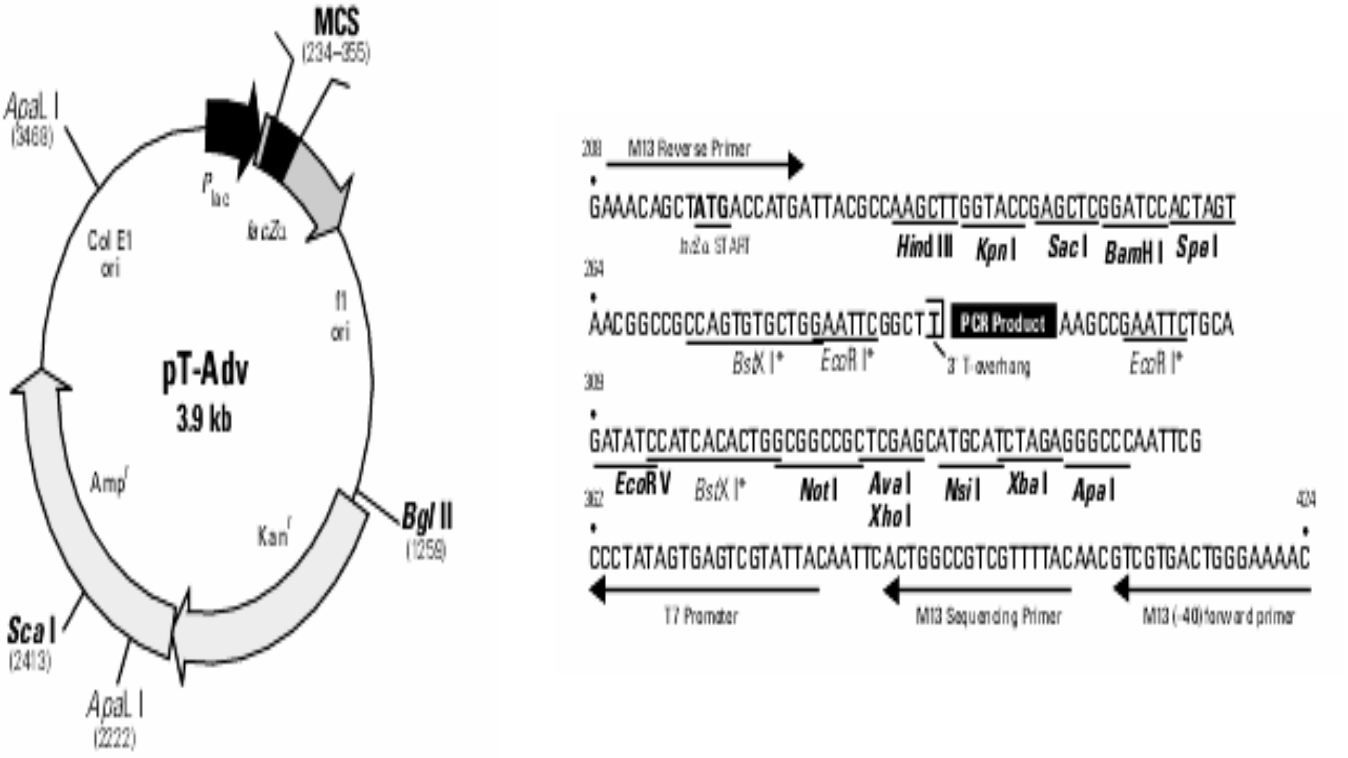


Fig. 13: Restriction Map and Multiple Cloning Site (MCS) of pT-Adv (Promega)**.** Unique restriction sites are in bold. Restriction sites with asterisks (*) are present only in the MCS and can be used to excise the inserted PCR product.

2.4 In situ hybridization of H1t in equine testis sections

In-situ hybridization, also referred to in hybridization histochemistry, was introduced in 1969 (Buongiorno-Nardelli and Amaldi, 1969; John et al., 1969). The basic technique utilizes the fact that DNA and RNA will undergo hydrogen bonding to complimentary sequences of DNA or RNA. By labeling sequences of DNA or RNA of sufficient length (approximately 50-300 base pairs), selective probes can be made to detect particular sequences of DNA or RNA. The application of these probes to tissue sections allows DNA or RNA to be localized within tissue regions and cell types. In-situ hybridization is a powerful technique and unique in the way that it allows studying the distribution and cellular localization of DNA and RNA sequences in a heterogeneous cell population.

2.4.1 Production of Digoxigenin (DIG)-labeled cRNA probes

DIG-labeled cRNA-probes were generated as described previously (Steger et al., 1998). Briefly, the 201 bp RT-PCR-product, which was generated with the H1t_stallion_F/R oligonucleotide primers from RNA of equine testis was subcloned into the pGEM-T vector (Promega, Mannheim) (Fig. 14). Plasmids were transformed in the XL1-Blue E. coli strain (Stratagene, Heidelberg) and extracted by column purification, according to the manufacturers' instructions (Qiagen, Hilden).

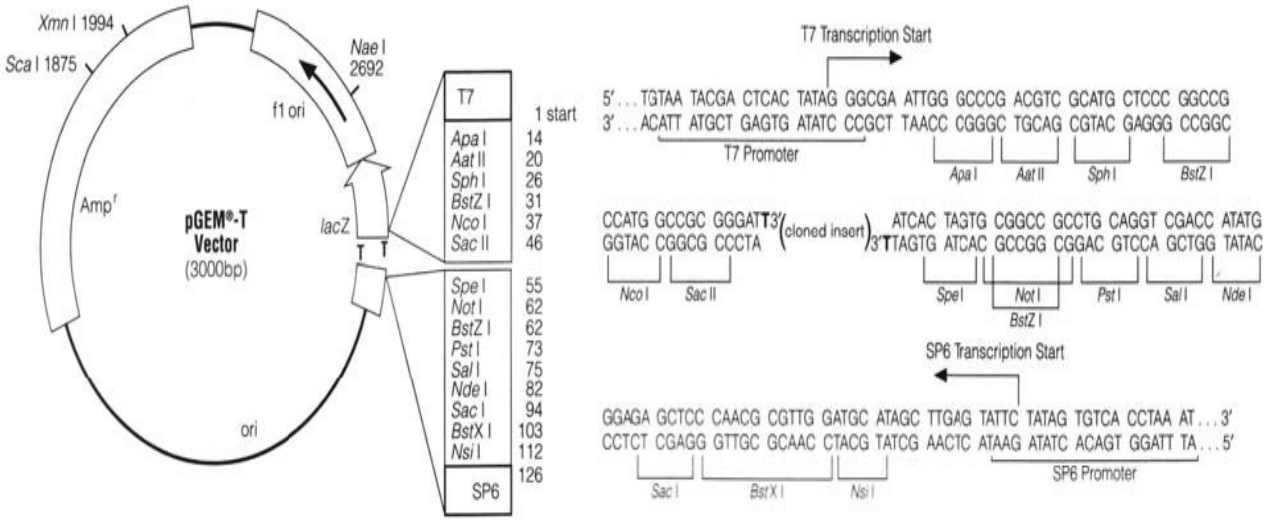


Fig. 14: Restriction Map and Multiple Cloning Site (MCS) of pGEM-T vector (pGEM-T (Promega) 3,0 kb Gesamtlänge, *lac*-Operon, Amp^r, T7-Promotor, SP6-Promotor, MCS (*Apa*I, *Aat*II, *Aph*I, *Nco*I, *Sac*II, *Spe*I, *Not*I, *Pst*I, *Sal*I, *Nde*I, *Sac*I, *Bst*XI, *Nsi*I).

A. Production of the H1t-sense-cRNA and H1t-anti-sense-cRNA

After sequencing, positive clones were digested with NcoI and NotI (New England Biolabs, Frankfurt) (Table 5) for the production of the H1t-sense-cRNA and H1t-antisense-cRNA, respectively (Fig. 15).

Table 5: Summarized formula for the single digestion protocol

Not	NCO
Plasmid 5 µl	Plasmid 5 µl
Enzyme 1 µl	Enzyme 1 µl
Buffer 3 2 µl	Buffer 4 2 µl
BSA 1 µl	--
DEPC water 11 µl	DEPC water 12 µl
Total 20 µl	Total 20 µl

- 1 hour at 37 °C
- 20 minutes at 65 °C
- DNA agarose gel for control
- Stocking at minus 20 °C

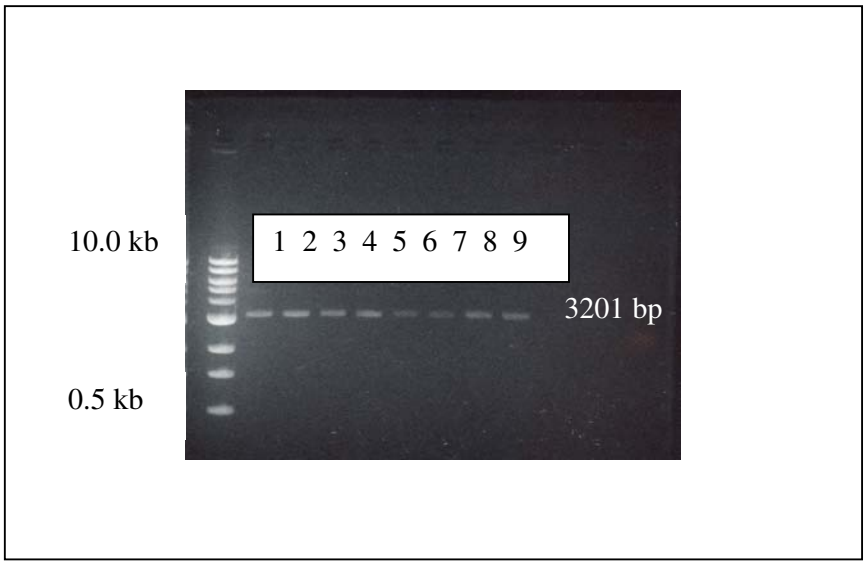


Fig. 15: H1t pGEM-T plasmid was digested for the production of cRNA sense and cRNA antisense :(1) K3 Not, (2) K3 NCO, (3) K4 Not, (4) K4 NCO, (6) K6 Not, (7) K6 NCO, (8) K7 Not, (9) K7 NCO.

B. In vitro transcription

Subsequently, in-vitro transcription was performed using the 10x RNA-DIG Labeling-Mix (Boehringer Mannheim, Mannheim) and T7 and SP6 RNA polymerases (Promega, Mannheim). The in vitro transcription was prepared according to the protocol of the Veterinary Anatomy Institute (Table 6).

Table 6: Summarized formula for the in vitro transcription protocol

DEPC water	8 µl
5 x transcription buffer	4 µl
10 x Dig RNA labeling mix	2 µl
100 x DTT	2 µl
Plasmid (single digestion)	2 µl
RNA polymerase SP6 / T7	2 µl

- 1 hour at 37 °C
- 0. 5 µl 0. 5 M EDTA solution (Sigma) to prevent degradation of RNA
- 1. 2 µl 8 M LiCL solution (Sigma) to precipitate the RNA
- 70. 7 µl 96 % ethanol minus 20 C to precipitate the RNA
- 1 hour at minus 80 °C
- Centrifugate for 20 minutes at 4 °C (13 000 UpM)
- Supernatant suck off
- 200 µl 75 % ethanol minus 20 °C
- Centrifugate for 15 minutes at 4 °C (13 000 UpM)
- Supernatant suck off
- Dry the pellet and resuspend the pellet with 50 µl DEPC water at 70 °C
- Stocking at minus 20 °C

2.4.2 In-situ hybridization

In-situ hybridization, as the name suggests, is a method to localize, either mRNA within the cytoplasm or DNA within chromosomes, by hybridizing the sequence of interest to a complimentary strand of a nucleotide probe. All fixations, washing and handling methods are run under sterile conditions to prevent RNase contamination.

A. Solution preparation

- 0. 2 N HCl: 5.2 ml 25 % HCl plus 200 ml dietylpyrocarbonate (DEPC) water
- 20 x SSC – permanent solution: in 1 l distilled water 88.23 g Natrium citrate ($C_6H_5O_7Na_3$ x $2H_2O$) plus 175.29 g NaCl, pH 7.0. Add 1 ml dietylpyrocarbonate (DEPC), shake; incubate overnight at 37 °C; autoclave
- 1 M $MgCl_2$- solution: 81.4 g $MgCl_2$ plus 400 ml dietylpyrocarbonate (DEPC) water
- PBSM-buffer: 200 ml dietylpyrocarbonate (DEPC) water plus 1 PBS-Tablet plus 1 ml 1 M $MgCl_2$-solution
- 0.2 % glycine-solution: 100 ml PBSM-Buffer plus 200 mg glycine
- 4 % paraformaldehyde solution: dissolve 25 g paraformaldehyde in 500 ml 1 x PBSM-buffer (under the flue with magnetic stirrer at 70°C). Add 4 N NaOH until the solution gets clear, maybe adjust the concentrated parameters with HCl to pH 7.0, aliquote and store at minus 20 °C
- 50 % dextran sulfate: in 10 ml dietylpyrocarbonate (DEPC) water plus 5 g dextran sulfate. For dissolving, leave a few days in the fridge, then aliquote and store at minus 20 °C
- Denhardt-reagent (BFP): in 10 ml dietylpyrocarbonate (DEPC) water solve 200 mg BSA plus 200 mg Ficoll 400 and 200 mg polyvinylpyrolidon, aliquote and store at minus 20 °C
- 10 x TNMT buffer: in 1 l distilled water 121.1 g Tris-HCl plus 58.4 g NaCl plus 4.17 g $MgCl_2$
- 5 x NTB buffer: 60.5 g Tris-HCl plus 29.2 g NaCl in 1 l distilled water dissolve, pH 9.6 adjust. Add 1 ml dietylpyrocarbonate (DEPC). Shake well and leave in the incubator overnight at 37 °C, autoclave

- 1 M levamisole: 10 ml 1 x NTB buffer plus 2.4 g levamisole, aliquote, store at minus 20 °C
- TBS buffer: 121.1 g Tris-HCl plus 58.4 g NaCl in 1 l distilled water, adjust to pH 7.4

B. Prearrangement at the previous day

Store slides and xylene cuvette at 60 °C into the incubator, overnight

C Preparation of the tissue sections

- Deparaffinize and rehydrate tissue sections with alcohol series
- Wash 5 minutes in xylene 60 °C
- Wash 2 x 5 minutes in xylene at room temperature
- Wash 2 x 5 minutes in 100 % ethanol
- Wash 1 x 5 minutes in 96 % ethanol
- Wash 1 x 5 minutes in 70 % ethanol
- Subsequently, take a steril cuvette
- Wash the tissue section with 1 x in dietylpyrocarbonate (DEPC) water
- Incubate 20 minutes in 0.2 N HCl
- Incubate 15 minutes at 70 °C in 2 x SSC
- Finally wash shortly in PBSM-buffer

D. Digestion with proteinase K

To promote an access of the labelled probes into the cells, the tissues were permeabilized with RNase-free proteinase K.

- Incubate the slides in a humid chamber with proteinase K (20 µg/ml) for 25 minutes in the incubator at 37 °C
- Stop the reaction incubate 1 x 5 minutes 0.2 % glycine

E. Blocking of endogenous phosphatase

- Wash the slides 15 seconds with 20 % acetic acid in dietylpyrocarbonate (DEPC) water
- Wash shortly in PBSM-buffer

F. Post fixation

In order to better fix the mRNAs onto slides, post fixation was carried out.

- Incubate the slides 10 minutes in 4 % paraformaldehyde solution
- Wash shortly in PBSM-buffer
- Incubate the slides one hour in 20 % glycerol in dietylpyrocarbonate (DEPC) water (prehybridization step)

G. Preparing the H1t cRNA probe mix

cRNA mix: the dilution of the H1t cRNA was 1:50

- 4 µl dietylpyrocarbonate (DEPC) water
- 4 µl Salmon-sperm-DNA (1 mg/ml)
- 8 µl Yeast-t-RNA (1 mg/ml)
- 4 µl DIG-cRNA

H. Preparing the hybridization buffer

- 52 µl dietylpyrocarbonate (DEPC) water
- 40 µl 20 x SSC
- 80 µl 50 % dextrane sulfate
- 8 µl Denhardt reagent
- 200 µl formamide

The labeled probes and hybridization buffer were mixed and put on slices (50 µl per slides).
Place the slides on the heating surface for 10 minutes at 70 °C (denaturation).
Afterwards put the slides directly onto the ice surface to cool down (stabilisation) and incubate into the incubator at 37 °C, overnight in a humid chamber with dietylpyrocarbonate (DEPC) water.

I. Post hybridization step

This step is funded by stringent washing, in order to eliminate non-specific cRNA binding. The non-specific background will be reduced by this procedure

- Wash 3 x 10 minutes in 4 x SSC at room temperature
- Wash 4 x 5 minutes in 4 x SSC at 42 °C
- Wash 1 x 15 minutes in 2 x SSC at 60 °C
- Wash 1 x 15 minutes in 0.2 x SSC at 42 °C
- Wash 1 x 5 minutes in 0.1 x SSC at room temperature
- Wash 1 x 5 minutes in 2 x SSC at room temperature

J. Immunohistochemistry

Buffer preparation:

1 x TNMT: 100 ml 10 x TNMT plus 900 ml distilled water

- Wash the slides 10 minutes in 1 x TNMT-buffer at room temperature
- Incubate one hour at room temperature in 3 % BSA plus 1 x TNMT buffer
- Preparing anti-DIG-antibody (Anti-Digoxigenin-AP Fab-fragment): Diluition 1:500 in TNMT-buffer
- Incubated with anti-DIG-antibody in humid chamber overnight at 4 °C

L. Detection

Buffer preparation:

1 x NTB: 20 ml 5 x NTB plus 5 ml 1 M $MgCl_2$ plus 75 ml distilled water

1 x TNMT: 100 ml 10 x TNMT plus 900 ml distilled water

- Wash the slides 2 x 10 minutes in 1 x TNMT buffer
- Wash 5 minutes in 1 x NTB buffer
- Wash 5 minutes in 50 ml 1 x NTB plus 250 µl 1M levamisole
- Develop with NBT-BCIP in a dark humid chamber (30 minutes or 1 hour)
- Stop with 1 x NBT
- Cover the slides with DAKO Glycergel® Mounting Medium

2.5 Antibody generation and verification of specificity

The cloned equine H1t cDNA fragment was in silico translated into protein (GenBank Accession No. CAI26255) and used for antibody production. Briefly, the synthetic peptide KPPAKKRGKKPVGL (Fig. 15) was coupled via the MAP residue to keyhole limpet hemocyanin and used to immunize two rabbits (Invitrogen, London). Antigenity of the rabbit serum was confirmed by Elisa analysis using the synthetic peptide as antigen and the polyclonal antiserum was affinity-purified (Invitrogen, London). An overview of the multiple sequence alignment and eH1t peptide are shown in the figure 16.

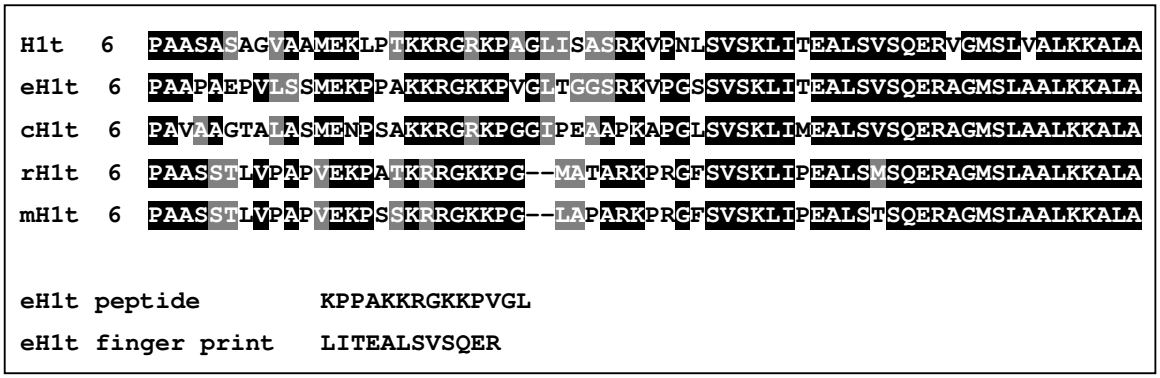


Fig. 16: Multiple alignment of human (H1T), rat (rH1t), mouse (mH1t), canine (cH1t) and equine (eH1t) H1t protein sequences. Deduced amino acid sequences were aligned using EBI *ClustalW* algorithm and alignment was visualized by BOXSHADE 3.21. Amino acid identity is displayed with *black* shading, while amino acid similarities are highlighted in *grey*. Amino acid sequences were deduced from the following GenBank Accession Nos.: rH1t (NM_012579), mH1t (NM_010377), H1T (NM_005323), and eH1t (AJ865320). Amino acid sequence of cH1t was derived from Ensembl (http://www.ensembl.org) orthologue prediction.

The H1t protein was separated in a preparative SDS 12 % gel. The appropriate band was excised from the gel and sequenced. In order to indentify the H1t protein, a peptide mass fingerprint analysis MS/MS was performed. In this method, the unknown protein of interest is first cleaved into smaller peptides, whose absolute masses can be accurately measured with a mass spectrometer such as MALDI-TOF or ESI-TOF. Thus, the Peptide Mass Fingerprint-Analysis MS/MS (TopLab, Martinsried) showed positive reaction for H1t (LITEALSVSQER) (Fig. 17).

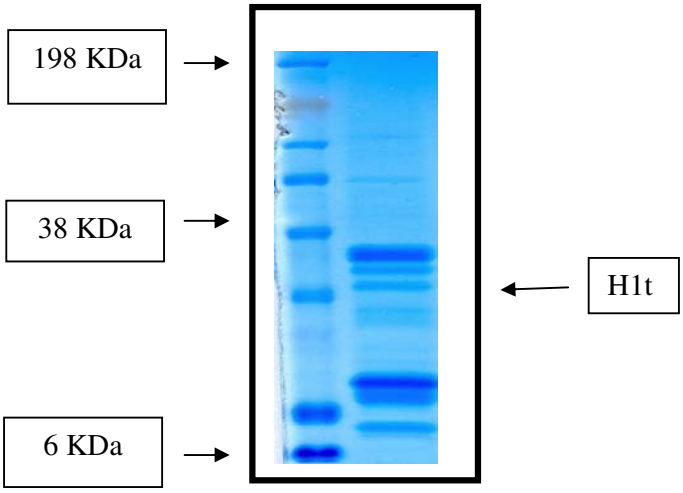


Fig. 17: SDS 12 % gel. The band number 4 was identified and sequenced. The H1t protein has a molecular weight of 29 kDa.

2.5.1 Histone isolation

For the isolation of histones from the nuclei, sulphurous acid must be used. This acid extraction removes histones from DNA and separates the core histone and linker histone from each other inside the nucleosome.

Buffer preparation:

Lysis Buffer:

- Tris - HCl 0.605 g
- $NaHSO_3$ 2.6 g
- Triton X-100 5 ml

- $MgCl_2$ 1.02 g
- Saccharose 43 g
- With distilled water in 500 ml fill and dissolving (pH 6.5 adjust)

Tris-EDTA Buffer:

- Tris 0.242 g
- EDTA 0.967 g
- Fill with 70 ml distilled water. Adjust the pH to 7.4
- 100 - 120 mg testicular tissue was homogenized and dissolved in 1 ml ice-cold lysis buffer
- Collect nuclei by centrifugation (18.000 g) for 10 minutes at 4 ºC
- Wash 3 x with lysis buffer
- Suspend nuclei with 100 µl Tris-EDTA buffer
- Add 1.1 µl conc. H_2SO_4 to the pellet and vortex. Incubate at 4 ºC for more than one hour to extract the histones from the nuclear pellet
- Take supernatant after high-speed centrifugation (18.000 g) for 10 minutes at 4 ºC
- Add 1 ml acetone to the supernatant
- After overnight incubation at 20 ºC, centrifugate (8.000 g) for 10 minutes at 4 C and wash 1x with acetone
- Air-dry at 37 ºC to give white powder of histone mixture containing H1, H1t, H2A, H2B und H4

Histones were then resuspended in 1 % sodium dodecyl sulphate (SDS)

2.5.2 Western blot analysis

Western blot is a method in molecular biology/biochemistry/immunogenetics to detect protein in tissue homogenate or extract. It uses gel electrophoresis to separate denatured proteins by mass. The proteins are then transferred out of the gel and onto a membrane (typically nitrocellulose), where they are "probed" using antibodies specific to the protein. As a result, researchers can examine the amount of protein in a given sample and compare levels between several groups (Towbin et al., 1979).

A. Prepare samples

The protein samples containing histone are boiled 5 to 10 minutes in a buffer solution (Laemmli's buffer - known as "sample buffer"), containing a buffer substance, normally tris base, dye, a sulfhydryl compound (typically beta-mercaptoethanol or Dithiothreitol (DTT - for reducing disulfide bonds), an anionic lipophilic detergent (sodium dodecyl sulfate - SDS) and glycerol to increase its buoyant density. The boiling denatures the proteins, unfolding them completely. SDS then surrounds the protein with a negative charge and the beta-mercaptoethanol prevents the reformation of disulfide bonds. The glycerol increases the density of the samples vs. the upper buffer in the gel tank and thus faciliates loading the samples as they will sink to the bottom of the gel pockets (Renart et al., 1979; Towbin et al., 1979; Burnette, 1981). The electrophoresis was prepared according to the instructions of the kit supplier by Invitrogen (Table 7).

Table 7: Summarized formula for the eletrophoresis protocol

Reagent	Reduced Sample	Non-Reduced Sample
Sample	X µl	X µl
NuPAGE LDS sample buffer (4 x)	2.5 µl	2.5 µl
NuPAGE Reducing Agent (10)	1 µl	--
Deionized water	To 6.5 µl	To 7.5 µl
Total volume	10 µl	10 µl

- Heat samples at 70 °C for 10 minutes
- Prepare 1 x SDS running buffer by adding 50 ml 20 x NuPAGE MOPS SDS running buffer to 950 ml of deionized water
- Load the appropriate concentration of your protein sample onto the gel
- Fill the upper buffer chamber with 200 ml 1 x NuPAGE SDS running buffer
- For reduced samples use 200 ml 1 x NuPAGE SDS running buffer containing 500 µl NuPAGE Antioxidant. Fill the lower buffer chamber with 600 ml 1 x NuPAGE SDS running buffer

Run conditions: Voltage: 200 V, run time: 50 minutes, Expected Current: 100-125 mA/gel (start); 60-80 mA /gel (end)

B. Electrophoresis of NuPAGE Gels (Procedure using Xcell SureLock Mini-Cell)

The proteins of the sample are separated according to molecular weight using gel electrophoresis. Gels have various formulations depending on the lab, molecular weight of the proteins of interest, and buffers available. Polyacrylamide gels are most common. Since the proteins travel only in one dimension along the gel, samples are loaded side-by-side into "wells" formed in the gel. Proteins are separated by mass into "bands" within each "lane" formed under the wells. One lane is reserved for a "marker" or "ladder," a commercially available mixture of proteins having defined molecular weights (Renart et al., 1979; Towbin et al., 1979; Burnette, 1981).

- Remove the NuPAGE gel from the pouch
- Rinse the gel cassette (12 % Bis-Tris gel) with deionized water. Peel off the tape from the bottom of the cassette
- In one smooth motion, gently pull the comb out of the cassette
- Rinse the sample wells with 1 x NuPAGE SDS running buffer
- Invert the gel and shake to remove the buffer. Repeat two more times
- Orient the two gels in the Mini-Cell such that the notched “well” side of the cassette faces inwards toward the buffer core
- Seat the gels on the bottom of the Mini-Cell and lock into place with the gel tension wedge
- Fill the upper chamber with a small amount of the running buffer to check for tightness of the seal
- Fill the upper buffer chamber (inner) with the appropriate 1 x running buffer
- The buffer level must exceed the level of the wells
- Load an appropriate volume of sample at the desired protein concentration onto the gel
- Load appropriate protein molecular weight markers
- Fill the lower (outer) chamber with 600 ml of the 1 x MOPS running buffer
- After electrophoresis is complete, shut off the power, disconnect electrodes, and remove gel from the Xcell *SureLock* Mini-Cell

- Separate each of the three bonded sides of the cassette by inserting the knife into the gap between the cassette two plates. The notched ("Well") side of the cassette should face up
- Push down gently on the knife handle to separate the plates. Repeat on each side of the cassette until the plates are completely separated
- Carefully remove and discard the top plate, allowing the gel to remain on the bottom (slotted) plate

C. Blotting (Using 20 x NuPAGE Tranfer buffer for one gel)

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or PVDF. The membrane is placed face-to-face with the gel, and current is applied to large plates on either side. The charged proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection. Both varieties of membrane are chosen for their non-specific protein binding properties (i.e. binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeat probings (Renart et al., 1979; Towbin et al., 1979; Burnette, 1981). The blotting was prepared according to the instructions of the kit supplier by Invitrogen (Table 8).

Table 8: Summarized formula for the blotting protocol

	Reduced Samples	**Non-reduced Samples**
NuPAGE Tranfer buffer	50 ml	50 ml
NuPAGE Antioxidant	1 ml	--
Methanol	100 ml	100 ml
Deionized Water	849 ml	850 ml
Total Volume	1000 ml	1000 ml

- Use about 700 ml of 1 x NuPAGE transfer buffer to soak the pads until saturated
- Remove the air bubbles by squeezing the pads while they are submerged in buffer
- Removing the air bubbles is essential as they can block the transfer of biomolecules if they are not removed

- Take a nitrocellulose membrane and place the membrane directly into a shallow dish containing 50 ml of 1 x NuPAGE transfer buffer for several minutes
- After opening the gel cassette, remove wells with the knife
- Place a piece of pre-soaked filter paper on top of the gel, and lay just above the slot in the bottom of the cassette, leaving the "foot" of the gel uncovered
- Keep the filter paper saturated with the transfer buffer and remove all trapped air bubbles by gently rolling over the surface using a glass pipette as a roller
- Turn the plate over so the gel and filter paper are facing downwards over a gloved hand or clean flat surface
- Use the knife to push the foot out of the slot in the plate and the gel will fall off.
- When the gel is on a surface, cut the foot off the gel with the knife
- Wet the surface of the gel with transfer buffer and position the pre-soaked transfer membrane on the gel, ensuring all air bubbles have been removed
- Place another pre-soaked anode filter paper on top of the membrane. Remove any trapped air bubbles (Fig. 18)
- Place two soaked blotting pads into the cathode (-) core of the blot module. The cathode core is the deeper of the two cores and the corresponding electrode plate is a darker shade of gray. Carefully pick up the gel membrane assembly and place on blotting pad in the same sequence, such that the gel is closed to the cathode core
- Add enough pre-soaked blotting pads to rise to 0.5 cm over rim of cathode core. Place the anode (+) core on the top of the pads. The gel membrane assembly should be held securely between the two halves of the blot module ensuring complete contact of all components
- Position the gel/membrane assembly and blotting pads in the cathode core of the Xcell II Blot Module to fit horizontally across the bottom of the unit. There should be a gap of approximately 1 cm at the top of the electrodes when the pads and assembly are in place
- Hold the blot module together firmly and slide it into the guide rails on the lower buffer chamber. The blot module will only fit into the unit one way, so the (+) sign can be seen in the upper left hand corner of the blot module. Properly placed, the inverted glod post on the right hand side of the blot module will fit into the hole next to the upright gold post on the right side of the lower buffer chamber

- Place the gel tension wedge so that its vertical face is against the blot module. Lock the gel tension wedge by pulling the lever forward
- Fill the blot module with 1 x NuPAGE transfer buffer until the gel / membrane assembly is covered in this buffer. Do not fill all the way to the top as this only generate extra conductivity and heat
- Fill the outer buffer chamber with deionized water by pouring approximately 650 ml in the gap between the front of the blot module and the front of the lower buffer chamber. The water level should reach approximately 2 cm from the top of the lower buffer chamber
- Place the lid on top of the unit

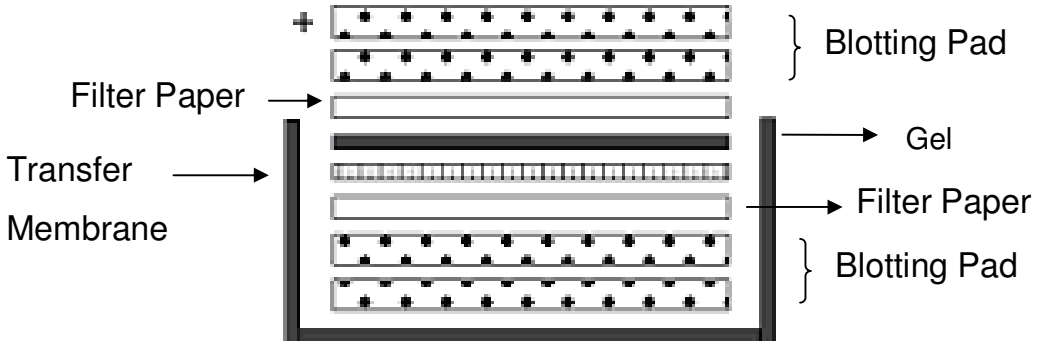


Fig. 18: Scheme of membrane blotting. According to Invitrogen protocol.

D. Transfer conditions for NuPAGE gels using the Xcell II Blot Module

Gel	Transfer Buffer	Membrane	Power Conditions
NuPAGE Novex Bis-Tris Gel	1 x NuPAGE transfer buffer with 10 % methanol	Nitrocellulose	30 Volts constant for 1 h Expected current: Start: 170 mA End: 110 mA

E. Coomassie Staining

Coomassie (also known as Brilliant Blue, Brilliant Blue G, Acid Blue 90, C.I. 42655, or Brilliant Blue G 250) is a blue dye commonly used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel is soaked in the dye for thirty minutes. Afterwards put the gel thirty minutes or more into a solution for decolourisation. This treatment allows the visualization of bands onto the gel. The visualization on the gel usually contains a set of molecular weight marker so that protein can be determined in a known position.

Staining solution:

- 2.5 g coomassie brilliant blue
- 455 ml methanol
- 455 ml deionized/distilled water
- 90 ml glacial acetic acid

Destaining solution:

- 455 ml methanol
- 455 ml deionized/distilled water
- 90 ml glacial acetic acid (but it can also be destained using only distilled water and heating)
- After blotting incubate the gel in this staining solution overnight at room temperature by gentle shaking
- Decant staining solution and add the decolourisation solution. Incubate the gel overnight at room temperature by shaking gently. The gel will have a clear background.

F. Immunodetection of proteins

Since the membrane has been chosen for its ability to bind protein and both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically bovine serum albumin (BSA) or non-fat dry milk, with a minute percentage of detergent such as tween 20. The protein in the dilute solution attaches to non-specific binding sites on the membrane. This reduces "noise" in the final product of the Western blot, leading to clearer results and eliminates false positive results.

During the detection process the membrane is "probed" for the protein of interest with antibodies, and links them to a reporter enzym, which drives a colorimetric or photometric signal. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications (Renart et al., 1979; Towbin et al., 1979; Burnette, 1981). The immunodetection of the proteins was prepared according to the instructions of the kit supplier by Invitrogen (Table 9).

Table 9: Summarized formula for the buffer preparation

PBS buffer	**PBS wash buffer + tween**	**PBS wash buffer - tween**	**Tris wash buffer**	**NTB buffer**
1 dragée PBS	100 ml PBS	200 ml PBS	50 ml Tris	40 ml 5 x NTB
200 ml deionized water	1 g BSA	2 g BSA	4.25 g NaCl	160 ml deionized water
-	1 ml Tween	-	0.5 ml Triton X-100	-
-	-	-	500 ml deionized water	-

- After the blotting, the membrane was blocked with 5 % non-fat dried milk dissolved in 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 0.1 % tween for 30 minutes by gentle agitation
- Wash the membrane 1 x short and 3 x 5 minutes in PBS wash buffer without tween
- Incubate the membrane with the primary polyclonal anti-H1t antibody overnight (1:100; Invitrogen, Karlsruhe)
- Wash the membrane 1 x shortly and 3 x 5 minutes at room temperature in PBS wash buffer with tween by gentle agitation
- Incubate the membrane with the secondary antibody (mouse - anti-rabbit/1:500; DAKO, Hamburg) at room temperature during 45 minutes
- Wash the membrane 1 x shortly and 2 x 5 minutes in PBS wash buffer with tween

- Incubate the membrane with the secondary rabbit anti-mouse dissolved 1:250 in 1 % PBS /1 % BSA / 1 % tween at room temperature during 45 minutes
- Wash the membrane 1 x shortly and 3 x 5 minutes in Tris wash buffer with tween
- Finally, incubate the membrane with APPAP 1:500 at room temperature during 45 minutes
- Wash the membrane 1 x shortly and 3 x 5 minutes in NTB wash buffer
- Incubate the membrane with NBT / BCIP for 20 minutes at room temperature
- Wash the membrane 2 x 5 minutes with deionized water
- Air dry the membrane at 37 °C

2.5.3 Immunohistochemistry

Immunohistochemistry refers to the process of localizing proteins in cells of a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. Visualising an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore, such as FITC, rhodamine, or Texas Red. The latter method is of great use in confocal laser scanning microscopy, which is highly sensitive and can also be used to visualise the interactions between multiple proteins. In 1981 a new generation of immunohistochemical methods emerged with the advent of the avidin-biotin methods, which remains widely used today (Hsu et al., 1981).

All avidin-biotin methods rely on the strong affinity of avidin or streptavidin for the vitamin biotin. Streptavidin (from Streptomyces avidinii) and avidin (from chicken egg) both possess four binding sites for biotin. The biotin molecules may be conjugated easily to antibodies and enzymes. In the avidin-biotin complex (ABC) method secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex (Heras et al., 1995) (Fig. 19).

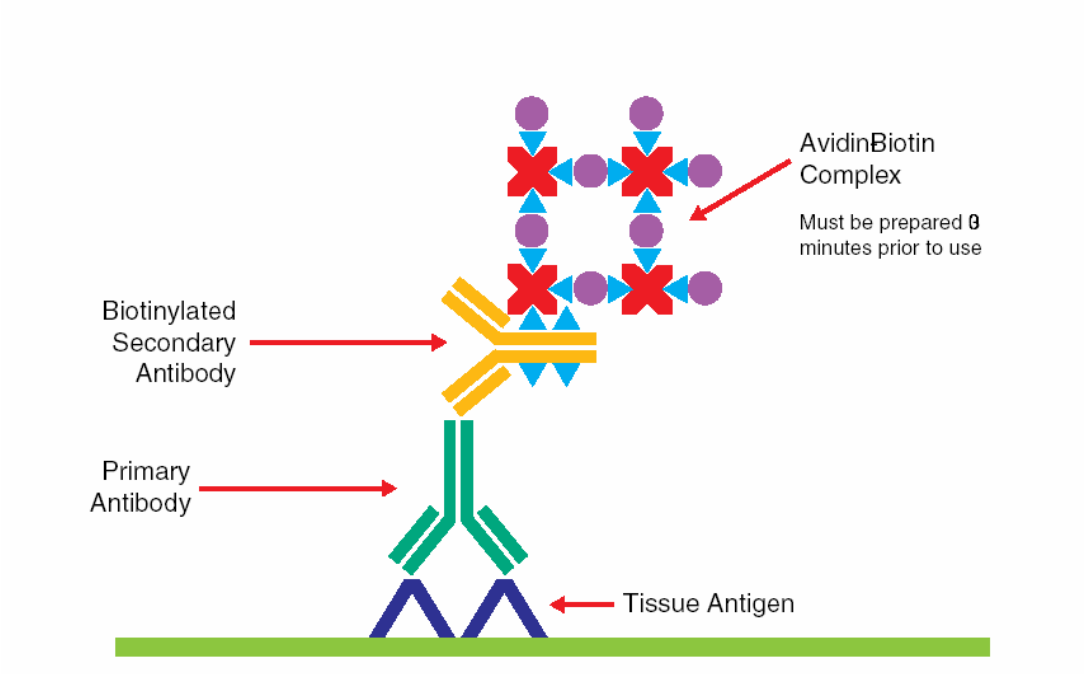


Fig. 19: Avidin-biotin complex (DAKO).

Table 10: Summarized formula for the buffer preparation

10 x Tris HCl buffer	**1 x Tris-HCl wash buffer**	**Block buffer**	**Proteinase K stock solution**	**20 % acetic acid**
121.1 g Tris HCL	100 ml 10x Tris-HCl	10 g BSA	10 mg proteinase K	160 ml DEPC water
58.4 g NaCL	1 ml Triton X.100	200 ml 1 x Tris-HCl	1 ml 1 x PBSM buffer	40 ml glacial acetic acid
1 l distilled water	900 ml distilled water	-	20 µl aliquots	-
pH 7.4	pH 7.6	-	-	-

A. Prearrangement at previous day

Leave the slices into the incubator at 37 °C overnight in order to obtain sufficient adhesion of the tissue sections.

B. Preparation of the tissue sections

Deparaffinize and rehydrate tissue sections with alcohol series:

- Wash 10 minutes in xylene (2x)
- Wash 2 x 5 minutes in 100 % ethanol
- Wash 1 x 5 minutes in 96 % ethanol
- Wash 1 x 5 minutes in 70 % ethanol
- Subsequently, take a sterile cuvette
- Wash the tissue section with 1 x distilled water
- Finally wash 10 minutes with 1 x Tris-HCl-buffer

C. Incubation with proteinase K

- Incubate the slides in a humid chamber with proteinase K (1.5 µl in 1000 µl) for 20 minutes in the incubator at 37 °C.
- Wash 3 x 5 minutes with 1 x Tris-HCl-buffer

D. ABC method

- Incubate the slides with 3 % of H_2O_2 in methanol for 30 minutes
- Finally wash 3 x 5 minutes with 1x Tris-HCl-buffer

E. Block

- Incubate the slides with 5 % BSA buffer during 30 - 60 minutes

F. Incubation of the primary antibody

- By polyclonal antibody: Incubate overnight at 4 °C (1:800)

G. Incubation of the secondary antibody

- Wash 3x 5 minutes with 1x Tris-HCl-buffer
- Incubate with goat-anti-rabbit BIOT (1:250) for 60 minutes
- Wash 3 x 5 minutes with 1x Tris-HCl-buffer

H. ABC complex

- Incubate with ABC complex during 60 minutes
- Wash 3 x 5 minutes with 1x Tris-HCl-buffer

I. Detection

- Develop with 3,3´- diaminobenzidinetrahydrochloride (DAB) or 3-amino-9-ethylcarbazole (AEC) in a dark humid chamber (2-10 minutes)
- Stop with distilled water
- Cover with DAKO Glycergel® Mounting Medium

2.7 General chemicals and reagents

(3-Aminopropyl) triethoxysilane 3-Aminopropyl-ethoxysilane (APEX); Sigma-Aldrich Chemie GmbH, Munich

Bovine Serum Albumin (BSA); Fluka Chemie AG Buchs, Switzerland

100x BSA solution; New England Biolabs, Frankfurt

Chloroform; Roth, Karlsruhe

4',6-diamidino-2-phenylindole (DAB); Research Genetics, Karlsruhe

Dextrane sulfate; Sigma-Aldrich Chemie GmbH, Munich

Dietylpyrocarbonate (DEPC); Sigma-Aldrich Chemie GmbH, Munich

Dimethyl formamide; Sigma-Aldrich GmbH, Taufkirchen

DNA-Purification systems QIAEX II, Qiagen, Hilden

DNA-Ligase; Promega, Mannheim

DTT (Dithiothreitol); Sigma-Aldrich GmbH, Taufkirchen

EDTA (ethylenediaminetetraacetic acid); Sigma-Aldrich, Steinheim

Acetic acid; Merck, Darmstadt

Ethyl alcohol; Schmitt, Dillenburg

First strand buffer; Gibco BRL, Eggenstein

Formamid; Sigma-Aldrich GmbH, Taufkirchen

Formalin 35%; Sigma-Aldrich GmbH, Taufkirchen

Glycine; Sigma-Aldrich GmbH, Taufkirchen

HCl; Merck, Darmstadt

Isopropanol; Roth, Karlsruhe

Laemmli-Sample Buffer; Sigma-Aldrich GmbH, Taufkirchen

LB-Medium; Quantum Appligene, Heidelberg

LB-Agar-Medium; Quantum Appligene

Levamisole; Sigma-Aldrich GmbH, Taufkirchen

Ligase-Buffer; Promega, Mannheim

Lithium Chloride-Solution (LiCl); Sigma-Aldrich GmbH, Taufkirchen

Magnesium Chloride ($MgCl_2$), Serva, Heidelberg

Methanol; Merck, Darmstadt

Milk powder; Milchhof, Marburg

$MgCl_2$; Promega, Mannheim

Sodium Citrate ($C_6H_5O_7Na_3$ x $2H_2O$); Sigma-Aldrich GmbH, Taufkirchen

Sodium Chloride (NaCl); Merck, Haar

NBT/BCIP (Nitro-Blue-Tetrazolium / Bromo-Chloro-Indolyl-Phosphat) (Phosphatase Substrat); KPL, Wedel

Paraformaldehyde; Merck, Haar

Phosphate buffered saline (PBS); Sigma-Aldrich GmbH, Taufkirchen

PCR-Puffer; Gibco BRL, Eggenstein

Picric acid; Riedel-deHaen, Seelze

Polyvinyl pyrrolidone; Sigma-Aldrich GmbH, Taufkirchen

Prestained Precision Protein Standard; Bio-Rad, Munich

Salmon-sperm-DNA; Sigma-Aldrich GmbH, Taufkirchen

Tetracyclin (1.5 mg); Stratagene, Heidelberg

Tris-HCl; Sigma-Aldrich GmbH, Taufkirchen

Triton X-100; Merck, Darmstadt

TRIzol®; Invitrogen, Karlsruhe

Tween 20-R, Roth, Karlsruhe

5-bromo-4-chloro-3-indolyl-beta-D-galactosidase (X-Gal); Invitrogen, Karlsruhe

Xylene; Roth, Karlsruhe

Yeast-t-RNA; Sigma-Aldrich GmbH, Taufkirchen

Goat serum; Vector, Burlingame, USA

2.7.1 Antibodies

Anti testis-specific histone (horse); Invitrogen, Karlsuhe

Anti-Digoxigenin-AP Fab-Fragment; Roche, Mannheim

Goat-Anti-Rabbit secondary antibody; DAKO, Hamburg

2.7.2 Equipment

Blotting apparatus (Trans-Blot® SD-Dry Transfer Cell); Bio-Rad, Munich

Embedding machine (Leica EG 1160); Leica, Bensheim

Dewatering apparatus (Leica TP 1050); Leica, Bensheim

Vacuumtissue infiltation automat; Leica, Bensheim

Gel electrophoresis apparatus Agagel Mini®; Biometra, Göttingen

Hot plate; MAGV, Rabenau-Londorf

Light microscope (Leica DM LB); Leica Wetzlar

3-CCD Color Video Camera KY-F55B; JVC Friedberg

Mini-Protean 3® Electrophoresis Cell-Systems; Bio-Rad, Munich

Scanner AV630; Avision Inc, Taiwan

Microtome, Leica SM 2000 R; Leica, Bensheim

Sterilisator; Heraeus, Rabenau

PowerPac 200 Power Supply; Bio-Rad, Munich

T3-Thermocycler; Biometra, Göttingen

Dryer; Memmert, Schwabach

Ultra-Turrax-homogenisator T8; IKA Labortechnik, Staufen

Water bath; Memmert, Schwabach

Centrifuge MIKRO 22R; Hettich, Tuttlingen

2.7.3 Others materials

Single cuvette (Eppendorf UVetten®); Eppendorf, Hamburg

Digoxigenin RNA-Labelling-Mix; Boehringer, Mannheim

DNase I; Roche, Mannheim

DNA-Ladder („100 bp“); New England Biolabs, Frankfurt

dNTP; Promega, Mannheim

Primer Oligo dT-15; Promega, Mannheim

PVDF-Membran (Westran® Schleicher & Schuell); Schleicher & Schuell GmbH, Dassel

Filter paper; Whatman 3MM; Whatman GmbH, Rothenburg

pGEM-T Vector; Promega, Mannheim

Escherichia coli, Eco XL-1 Blue; Stratagene, Heidelberg

Restriction enzyme (Not-I, Nco-I); New England Biolabs, Frankfurt

Superscript II Reverse Transcriptase; Gibco BRL, Eggenstein

RNA-Polymerasen (T7 and SP-6); Promega, Mannheim

Taq DNA-Polymerase; Gibco BRL, Eggenstein

Transcription buffer; Boehringer Mannheim, Mannheim

2.7.4 Kits

qReal Time PCR; EUROGENTEC, Seraing, Belgium

QIAEX II Kit; Qiagen, Hilden

QIAprep Spin Plasmid Kit; Qiagen, Hilden

2.8 Abbreviations

%	Per cent
° C	centrigade
ABC	Avidin biotin complex
bp	Base pair
BSA	bovine serum albumin
cDNA	Complementary DNA
cRNA	Complementary RNA
DEPC	Diethylpyrocarbonate
DNA	Desoxyribonucleic acid
dNPTs	Deoxyribonucleoside triphosphate
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic
Fig.	Figure
g	gram
h	hour
kb	kilo base
kDa	kilo Dalton
l	liter
M	molar
MCS	multiple cloning site
MDR	multidrug-resistance
Min	minute
MOPS	3-N(Morpholino) Propane Sulfonic Acid
mRNA	messenger RNA
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	rotations per minute
RT	Room temperature
RT-PCR	reverse transciptase PCR

s	second
SDS	Sodium docecyl sulphate
SSC	Sodium choride – Sodium citrat solution
TAE	Tris-acetate- EDTA buffer
Tm	melting temperature of a primer
Tris	trishydroxymethylaminomethane
Triton x-100	octyl phenol ethoxylate
U	Unit
UV	Ultraviolet

3 Results

3.1 The testis specific histone (H1t) and its expression in the testis

3.1.1 H1t sequence and Genbank entreis

➔ LOCUS aJ865320 198 bp mRNA linear MAM 24-NOV-2004

DEFINITION Equus caballus partial mRNA for testis-specific histone H1t (h1t gene).

ACCESSION AJ865320

VERSION AJ865320.1 GI: 56237696

KEYWORDS h1t gene; testis-specific histone.

SOURCE Equus caballus (horse)

ORGANISM Equus caballus

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Perissodactyla; Equidae; Equus.

Translation="PAAPAEPVLSSMEKPPAKKRGKKPVGLTGGSRKVPGSSVSKLIT
EALSVSQERAGMSLAALKKALA"

ORIGIN

1 ccagcagccc cagccgaacc tgttttatct tctatggaga agcctccagc caagaagcga

61 gggaagaagc cggttggctt gacgggtgga agtcgcaaag ttcctggttc gtctgtgtcc

121 aagttgatca ctgaggctct ctcagtgtcc caggagcgag cgggcatgtc gctggccgct

181 ctcaagaagg cgctggca

In order to classify the new *Equus caballus* H1t protein sequence BLAST analyses were performed of the NCBI/EBI/DDBJ databases with the H1ST-domain sequence of subfamily-H1T as query. We obtained a list of human sequences, exhibiting a certain homology with equine H1t protein. Multiple amino acid sequence alignment was performed with *ClustW* algorithm (http://www.ebi.ac.uk/ckustalw/) and the phylogenetic tree was visualized using TreeFiew (Fig. 20).

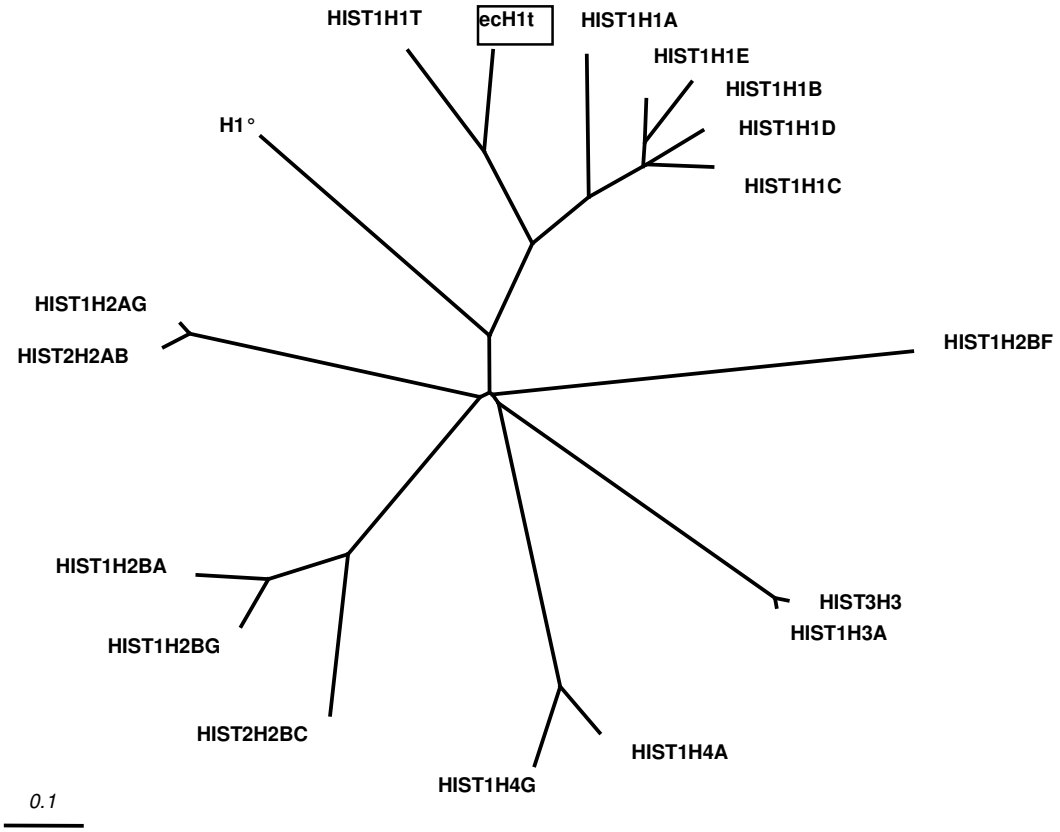


Fig. 20: Unrooted protein tree of selected members of the HIST-family and assignment of the *Equus caballus* H1t protein (ecH1t) sequence to HIST-subfamily 1H1T. The scale bar indicates the distance as calculated from the multiple alignments.

Using oligonucleotide primers derived from the human H1t sequence (GenBank Accession No. M97755), a RT-PCR product of the expected size was obtained from stallion testis RNA (Fig.21). This fragment was cloned and the sequence was deposited in the GenBank database with Accession No. AJ865320. According to this sequence, equine H1t-specific oligonucleotide primers were designed and used to investigate the stage-specific and quantitative expression of the H1t gene during normal spermatogenesis.

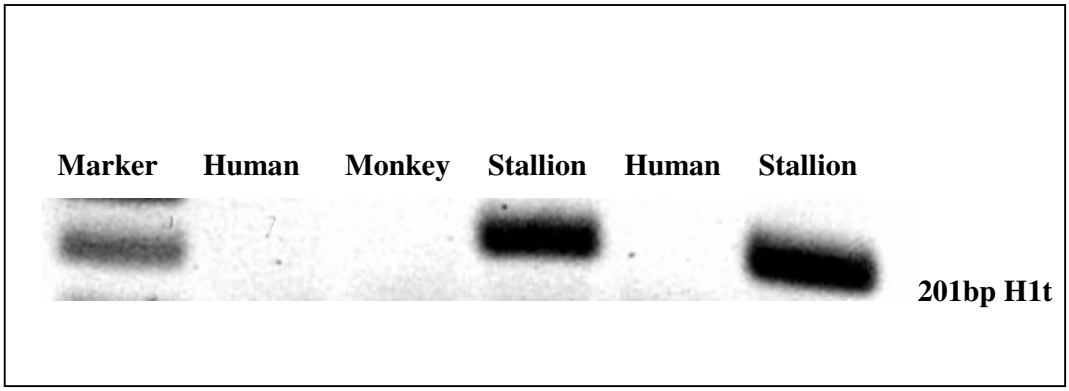


Fig. 21: RT-PCR analysis of H1t transcripts from stallion testes, monkey testis, and human testes as indicated using the specific primers. The bands were checked by sequencing.

3.1.2 Specific expression of the equine H1t mRNA

Using RT-PCR, we demonstrated the presence of H1t mRNA in testes containing complete spermatogenesis as well as in testes containing spermatogenesis development up to primary spermatocytes and in testes containing spermatogenesis development up to spermatogonia (Fig. 22).

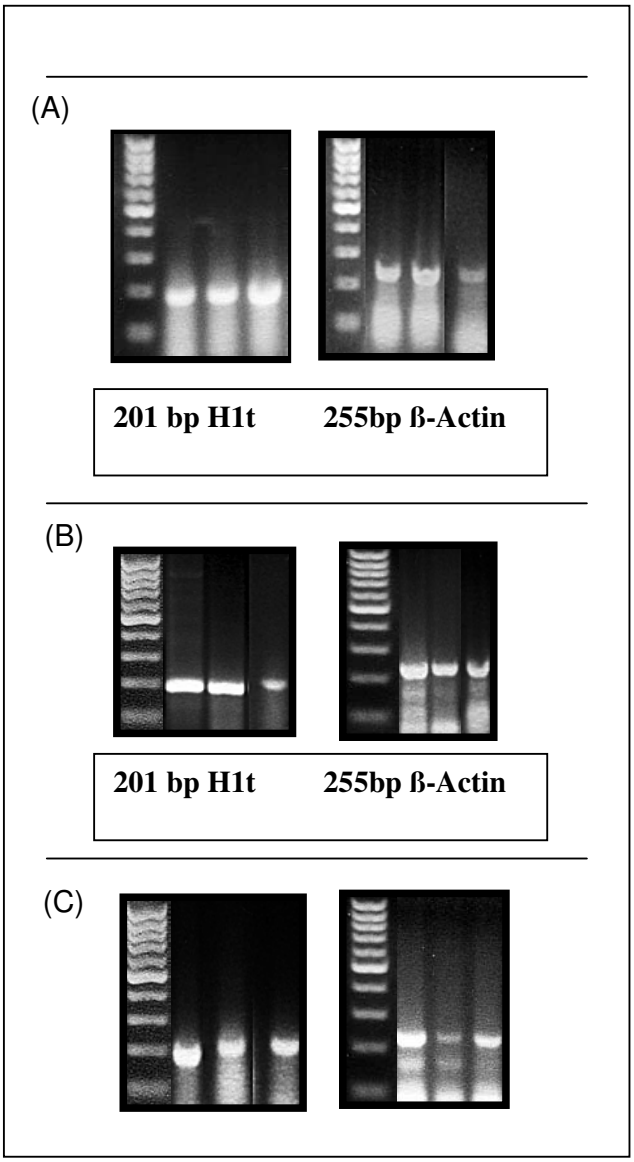


Fig. 22: Analysis of RT-PCR products of H1t in a 2 % electrophoretic agarose gel. (A) testes containing complete spermatogenesis, (B) testes containing spermatogenesis development up to primary spermatocytes, (C) testes containing spermatogenesis development up to spermatogonia. ß-actin was used as a loading control.

3.1.3 Quantitative H1t mRNA expression and statistical analysis

Quantitative analysis revealed in testes with histologically complete spermatogenesis as well as prepubertal testes containing spermatogenic development up to primary spermatocytes (leptotene, zygotene primary spermatocytes) and cryptorchid testes containing spermatogenic development up to spermatogonia, positive bands for H1t. The melting curve (not shown) indicated that the amplification products were H1t specific as single melting peaks characteristic of the amplicon and band sizes of approximately 98 bp were obtained for all the isolates tested.

The analysis of variance revealed that the values of H1t mRNA transcripts were significant different between these groups ($p = 0.0043$). Tukey test showed no significant differences can be shown between complete spermatogenesis (abundant pachytene spermatocytes) and primary spermatocytes (leptotene, zygotene).

However, a statistically significant difference existed between complete spermatogenesis (abundant pachytene spermatocytes) and prespermatogonia/spermatogonia with $p < 0.01$.

The difference between primary spermatocytes and prespermatogonia/spermatogonia were significant with $p < 0.05$ (Fig. 23).

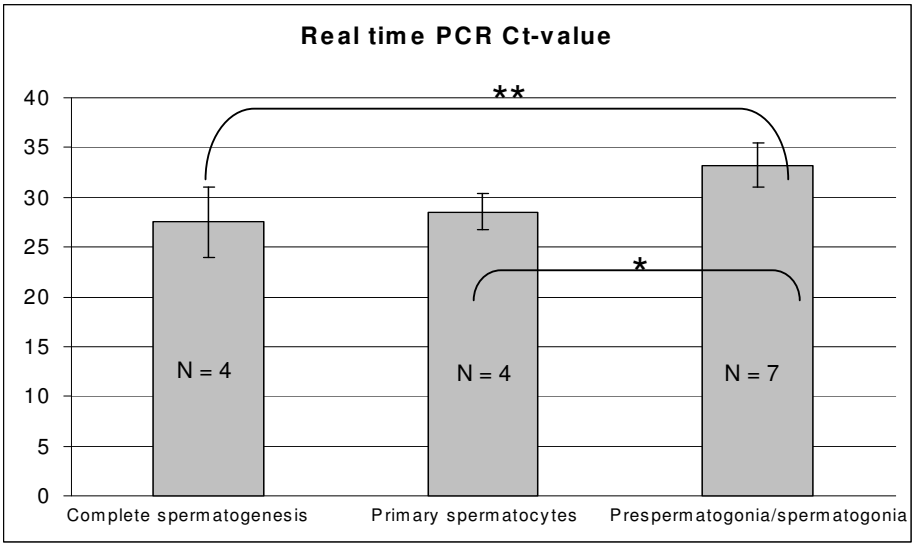


Fig. 23: Quantitative evaluation and statistical analysis of Real Time RT-PCR. The asterisk indicates * $p < 0.05$, ** $p < 0.01$.

3.1.4 Cell-localization of H1t cRNA in testes sections

In order to investigate the cell distribution pattern of the equine H1t mRNA in testis tissue, *in-situ* hybridization was performed using a specific digoxigenin-labeled H1t sense and antisense RNA probes. It was found by in situ hybridization that the H1t mRNA was expressed in spermatogonia and primary spermatocytes, but not in prespermatogonia (Fig. 24A-F).

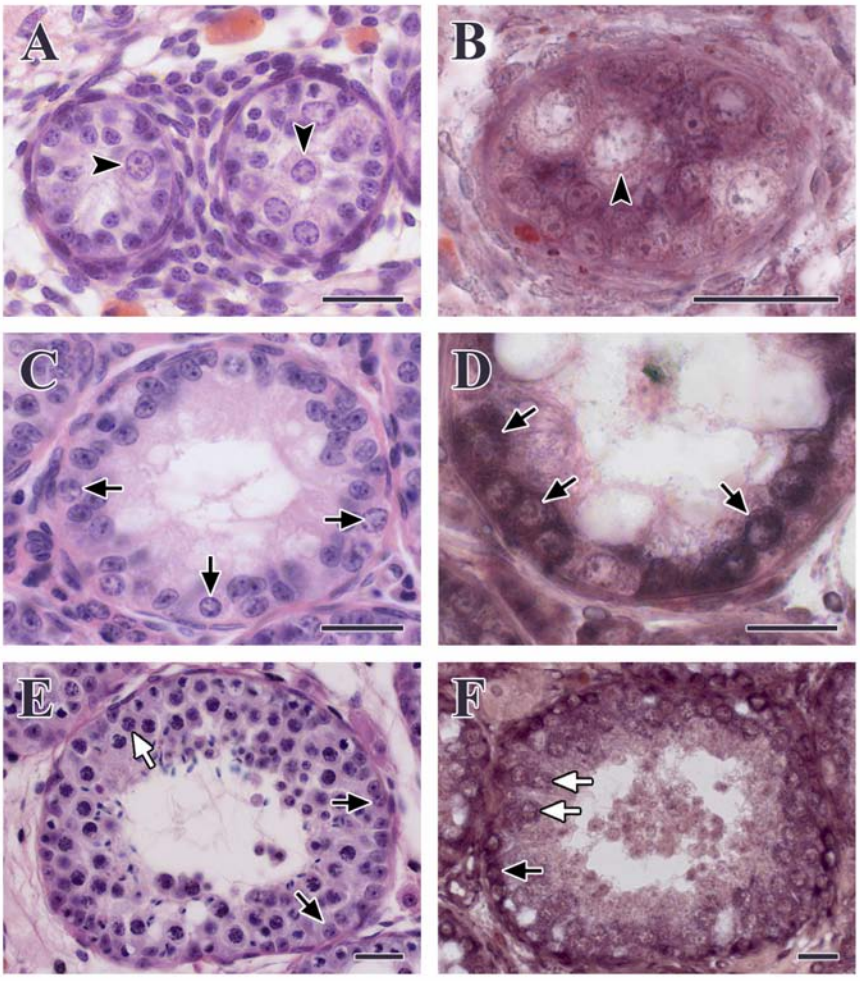


Fig. 24: (**A**) Haematoxylin-eosin staining of 6 month old stallion testis showing prespermatogonia (arrowhead). (**B**) In situ hybridization with a digoxigenin-labeled complementary RNA probe against H1t showing no reaction in prespermatogonia (arrowhead). (**C**) Haematoxylin-eosin (H&E) staining of 1 year old stallion testis showing spermatogonia (arrows).

(**D**) In situ hybridization showing positive spermatogonia (arrows). (**E**) Haematoxylin-eosin staining of 1.5 year old stallion testis showing qualitatively intact spermatogenesis. Spermatogonia (black arrows) and pachytene spermatocytes (white arrows). (**F**) In situ hybridization showing positive reaction in spermatogonia (black arrow) and in primary spermatocytes (white arrows). Bar = 25 µm.

Testes of four years old animals exhibit fully developed spermatogenesis and all stages of spermatogenesis can be identified (Fig. 25 A). H1t mRNA was found to be expressed by in-situ hybridization in spermatogonia and in primary spermatocytes from preleptotene at stage VIII up to midpachytene spermatocytes at stages VIII and I (Fig. 25 B).

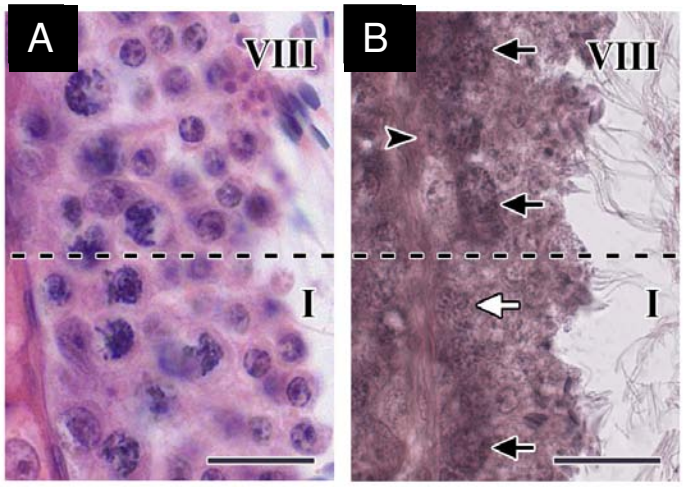
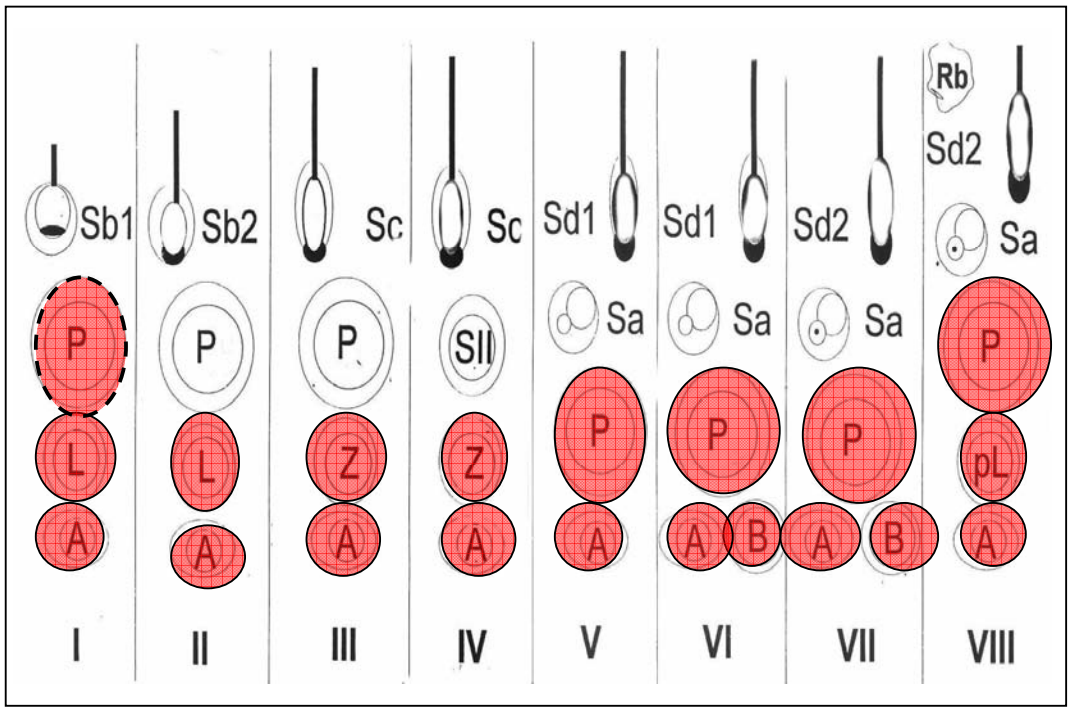


Fig. 25: (**A**) 4 year old animal: seminiferous epithelium at stage VIII and stage I. Haematoxylin-eosin (H&E) staining. (**B**) In situ hybridization of the seminiferous epithelium at stages VIII and I showing positive spermatogonia (arrowhead) and positive primary spermatocytes up to mid-pachytene at stage VIII (black arrows), and negative late pachytene spermatocytes at stage I (white arrow). Bar = 25 µm. Data are summarized in Table 11.



Positive

Negative

Sometimes positive

Table 11: H1t mRNA expression in the course of the spermatogenic cycle. (A) spermatogonium type A, (B) spermatogonium type B, (pL) preleptotene spermatocyte, (L) leptotene spermatocyte, (Z) zygotene spermatocyte, (P) pachytene spermatocyte, (SII) second spermatocyte, (Sa) round spermatid, (Sb1, Sb2, Sc, Sd1, Sd2) spermatids, (Rb) residual body. Stages according to Johnson et al., 1990.

3.1.5 H1t Protein expression and cell-protein-localization

In order to analyze the specificity of the H1t antiserum, western blot analyses was performed with protein extracts from various equine tissues.The results showed a specific band in the testis tissue. This band was detected at 29 kDa. Additionally was identified a weak signal in equine ovar and liver tissue. This may be explained by the fact that the member H1 family has multiple isoforms which was recently reported in the literatur, indicating cross reaction of the H1t antibody with the other H1 subtypes (Fig. 26).

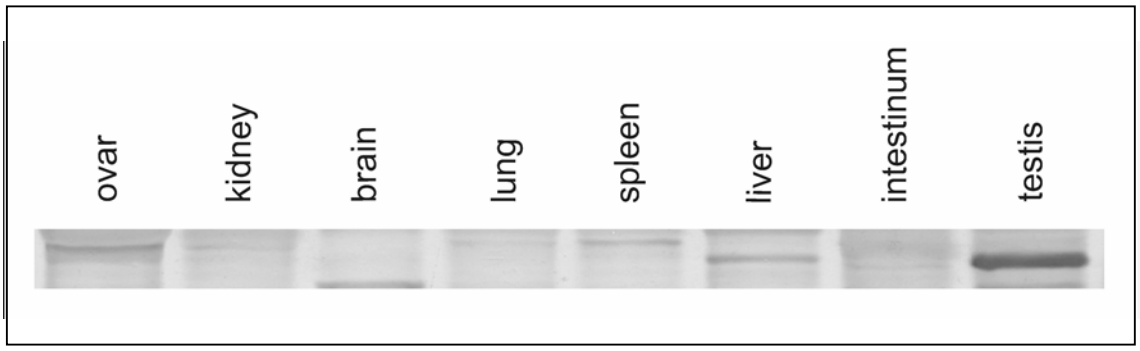


Fig. 26: Expression profile: Protein samples (20 µg/lane) prepared from various adult equine tissues were examined with antiserum against H1t. Western blot analysis of the extracts from various equine tissue showed that the H1t is expressed only in the testis.

The H1t protein was first detected in prepubertal testes in 1 year old animals together with the beginning of occurrence of primary spermatocytes (Fig. 27a), while cryptorchid testes containing only prespermatogonia/spermatogonia were negative (Fig. 27b). Testes containing complete spermatogenesis development were used as positive control.

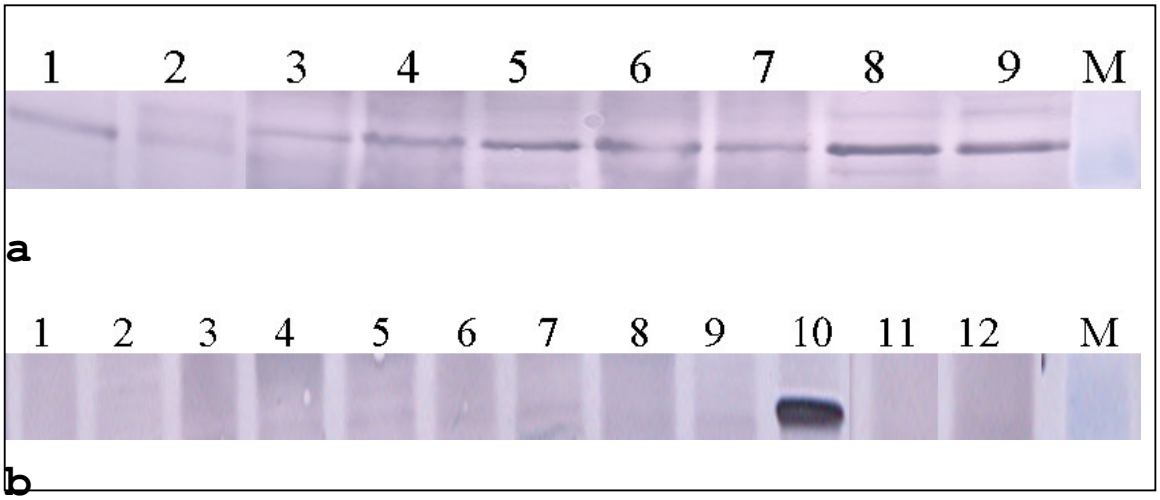


Fig. 27: a. Western blot analysis with antibody against H1t in prepubertal stallion testes (1, 2, 3, 4), and stallion testes showing complete spermatogenesis (5, 6, 7, 8, 9). b. Western blot analysis with antibody against H1t in inguinal cryptorchid testes (1, 2, 3, 4, 5, 6, 7, 8, 9) and stallion testis with complete spermatogenesis as positive control (10). Horse kidney tissue (11) und pig testis (12) were used as negative control.

In order to identified the H1t distribuition pattern during the equine spermatogenesis, cell-protein-localization studies were performed for equine H1t. Applying a polyclonal antibody against the equine H1t, immunohistochemistry analyses resulted in a strong signal in the nuclei of primary spermatocytes up to Sd1 elongated spermatids (Fig. 28).

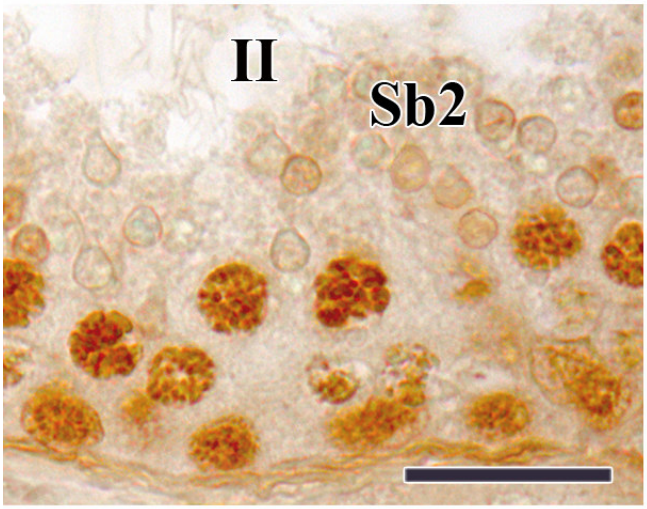
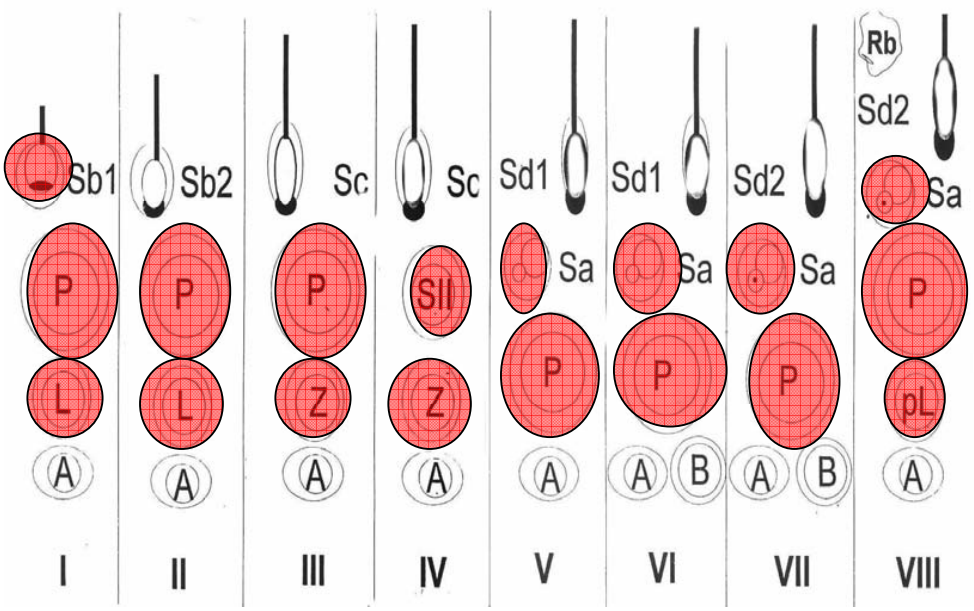


Fig. 28: Immunohistochemistry using rabbit anti-horse H1t antibody in a seminiferous epithelium of adult equine testis at stage II showing positive signals in primary spermatocytes up to Sd1 elongated spermatids. Note: The elongating spermatids (Sb2) are negative. Primary magnification x 40 µm. Data are summarized in Table 12.



Positive ◍ Negative ○

Table 12: H1t protein expression during adult stallion spermatogenesis. (A) spermatogonium type A, (B) spermatogonium type B, (pL) preleptotene spermatocyte, (L) leptotene spermatocyte, (Z) zygotene spermatocyte, (P) pachytene spermatocyte, (SII) second spermatocyte, (Sa) round spermatid, (Sb1, Sb2, Sc, Sd1, Sd2) spermatids, (Rb) residual body. Stages according to Johnson et al., 1990.

3.2 Age-dependent expression of the testis-specific histone (H1t)

3.2.1 Age-dependent H1t mRNA expression by RT-PCR

Expression analysis of equine H1t mRNA was performed with RT-PCR. The RT-PCR showed presence of H1t mRNA transcripts in ≤ 2.5 years testes as well as ≥ 3 years testes. Using the equine specific primers we obtained a PCR product of 201 pb. We used the β-actin as positive control of the cDNAs (Fig. 29).

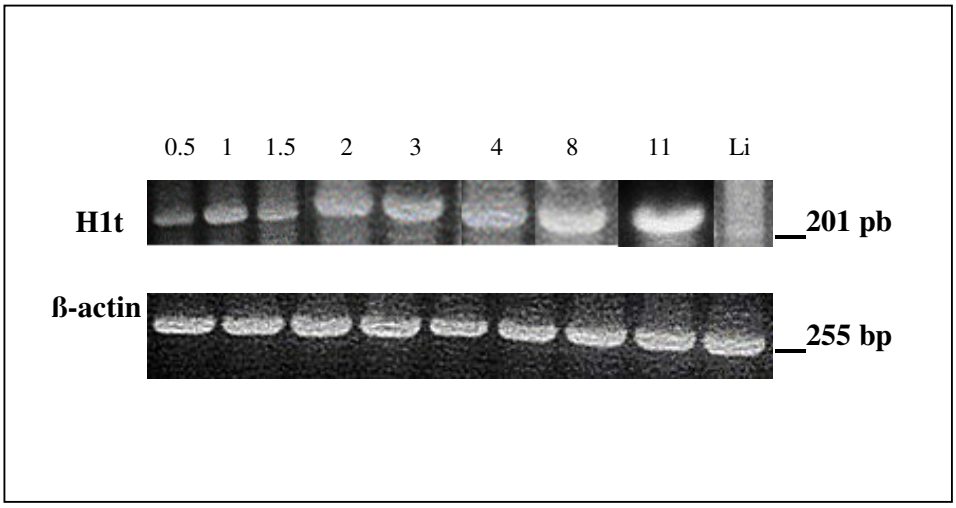


Fig. 29: RT-PCR of H1t mRNA showed a band at the level of 201 bp in stallion testes with different ages (6 month; 1 year old; 1. 5 years old; 2 years old; 3 years old; 4 years old; 8 years old; 11 years old; Li – liver as negative control).

3.2.2 Quantitative H1t mRNA expression and statistical analysis

Quantitative expression analysis of H1t mRNAs transcripts was performed with Real-Time RT-PCR. Using qPCR, equine H1t expression was highest in animals with more than 3 years old and containing a complete spermatogenic development. A moderate H1t mRNA expression was observed in animals at the age up to 2.5 years old.

The statistical analysis showed a statistical significant differences between the groups ≤ 2.5 years and ≥ 3 years ($p = 0.0055$). The values (arithmetic mean ± S.D) for the ≤ 2.5 years group were 30.67 ± 2.79. The ≥ 3 years group had a lower mean Ct-value (26.45 ± 1.78). The decrease of the Ct-value with increasing age is also presented in form of a scattergram and non-linear regression line (Fig. 30).

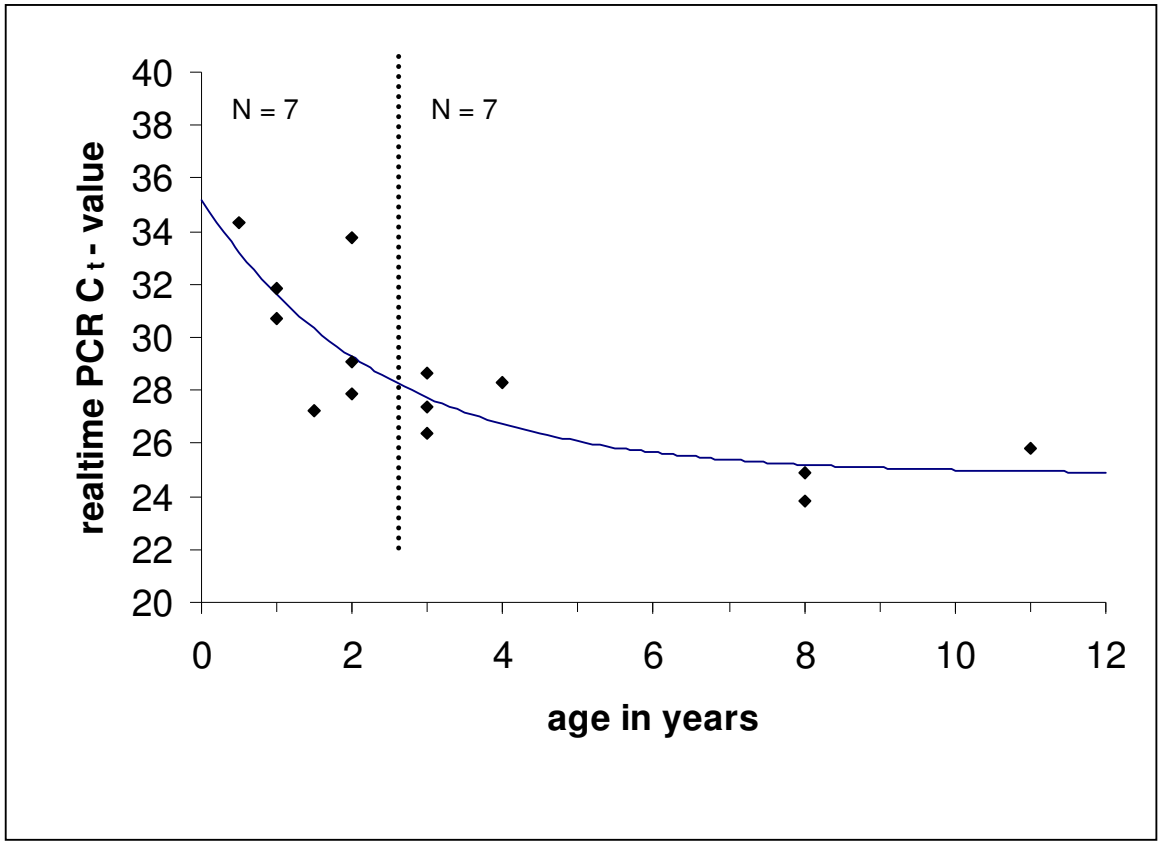


Fig. 30: Quantitative Real Time RT-PCR and statistical results were represented in form of regressions analysis of mean Real-Time PCR Ct-value in dependency of the age (scattergram with non-linear regression function).

3.2.3 Age-dependent H1t cRNA expression in testes sections

In order to observe the stage specific expression of H1t mRNA in the cycle of the seminiferous epithelium by in-situ hybridization and the possible difference between peripubertal stallion and adult stallion, it was investigated the H1t mRNA expression in testes with 2 years old as well as in testes with four years old (Fig. 31A-H). We did not observe any differences between the stage cell-specific expressions of H1t mRNA in testes in age of 2 years compared to 4 years old stallion.

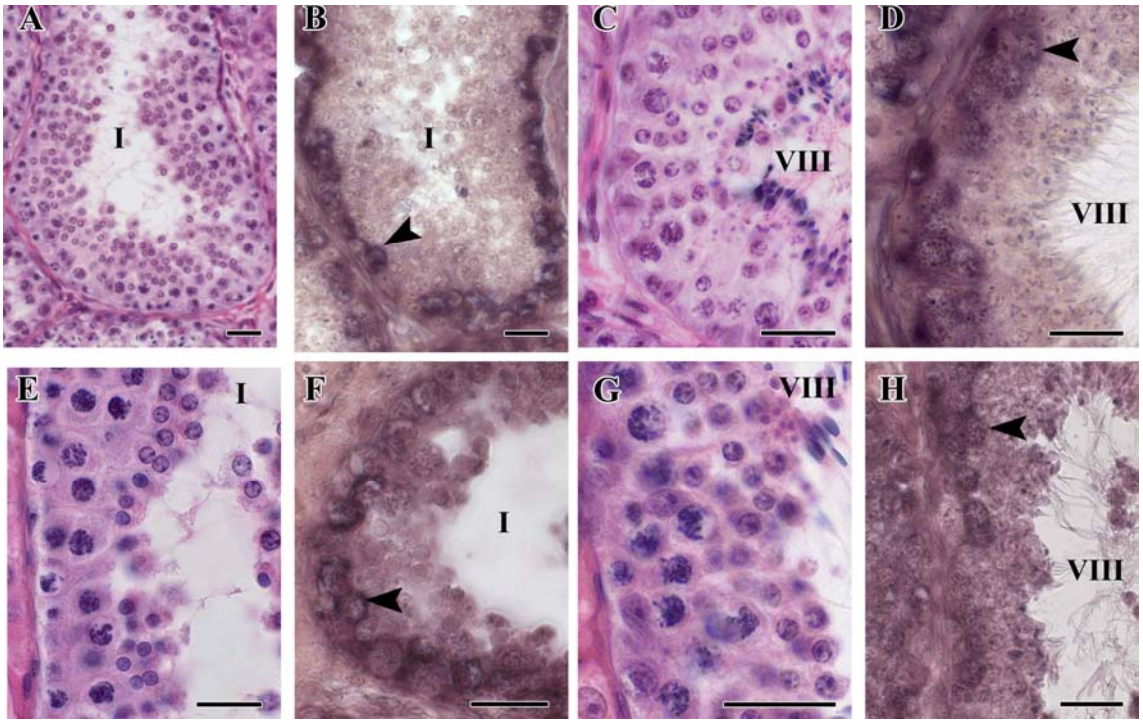


Fig. 31: (**A**) Histology of the seminiferous epithelium applying haematoxylin-eosin staining: Seminiferous epithelium at stage I from 2 years old testis showing a qualitative intact spermatogenesis. (**B**) In-situ hybridization of the seminiferous epithelium at stage I showing positive reaction in spermatogonia up to primary spermatocyte (arrowhead). (**C**) Histology of the seminiferous epithelium at stage VIII from 2 years old testis showing complete spermatogenesis. (**D**) In-situ hybridization of the seminiferous epithelium at stage VIII showing positive reaction in spermatogonia up to pachytene spermatocytes (arrowhead). (**E**) Histology of the seminiferous epithelium at stage I from 4 years old testis showing complete spermatogenesis. (**F**) In-situ hybridization of the seminiferous epithelium at stage I showing positive reaction in spermatogonia up to primary spermatocytes (arrowhead).

(**G**) Histology of the seminiferous epithelium at stage VIII from 4 years old testis showing complete spermatogenesis development. (**H**) In-situ hybridization of the seminiferous epithelium at stage VIII showing positive reaction in spermatogonia up to primary spermatocytes (arrowhead). Primary magnification x 40 µm.

3.2.4 Age-dependent H1t protein expression

H1t protein levels were detectable from 1 year up to 11 years old testes. This could be explained though the fact that in these testes the presence of early, mid- and late pachytene spermatocytes are abundant (Fig. 32).

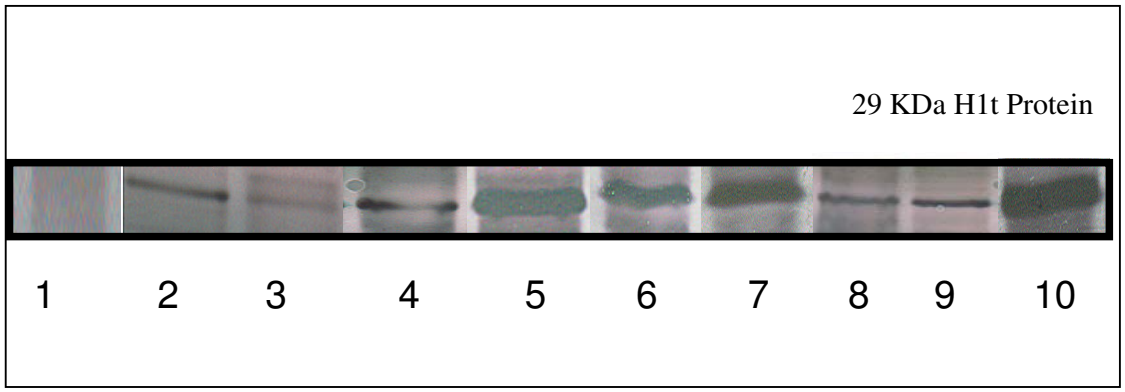


Fig. 32: H1t protein expression in testes at different ages. (1) 6 months old testis, (2) 1 years old testis, (3) 1 years old testis, (4) 1.5 years old testis, (5) 2 years old testis, (6) 3 years old testis, (7) 4 years old testis, (8) 8 years old testis, (9) 8 years old testis, (10) 11 years old testis.

In order to investigate the H1t cell-protein-localization in peripubertal testes, immunohistochemistry analyses were performed applying a polyclonal antibody against the equine H1t. A strong immunoreactivity against the equine H1t protein was observed in primary spermatocytes up to elongated spermatids. This result indicated a prolonged H1t protein expression in peripubertal animals compared with adult stallions (Fig. 33). Data summarized in Table 13.

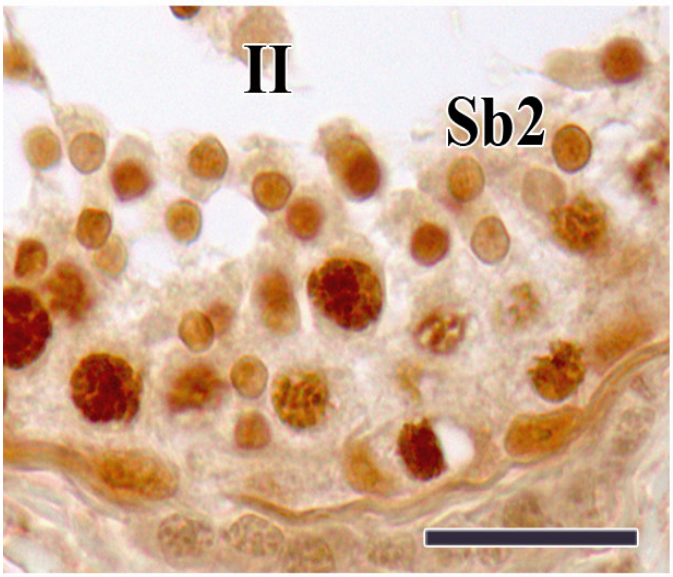
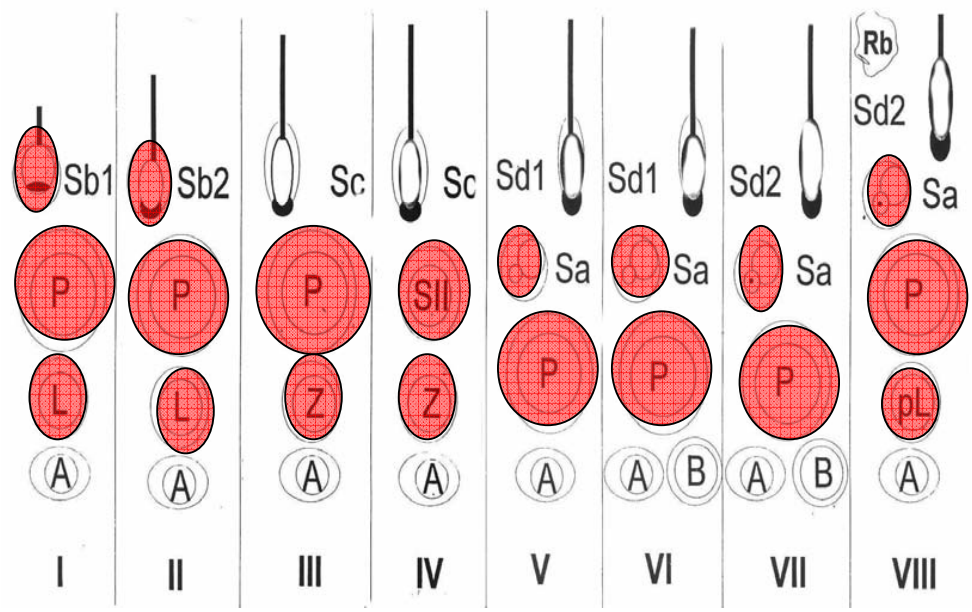


Fig. 33: Immunohistochemistry using rabbit anti-horse H1t antibody. In peripubertal equine testes, a prolongation of the H1t protein expression up to elongating spermatids (Sb2) was obseved in stage II. Primary magnification x 40 µm. Data are summarized in Table 13.



Positive ● Negative ○

Table 13: H1t protein expression during peripubertal stallion spermatogenesis. (A) spermatogonium type A, (B) spermatogonium type B, (pL) preleptotene spermatocyte, (L) leptotene spermatocyte, (Z) zygotene spermatocyte, (P) pachytene spermatocyte, (SII) second spermatocyte, (Sa) round spermatid, (Sb1, Sb2, Sc, Sd1, Sd2) spermatids, (Rb) residual body. Stages according to Johnson et al., 1990.

4 Discussion

4.1 H1t gene expression

This is the first study identifying, cloning and sequencing a fragment of the histone H1t mRNA in the equine testis. As the expression of H1t gene in testis has been found in several different species, such as human (Steger et al., 1998), rat (Meistrich et al., 1985; Seyedin and Kistler, 1980), mouse (Drabent et al., 1996) and monkey (Koppel et al., 1994), expression is also likely to be present in stallions.

A fragment of the equine histone H1t was amplified using reverse transcriptase polymerase chain reaction and was cloned. Whereas the H1t gene was found in several different species as was expected also in horses with normal spermatogenesis and with premature infertility.

In this study, specific amplification of the equine H1t gene using cDNA samples via RT-PCR have been shown that the equine H1t gene is expressed also in spermatogonia and in primary spermatocytes especially in pachytene spermatocytes. These findings are in part similar to the study of Seyedin and Kistler (1980) and Seyedin et al. (1981), who suggested that the H1t gene is expressed only in mid to late pachytene spermatocytes in the rat. Meistrich et al. (1985) measured the levels and synthesis of histone variants directly in spermatogonia (histones H1d, H1de, H1a, H1c) and in various stages of primary spermatocytes (histone H1t) purified from the rat testis.

Quantitative measurements of equine H1t mRNA analyses showed lower amount of the H1t mRNA in testes containing spermatogenic development up to spermatogonia which indicated that the equine H1t mRNA expression begin at mRNA level in spermatogonia. This is in line with a study showing low levels of H1t mRNA in prepubertal mouse tests, also demonstrating H1t mRNA expression by electron microscopy in situ hybridization (Drabent et al., 1998). Therefore, this indicates that the H1t gene is expressed at premeiotic stages, albeit at a low level. Our results concerning the H1t mRNA expression in spermatogonia do not agree with human (Steger et al., 1998).

In-situ hybridization with the H1t cRNA of the equine testis-specific histone variant was done in testes of stallions at four different developmental stages (testes showing spermatogenic development up to spermatogonia, testis showing spermatogenic development up to primary spermatocytes, testes showing spermatogenic development until elongated spermatids and

testes showing complete spermatogenesis) in order to investigate in which cells start the expression of the H1t mRNA. Higher levels of hybridization reactions were observed in spermatogonia, leptotene, zygotene and pachytene spermatocytes at stage I-VIII of the seminiferous epithelium cycle and were absent in round spermatids, elongated spermatids and sperm. These results were similar to Kremer and Kistler (1991), whereas it is suggested that the rat H1t mRNA was identified by in-situ hybridization in the mid and late pachytene spermatocytes at stages VII to XIII of the seminiferous epithelial cycle.

In 1996, Drabent et al. proposed a maximal expression of the mouse H1t mRNA in the late and mid-pachytene spermatocytes at stage VII of the seminiferous epithelium cycle. Later, Drabent et al. (1998) have extended their analysis to more sensitive approaches and demonstrated by RNase protection and electrone-microscopic in-situ hybridization, that mouse H1t mRNA is detectable even in spermatogonia.

Our findings indicate a variable difference between the mammalian species concerning the stage specific expressions of H1t mRNA. This could be explained by the fact that the cycle of the seminiferous tubules is not identical from one species to another, or even among animals of different strains of the same species.

Concerning again to the stage specific expression of equine H1t mRNA, it was detectable in spermatogonia and primary spermatocytes from preleptotene spermatocytes at stage VIII up to midpachytene spermatocytes of stage VIII and I.

Additionally, a highly specific polyclonal antibody was obtained to indentify electrophoretically pure histone. Western blot analysis demonstrated that the rabbit anti-equine H1t polyclonal antisera that produced only recognized a 29 kDa protein band from the stallion testis in accordance with (Ramesh et al., 2006), which in their study detected also a specific band for H1t at the level of 29 kDa. In testis homogenate from pig and other tissues (used as negative control), the H1t protein was undetectable indicating the high specificity of the antibody.

The histone H1t seems to be truly a male germ cell-specific described by Lennox and Cohen (1983). In our expression profile experiment with different horse tissues was observed a specific band in the testis tissue. The H1t is the only tissue-specific member of the mammalian H1 histone family cited by Bartell et al. (1996), in contrast to the other H1 subtype genes found in male germ cells which are also expressed in somatic cells (Franke et al., 1998).

Consequently, by western blot analysis, the H1t protein was first detected in prepubertal testes of one year old animals together with the initiation of spermatogenesis and the occurrence of primary spermatocytes, while testes containing only prespermatogonia/spermatogonia were negative. These findings suggest that the H1t protein expression is high in these cells (Drabent et al. 1996).

In the present study, there was a strong immunohistochemical signal for the testis specific histone protein (H1t) in the equine primary spermatocytes at stages I-VIII of the seminiferous epithelium cycle as well as in the nuclei of equine round spermatids at stage VIII of the seminiferous epithelium cycle. However, immunohistochemically, the equine H1t protein was not detected in prespermatogonia/spermatogonia. These data were similar to the data obtained in mouse (Drabent et al., 1996, 1998) and human (Steger et al., 1998).

Considering the mRNA expression pattern, the protein expression results indicated that the H1t gene may be transcribed during a short period in spermatogonia and the mRNA may be preserved until later stages as pachytene spermatocytes but the synthesis of the protein must occur during the early pachytene spermatocytes stage and persists until the stages of round spermatids. These data represent a strong temporal correlation between the onset of H1t gene transcription and synthesis of the H1t protein (Steger et al. 1998).

4.2 Age-dependent expression of the H1t gene during the different pubertal development

Reproductive efficiency in horses varies with the season (Sutovsky et al., 2003). For most equine breeds, selection of breeding stallions is based primarily on pedigree, athletic performance, or conformation with little consideration given to reproductive potential. After these stallions are chosen for a breeding career, many are found to be subfertile. Some stallions pass a routine breeding examination, yet are unable to impregnate mares, or do so very inefficiently (Dickson et al., 2001). This problem could be involved with the observation of recent studies that have already been described in horses about the influence of age on testicular size, hormone concentration, puberty, sexual behaviour, and daily sperm production (Melo et al., 1998). Douglas et al. (2001) cited that the histone-protamine replacement is a late-spermiogenesis event, along with acrosome formation, membrane remodeling, and other significant morphological and biochemical events that are necessary for normal sperm function.

Rizgalla (2002) described for the first time that scrotal testes of normal stallions at the age of 2 years showed by histological analysis normal spermatogenesis but at this age these animals have a prolonged H1 expression. Furthermore, in our study, the appearance of H1t gradually followed by the replacement of histone-protamin exchange was of particular interest.

Using RT-PCR, equine H1t cDNA expression was found in scrotal testes at different spermatogenesis development depending on age. H1t expression has been found in stallions between 6 months of age and 11 years of age.

Up to 2 years, it is possible to identify the cycle of the seminiferous epithelium in stallions spermatogenesis according to Rizgalla (2002) and in this period these animals already have a complete development of seminiferous tubules as in adults animals, but these animals still have sub fertility compared with adult stallions as was previously described by Melo et al. (1998).

In order to observe the stage specific expression of H1t in the cycle of the seminiferous epithelium by in-situ hybridization and the possible difference between peripubertal stallion and adult stallion, H1t mRNA expression was investigated in testes of 2 years old and in testes of four years old stallions. We did not observed any differences between the stage and cell-specific expressions of H1t mRNA in testes in age of 2 years compared to 4 years old stallions.

However, the quantitative Real-Time RT-PCR of the equine H1t mRNA from animals aged ≤ 2.5 years were significantly reduced in these animals compared with animals ≥ 3 years. In some of them it was possible to identify complete spermatogenesis, but at this age, the stage development of the seminiferous epithelium was variable and the efficiency of sperm production still low comparing to adult animals (Melo et al., 1998).

The H1t protein was first detected by western blot in prepubertal testes of one year old animals with initiation of spermatogenesis and the occurrence of primary spermatocytes, while testes (6 months) containing only prespermatogonia/spermatogonia were negative.

After applying a polyclonal antibody against equine H1t, immunohistochemical analysis in the testes of normal adult stallions resulted in a strong signal in the nuclei of primary spermatocytes in stages I-VIII up to round spermatids (Sa) in stage VIII, while in testes of peripubertal stallions, the H1t protein expression was detectable until elongated spermatids (Sb2) in stage II.

Therefore, the prolonged H1t-proteins expression during the peripubertal development could induce the following theory: these animals may have an incomplete chromatin condensation because the testis-specific histone (H1t) has a weak interaction with chromatin. In addition: if these animals have a deficit in H1t expression this means that the presence of H1a is more than H1t, as described by Khadake and Satyanarayana Rao (1995) that histones H1a and H1t are two major linker histone variants present at the pachytene interval of mammalian spermatogenesis. This theory could be consequently involved with a prolonged H1 protein expression as showed by Rizgalla (2002). In the human, Bedford et al. (1973) proposed that such structural or biochemical defects are thought to be associated with chromatin packaging in the sperm nucleus. Poor chromatin packaging and possible DNA damage may contribute to a failure of sperm decondensation and subsequently, fertilization failure or habitual abortion following fertilization (Zamboni, 1992; Haidl and Schill, 1994). Hofman and Hilscher (1991) investigated the presence of histone in the head of the spermatozoon with the aniline blue staining, because the degree of chromatin condensation can be assessed with acidic aniline blue staining, which discriminates lysine-rich histones from arginine-rich and cysteine-rich protamines. Histone-rich nuclei of immature spermatozoa are rich in lysine and consequently, take up the blue stain. Conversely, protamine-rich nuclei of mature spermatozoa are rich in arginine and cysteine and contain relatively lower amounts of lysine (Calvin, 1976; Gusse et al., 1986); thus, they do not stain with aniline blue. Dadoune et al. (1988) proved in sperm that by infertile males the probability to retain histone in the spermatozoon head is very high when compared to fertile males. This fact demonstrates that the incomplete histone protamine replacement could induce fertility impairment. In the context of our investigation, stallions in the peri- and postpubertal period do not have an adequate H1t expression during spermatogenesis. This fact may presume that the appearance of H1t-protein takes place later as normal compared with adults animals and this could be a reason for the prolonged H1 expression during spermatogenesis and consequently, incomplete histone protamine replacement. Dadoune et al. (1988) proved in humans for example that the retention of sperm head results in malformation of the sperm head (Fig. 34). Casey et al. (1997) and Gravance et al. (1997) observed in stallion with infertility problems, that their sperm show malformation of the head.

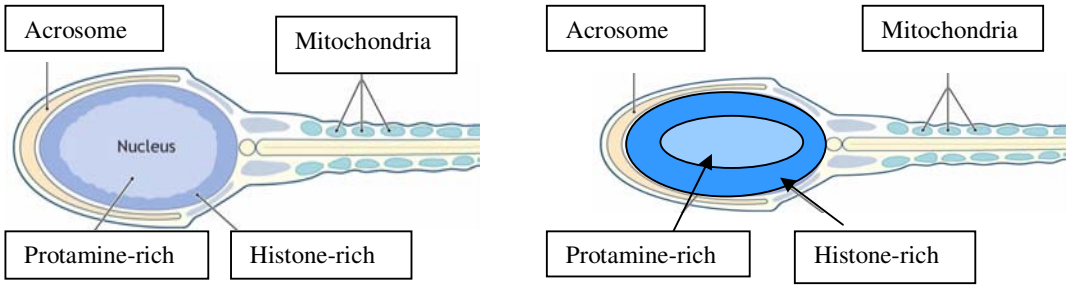


Fig. 34: The sperm. The sperm head contains the nucleus (DNA and proteins) and the acrosome (a vesicle rich in hydrolytic enzymes involved in egg penetration). Within the sperm nucleus, there is a histone-rich region that is localized peripherally and a protamine-rich region localized centrally. The sperm midpiece represents the proximal part of the sperm tail and it is rich in mitochondria. (A) Normal sperm head and (B) Sperm head with retention of histone protein.

However, the incomplete histone-protamine replacement in stallion spermatogenesis could be a factor for malformations of the sperm head and consequently the reason for subfertility or infertility. Another cause to cause sub fertility or infertility in stallion spermatogenesis is the chromatin denaturation in the sperm head. The chromatin denaturation occurs by the DNA and core protein denaturation. The DNA denaturation starts with the disaggregation of the double DNA-strand in the single-stranded DNA, it can be measured by SCSA (sperm chromatin structure assay) (Ballachey et al., 1988). For a spermatozoon to be fertile, it must be capable of undergoing decondensation at an appropriate time in the fertilization process (Amann, 1993). Infertile men manifest various nuclear alterations, including an abnormal chromatin structure, chromosomes with microdeletions, aneuploidies and DNA strand breaks (Hofman and Hilscher, 1991).

Studies by Love and Kenney (1998) in stallion spermatogenesis using SCSA showed that fertile stallions have a lower amount of chromatin denaturation, whereas stallions with subfertility have an explicit by higher number.

The H1t had by far the least condensing activity of any of the histone variants (Khadake and Satyanarayana Rao, 1995) and consequently the chromatin has a relax structure. Then the prolongation of the H1 expression and consequently the insufficient expression of H1t could induce chromatin denaturation.

Recapitulating the facts, the data presented in this study are similar to the situation in other mammals. The equine H1t gene is expressed at a high level in pachytene spermatocytes. Therefore, the statistically improvement in regression linear and T-test results confirm descriptively that the H1t gene expression in testes between 3-4 years old is not readily as in testes with more than 8 years old. These animals do not have a sufficient H1t mRNA expression comparing to adult animals. This observation could be one of different ways to explain the well-known premature subfertility during spermatogenesis of the stallion.

4.3 Conclusion

The present date described for the first time the H1t gene expression in the stallion testis. The equine H1t sequence was identified and published in the Genbank.

The stage- and cell-specific expression of testicular H1t mRNA during equine spermatogenesis was found by in-situ hybridization in spermatogonia and primary spermatocytes up to mid pachytene spermatocytes at stage I and VIII of the seminiferous epithelium cycle. The protein was first detected by western blot in testes with initiation of spermatogenesis and the occurrence of primary spermatocytes. Testes containing only prespermatogonia/spermatogonia were negative.

In testes of adult stallions, immunohistochemistry showed a strong signal in nuclei of primary spermatocytes up to round spermatids at stage I and VIII of the seminiferous epithelium cycle.

In younger stallions, real time RT-PCR revealed an increase of H1t mRNA expression with a range of individual variety between 3-4 years old animals indicating a stable expression in animals not before 4 years old. By in situ hybridization no differences between peripubertal and adult testes could be found. Immunohistochemistry revealed a strong signal in nuclei of primary spermatocytes at stage I and VIII up to elongating spermatids at stage II of the seminiferous epithelium cycle.

These data suggest the involvement of H1t gene expression in the well-known premature infertility the stallion.

5 Summary

The H1t gene is already known in several species such as mice, rat, monkey and human. In stallion, this gene was unknown so far. By means of bioinformatic analysis, were found an oligonucleotide from the same gene domain in this species. From equine testis homogenate a RT-PCR has been carried out. The product was sequenced and a fragment of equine H1t mRNA was cloned. This sequence was published in the GenBank (Accession no. AJ865320). The reverse transcriptase PCR together with in situ hybridization showed mRNA expression of H1t in all examined testes. H1t mRNA expression has been found in normal spermatogenesis in spermatogonia and primary spermatocytes up to mid pachytene. Ct value of Real-time RT-PCR showed that H1t mRNA is detected in testes containing spermatogenic development up to spermatogonia and in testes containing spermatogenic development up to mid pachytene spermatocytes. From protein-protein sequence homology (ClustalW http://www.ebi.ac.uk/clustalw/) between mice, rat, monkey and human, it was possible to generate a polyclonal antibody. The H1t protein was detected by Western Blot at 29 kDa using a polyclonal antibody specifically detecting the peptide LITEALSVSQER. The protein was identified and sequenced by fingerprint analysis with MALDI-MS/MS. By means of expression profile analysis with different equine organs, it could be demonstrated, that the detected H1t is testis specific. The H1t protein was first detected in prepubertal testes of one year old animals with initiation of spermatogenesis and the occurrence of primary spermatocytes, while testes containing only prespermatogonia/spermatogonia were negative. In testes of adult stallions, immunohistochemistry showed a strong signal in nuclei of primary spermatocytes up to round spermatids. Concerning the sub fertility of younger stallions, H1t mRNA expression was detected in all animals with different ages and spermatogenic development. Analyses of testes at different ages ($\leq$ 2 years and $\geq$ 3 years) by Real-Time RT-PCR revealed an increase of H1t mRNA expression with a wide range of individual variety between 3 - 4 years old animals indicating a stable expression in animals not before 4 years old. By in situ hybridization, no differences between pubertal and adult testes could be found. The H1t protein was only detected in testes showing primary spermatocytes. In addition, in peripubertal animals immunohistochemistry showed a prolonged expression of H1t protein that persists until elongated spermatids suggesting an impaired in nuclear protein exchange that could lead to an abnormal chromatin condensation. These data suggest the involvement of H1t gene expression in the well-known premature subfertility the stallion.

6 Zusammenfassung

Die Sequenz des H1t Gens der Spezies Maus, Ratte, Affe und Mensch ist bereits bekannt. Beim Pferd war die H1t Sequenz bisher unbekannt. Durch Bioinformatikanalysen wurden Primer aus dem mit Maus, Ratte, Affe und Mensch größten homologen Bereich des H1t ausgewählt. Aus equinem Hodenhomogenat wurde eine Nested PCR durchgeführt, das Produkt sequenziert und anschließend kloniert. Die partielle H1t Sequenz des Pferdes ist in der Genbank eingetragen (Accession AJ865320). Die mRNA Expression konnte durch Reverse Transkriptase PCR (RT- PCR) in Hodenhomogenaten sowie durch eine Digoxigenin-markierte H1t-mRNA Sonde mittels In-situ Hybridisierung an Paraffinschnitten nachgewiesen werden. Es hat sich gezeigt, dass in der normalen Spermatogenese H1t mRNA in spermatogonien and in frühen bis mittleren pachytänen Spermatozyten exprimiert wird. Aus einer Protein-Protein-Sequenz Homologie (ClustalW http://www.ebi.ac.uk/clustalw/) zwischen Maus, Ratte, Affe und Mensch wurde aus dem nicht konservierten Bereich eine Sequenz ausgesucht, um einen polyklonalen Antikörper herstellen zu lassen. Das H1t Protein ist durch Western Blot in der normalen Spermatogenese am Hodenhomogenat mit Hilfe eines polyklonalen Antikörpers gegen H1t Peptid LITEALSVSQER identifiziert und sequenziert worden (Peptidmassen – Fingerprint – Analyse mit MALDI – MS/MS). Das Protein hat ein Molekulargewicht von 29 kDa. In einem Expressionsprofil mit verschiedenen equinen Organen wurde festgestellt, dass H1t hodenspezifisch ist.

Im kryptorchiden Hoden zeigt das Keimepithel verschiedene Entwicklungsstadien der Keimzellen, die mit dem einen fetalen Hoden vergleichbar sind. Es handelt sich hier um Tubuli, in denen nur fetale Präspermatogonien und Spermatogonien vom Typ A vorhanden sind. Im präpubertären Hoden kann man Tubuli mit Keimzellen bis zur Stufe der frühen pachytänen Spermatozyten beobachten. Zum Nachweis des H1t Proteins wurde ein Western Blot durchgeführt. Die Ergebnisse zeigen, dass die Proteinexpression im Hodenhomogenat mit normaler Spermatogenese sehr stark ist. Dagegen gibt es kein Signal im Hodenhomogenat mit einem Arrest der Spermatogenese auf der Stufe von Spermatogonien in kryptorchiden Hoden. Im präpubertären Hoden konnte die Expression des H1t Proteins in frühen Spermatozyten nachgewiesen werden. Die Immunhistochemie zeigt in adulten Tieren ein starkes Signal im Zellkern von Primärspermatozyten bis hin zu runden Spermatiden. Um diese Ergebnisse auf mRNA Ebene quantitativ nachzuweisen, wurde eine Real-Time PCR durchgeführt. Die H1t mRNA wird in frühen Spermatozyten hochreguliert. Die bisherigen Daten zeigen, dass ebenso wie bei der Maus, das equine H1t auf RNA Ebene in geringen

Mengen bereits in Präspermatogonien exprimiert und mit dem Beginn der Meiose hochreguliert wird. Bezüglich der Subfertilität des Hengstes wurde die Untersuchung mittels RT-PCR und Real Time PCR altersabhängig (≤ 2 Jahre und ≥ 3 Jahre) untersucht. Die Real-time RT-PCR zeigte eine Zunahme der H1t mRNA Expression in einem großen Bereich individueller Variabilität zwischen drei und vier Jahre alten Tiere und eine stabile Expression bei Tieren, die älter als vier Jahre sind. Das H1t Protein wurde in Hoden mit der Initiierung der Spermatogenese und dem Erscheinen von primären Spermatozyten durch Western Blot detektiert. Die Zell-Proteinexpression mittels Immunhistochemie hat eine Verlängerung der H1t Proteinexpression in präpubertären Hoden von zwei Jahre alten Tieren ergeben. Diese Studien deuten darauf hin, dass zwischen der H1t Geneexpression und der „Subfertilität“ während der Spermatogenese des Hengstes ein Zusammenhang besteht.

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8 List of own publications

1. Original publications

• CAVALCANTI, M.C.O, RIZGALLA, M., GEYER, J., FAILING, K., LITZKE, L.-F., BERGMANN, M. Expression of histone 1 (H1) and testis-specific histone (H1t) gene during stallion spermatogenesis.

2. Book chapters

CAVALCANTI, M.C.O. (2007). Die Stammzellenforschung und ihre Bedeutung. In: Handbook of Stem Cells, Volume 2: Embryonic Stem Cells, (ed. Lanza, R.), Academic Press, San Diego, in press.

3. Abstracts

CAVALCANTI, M.C.O, RIZGALLA, M., GEYER, J., FAILING, K., LITZKE, L.-F., BERGMANN, M. Expression of histone 1 (H1) and testis-specific histone (H1t) gene during stallion spermatogenesis. 41. Jahrestagung Physiologie und Pathologie der Fortpflanzung/33. Veterinär-Humanmedizinische Gemeinschaftstagung, Giessen, Germany, Februar 28 - 29, 2008.

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CAVALCANTI, M.C.O, GEYER, J., BERGMANN, M.
Expression of testis-specific histone (H1t) gene in spermatogenesis of the stallion. Reproduction in Domestic Animals 41:1- 44, **Poster**, Hannover, Februar 2006.

CAVALCANTI, M.C.O, GEYER, J., BERGMANN, M.
Expression of testis-specific histone (H1t) gene in spermatogenesis of the stallion. Anatomische Gesellschaft – 100th Annual Meeting, **Poster**, Leipzig, März 2005.

4. Database entries

NCBI Gene Expression Omnibus (GEO) repository entries (www.ncbi.nlm.nih.gov/geo):
CAVALCANTI, M.C.O, GEYER, J, BERGMANN, M. Cloning of testis-specific histone H1t from stallion. GenBank, NCBI, November 2004.

5. Teaching

Laboratory Instructor: Institute of Anatomy, Histology and Embryology, University of Giessen, Giessen, Germany.
05/15/07 – 06/26/07: Research Internships in Science and Engineering (DAAD-RISE Internship programme).

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