Chromatin remodeling during mammalian spermatogenesis: Role of testis specific histone variants and transition proteins

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Chromatin remodeling during mammalian spermatogenesis: Role of testis specific histone variants and transition proteins

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Structure of chromatin undergoes extensive alteration during mammalian spermatogenesis. Several testis specific histone subtypes are synthesised and replace their somatic counterparts during premeiotic, meiotic and post meiotic stages of germ cell differentiation. Early work from our laboratory showed that pachytene spermatocyte nuclei as well as nucleosome core particle are more accessible to DNase 1 than the interphase liver nuclei. The higher order structure of chromatin in pachytene spermatocytes is also loosely packed due to poor DNA and chromatin condensing property of the testis specific linker histone H1t. A careful analysis of the amino acid sequence of histone H1t revealed the absence of DNA condensing domain containing SPKK/TPKK motifs in the C terminus of the histone H1t. The spermiogenesis process following the meiotic division is characterised by extensive remodeling of chromatin. Transition proteins, TP1 and TP2, unique to mammalian spermatogenesis play an important role in this spermiogenesis process. We have shown that TP1 is a DNA melting protein while TP2 is a DNA condensing protein. We have delineated the molecular anatomy of TP2 including the presence of two novel Zn finger modules, which are essential for the recognition of CpG islands in the genome. TP2 is also phosphorylated by sperm specific protein kinase A and phosphorylation/dephosphorylation cycle plays an important role in the chromatin condensation process.

Introduction

In eukaryotic cells the genome is packaged inside the nucleus due to the existence of a highly ordered nucleoprotein architecture called chromatin. In the first order of organization of chromatin structure, 146 bp of DNA is packaged into a nucleosome core particle around a histone octamer (van Holde, 1988; Wolfe, 1998). The linear array of the 10 nm polynucleosomal filament is folded into 30 nm irregular fibres (either solenoid or zig-zag structure) facilitated and/or stabilised by the linker histone H1.

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Spermatogenesis is a process in which spermatogonial stem cells undergo a series of biochemical and morphological changes resulting in the production of highly differentiated haploid cells called spermatozoa. The entire process of spermatogenesis can be divided into three phases: 1) stem cell renewal and differentiation, 2) meiosis and 3) spermiogenesis. In mammals, spermatogenesis is characterised by a unique chromatin remodeling process, in which somatic histones are sequentially replaced by testis specific variants, followed by the replacement of both somatic and testis specific histones with a class of basic proteins, transition proteins (TP1, TP2 and TP4). These transition proteins appear in a brief period of 2-3 days during stages 12-15 of spermiogenesis. Finally these transition proteins are replaced by protamines during stages 16-19. Although it was initially believed that spermatozoa contained only protamine, recent evidences have shown that 10% sperm chromatin still retains nucleosomal histones in both rodents and humans (Pittogi *et al.*, 1999). The packing of DNA in mammalian spermatozoa approaches the physical limits of molecular compaction making mammalian sperm chromatin the most condensed eukaryotic nucleus.

Our laboratory has been studying over past two decades the influence of testis specific histone variants on chromatin structure during meiotic prophase at pachytene interval as well as biochemical properties of transition protein TP1 and TP2 using rat spermatogenesis as the model system. A brief review of contributions from this laboratory is presented in this article.

Testis specific histone variants and chromatin structure of pachytene spermatocytes

Several testis specific variants of histones are expressed and assembled to nucleosome during meiotic prophase as well as in post meiotic cells (Figure 1). The testis specific histone TH2A gene is expressed only in testis. The H2A and TH2A histones differ in eight amino acid residues in the first half of the molecule and three consecutive changes are present in the C-terminal region. A testis specific variant of H2B (TH2B) differs by addition of three potential phosphorylation sites (Ser12, Thr23 and Thr34) and repositioning of two others (ser5 and ser60), resulting in a different phosphorylation map of the N-terminal tail (Kimmins and Sassone-Corsi, 2005). Among the five-histone classes, H1 histones exhibit the most diversity in its amino acid sequences. In mice and humans, there are eight previously described H1 subtypes, including the five somatic subtypes H1a–H1e, the replacement subtype H1o, the testis-specific linker-histone H1t (Lennox and Cohen, 1989) and the oocyte-specific H1 linker histone, H1foo (Tanaka *et al.*, 2001). H1t is first detected in mid-pachytene spermatocytes, where it rapidly integrates into the chromatin, replaces about 40 percent of the other somatic H1 subtypes, and persists until the elongating spermatid stage (Meistrich *et al.*, 1985). HILS1 (H1-like protein in spermatids 1), has been found recently in human and mouse (Iguchi *et al.*, 2003; Yan *et al.*, 2003). H1LS is detected later in elongating and condensing spermatids nucleus. Recently, Nishimune's group has identified and characterised a novel haploid germ cell-specific nuclear protein (HANP1) in the mouse testis, also designated HANP1/H1T2, which is implicated in the replacement of histones by protamines during spermiogenesis (Tanaka *et al.*, 2005).

Histone TH2B

The early work from the laboratory concentrated on studying the structural alterations in pachytene chromatin brought about by replacement by somatic histones by the testis specific nucleosomal core histones. Biophysical studies employing circular dichroism spectroscopy and thermal denaturation techniques revealed that nucleosomal core particle isolated from rat pachytene spermatocytes were less compact compared to nucleosomal core particle isolated from rat liver.
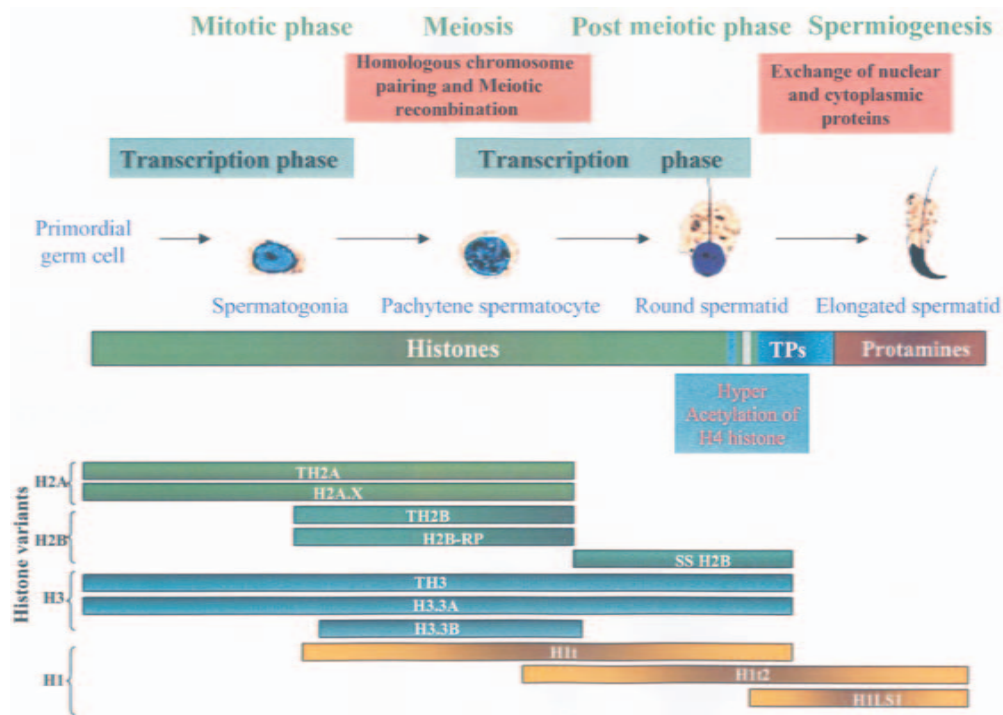
Fig.1 Chromatin dynamics during spermatogenesis: Spermatogenesis is characterized by sequential replacement of somatic histones by the testis specific histone variants at different stages of germ cell differentiation. The type of histone variants replacing the somatic counterpart are depicted in this figure including the recently discovered histone H1 variants in spermatids.

The nucleosomal core histones of pachytene chromatin had 80% of H2B replaced by the testis specific TH2B while about 10% of H2A was replaced by TH2A. It was concluded that the presence of testis specific core histones contributed to the less compact nature of the pachytene nucleosomal core particle (Rao *et al.*, 1983). This conclusion was further substantiated by DNase1 foot printing of pachytene nucleosome core particle showing increased sensitivity of pachytene nucleosomal core particle (Rao and Rao, 1987). The sensitive sites exactly mapped to H2B/ TH2B interacting site in the nucleosomal core DNA. Additional evidence came from the observation that subnucleosomal particles were generated, after micrococcal nuclease digestion, at a much faster rate from the pachytene chromatin than from liver chromatin (Rao and Rao, 1987). Based on these observations it was suggested that the nucleosome core is rendered less compact by the appearance of testis specific histone TH2B to facilitate DNA disentanglement for the DNA recombination between the paired homologous chromosomes. The amino acid sequence of testis specific histone H2B is now available, the comparison of sequences of H2B and TH2B reveals minor changes only in N-terminal eleven amino acids, which differs by addition of three potential phosphorylation sites (Ser12, Thr23 and Thr34) and repositioning of two others (ser5 and ser60), resulting in a different phosphorylation map of the N-terminal tail. It remains to be seen how such small changes in N terminal protein can influence the compaction of nucleosomal core particle. We also have to keep in mind the recent developments on

various covalent modifications that occur on histone tails. It remains to be seen, if such covalent modifications if any, might also influence compaction of pachytene chromatin at the nucleosomal level.

As mentioned in introduction the fifth linker histone H1 is necessary for stabilization of higher order structure of chromatin at the 30 nm chromatin fibre level. Histone H1t, the testis specific linker histone H1, is expressed during mid pachytene spermatocytes and comprises of almost 40% of total histone H1 in pachytene spermatocytes. Histone H1t gene has its own promoter elements to specify a tissue and stage specific expression pattern (Wilkerson et al., 2002). A series of biochemical and biophysical experiments have shown that histone H1t isolated from rat testis is a poor condenser of DNA and chromatin as compared to somatic histone H1 bdec from rat liver (Khadake and Rao, 1995). This observation is also corroborated by other studies (DaLucia *et al.*, 1994; Talasz *et al.*, 1998). We have argued that there has to be some information missing in the amino acid sequence of histone H1t, which is present in somatic histone H1s to render this with different condensing properties (Khadake and Rao, 1995). The structure of histone H1 can be divided into 3-sub domains a) N terminal tail, b) globular domain, c) C-terminal tail. The N terminal tail is implicated in influencing in higher order structure of chromatin while the globular domain is shown to be responsible for interaction of histone H1 with the nucleosome core particle. Among these structural domains, the globular domain is highly conserved among all histone H1s, while N terminal tail shows some sequence heterogeneity. The C terminal tail comprising about 100-120 amino acids is highly basic in nature and varies considerably in its amino acid sequence among all H1 histone subtypes.

The C-terminal domain of histone H1 is absolutely essential for generating 30 nm chromatin fibre and DNA condensation mediated by histone H1 is mainly due to its c-terminal domain (Allen *et al.,* 1986). Based on these facts, we compared the amino acid sequence of C-terminal tail of rat H1t and H1d and were surprised to see the presence of SPKK motifs only in the Histone H1d but not in H1t. Three SPKK motifs in Histone H1d are present within a stretch of 32 amino acids. SPKK motif has been shown to be a minor DNA binding motif (Suzuki, 1989). It was surmised that SPKK motifs might contribute to different DNA condensing properties of H1t and H1d. This prediction proved to be correct by showing that 16 mer synthetic peptide sequence containing two SPKK units could mimic all the DNA and chromatin condensing property of Histone H1d (Khadake and Rao, 1997). We have further shown that this domain indeed represents chromatin-condensing domain by several deletion mutants of histone H1d (Bharath *et al.*, 2002). We have analysed amino acid sequence of several histone H1 sub-types and find the presence of S/TPKK motif in most of histone H1 except H1t. A representative comparison of the sequence pattern is shown in Figure 2. We have included in this comparison the recently described oocyte specific H1 histone H1oo, histone H1 variant H1X, as well as testis specific H1 variants H1LS and H1T2. Interestingly, we find all the testis specific H1 histones lack 16 mer (S/TPKK) motif known to have DNA condensation properties.

The significance of absence of these motifs in this newly discovered H1s needs to be investigated. In summary, the presence of histone H1t in pachytene chromatin seems to loosen the chromatin structure even at higher order structure level. Thus it can be concluded that specific replacement of both core histones and linker histone H1 in pachytene chromatin renders chromatin to possess much more open structure probably influencing genetic recombination related events.
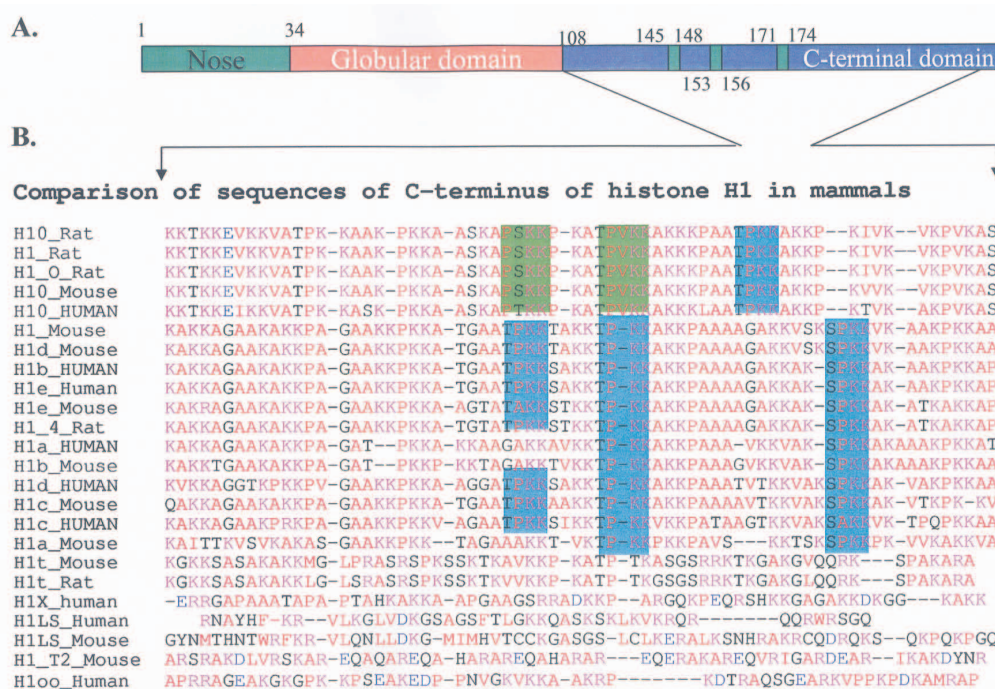
Fig. 2 A. Structural domains of histone H1. B. Multiple sequence alignment of the C-termnial domain of histone H1 subtypes highlighting the SPKK/TPKK motifs.

Chromatin dynamics during spermiogenesis

Role of Transition proteins (TPs) during final stages of spermiogenesis

The process of spermiogenesis in mammals, wherein the haploid round spermatids mature into highly condensed spermatozoa, can be broadly divided into three phases (Figure 3). In the first phase, encompassing stages 1–10, the round spermatids are transcriptionally active and contain nucleosomal chromatin. The second phase (stages 12–15) involves the replacement of nucleosomal histones by transition proteins TP1, TP2, and TP4. These transition proteins are exclusively localized to nuclei of elongating and condensing spermatids (Meistrich, 1989) and they constitute about 90% of the chromatin basic protein, with the level of TP1 being about 2.5 times those of TP2 (Yu *et al*., 2000). Finally, in the third phase, the transition proteins are replaced by protamines P1 and P2 during stages 16–19 (Meistrich, 1989). The biological significance of the evolution of transition protein genes and their physiological roles are not yet clearly understood. Both TP1-/- and TP2-/- knock out mice have been generated which are less fertile than normal and show abnormal chromatin condensation (Yu *et al*., 2000; Zhao *et al*., 2001). TP1 and TP2 double knockout mice are, however, sterile and spermatogenesis is severely impaired suggesting their important role in spermiogenesis (Zhao *et al*., 2004). Rat TP4 is a minor basic protein of 138 amino acid residues (2%) present in the step 12-15 spermatids. Akama *et al*., (1995) from their studies on the DNA binding properties of boar TP4, suggested that TP4 induces local destabilization of DNA and speculated that TP4 might contribute to the chromatin organization during spermiogenesis by helping the relaxation of the negatively super coiled DNA.
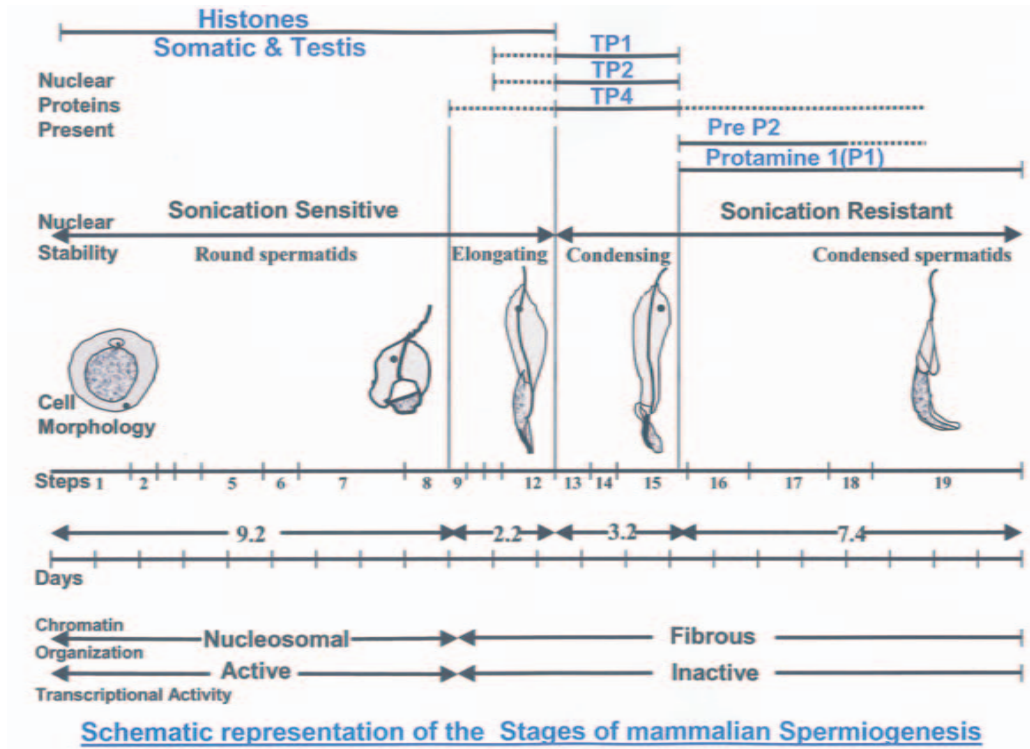
Fig. 3 Schematic representation of the stages of mammalian spermiogenesis: Morphological changes and the types of histones present at different stages of spermiogenesis are described. Stages 12-15 represent the interval during which transition proteins replace the histones.

Transition protein 1 (TP1)

In order to gain an insight into the significance of appearance of TP1, we have analysed the DNA binding properties using biophysical approaches like fluorescence quenching, thermal melting and UV absorption. From these DNA-protein interaction studies, we concluded that TP1 behaves as a DNA melting protein (Singh and Rao, 1987). It was speculated that the 2-tyrosine residues of TPl might intercalate between the nucleic acid bases resulting in local melting of the DNA duplex. Subsequently, it was shown that addition of TPl, decreases the compactness of DNA around the histone octamer destabilizes the nucleosome core particles suggesting that TP1 may be involved in displacement of histones from nucleosome type chromatin (Singh and Rao, 1988). Interestingly, TP1 has also been shown to stimulate DNA repair activity both *in vivo* and *in vitro* (Caron *et al.*, 2001), the significance of which needs to be further investigated.

Transition protein 2 (TP2)

TP2 is a basic protein of molecular mass of 13 kDa. Results from extensive biochemical and biophysical studies of TP2 from our laboratory have shown that TP2 has DNA and chromatin

condensation property (Baskaran and Rao, 1990; Brewer *et al.*, 2002). We have also shown that TP2 is a zinc metalloprotein and contains two atoms of zinc per molecule (Baskaran and Rao, 1991). Using circular dichroism spectroscopy technique, TP2 was shown to condense DNA with a preference to GC rich DNA in a zinc dependent manner (Kundu and Rao, 1995). It was interesting to study whether TP2 binds to CpG Island in the genome, which are natural GC rich sequences that are present in the 5' and 3' region of genes. By electrophoretic mobility shift assays, it was confirmed that TP2 preferentially binds to CpG island sequence. This interaction was abolished in the presence of EDTA and also upon methylation of C in the CpG dinucle-otide. We have concluded that TP2 recognizes CpG island in a zinc dependent manner and methylation of CpG island abolishes this specific interaction (Kundu and Rao, 1996). More recently by mutational analysis the domain architecture of TP2 was delineated and shown it to possess two structural and functional domains. N terminal domain having 2 zinc fingers, which is responsible for specific recognition of CpG islands (Meetei *et al.*, 2000) and C-terminal basic domain is rich in arginine and lysine residues, which is involved in DNA/ chromatin condensa-tion (Kundu and Rao, 1999). By employing extensive site directed mutational analysis, we have identified amino acid residues involved in zinc coordination. Based on our results, we have proposed novel zinc finger modules coordinating the two zinc atoms in TP2 (Meetei *et al.*, 2000). We would like to mention here that the identified zinc finger module of TP2 does not correspond to any of the known canonical zinc finger modules reported in literature.

We were interested in studying the *in vivo* localization of TP2 after ectopic expression in the absence of suitable *in vitro* cell culture and transfection system available for round spermatids, to study the effect of ectopic expression of TP2. We used cell line COS-7, for transfection studies of different deletion mutants. We have observed that wild type TP2 localizes to nucleo-lus containing ribosomal DNA, which is GC rich (Meetei *et al.*, 2000). By transfection studies of TP2 deletion mutants, we have delineated the Nuclear Localization Sequence (NLS) of TP2 to 87GKVSKRKAV95, which is rich in basic amino acid residues and closely resembles to the consensus monopartite NLS (Meetei *et al.*, 2000). TP2 is known to get phosphorylated immedi-ately after synthesis in the cytosol by protein kinase A. By site-directed mutagenesis the major phosphorylation sites in TP2 were demonstrated to occur at Thr-101 and Ser-109 residues in the C-terminal third of the protein (Meetei *et al.*, 2002). Further, we have demonstrated that the sperm-specific isoform of the catalytic subunit of protein kinase A (Cs-PKA) is involved in the phosphorylation of TP2 (Meetei *et al.*, 2002). Recently, it was found that the nuclear transport of TP2 is modulated by phosphorylation status of TP2 but is not required for its import into the nucleus (Ullas and Rao, 2003). Since phosphorylation sites are present in the C-terminal do-main of protein, which is known for DNA condensation property, we checked for condensing ability of phosphorylated and unphosphorylated TP2. Interestingly, phosphorylated TP2 is a poor condenser of DNA compare to unphosphorylated TP2 (Meetei *et al.*, 2002). Based on the above results, we have proposed a model that phosphorylation event temporarily masks the condensation property of the basic C-terminal domain, thus allowing lateral diffusion of TP2 along the chromatin to facilitate its zinc finger modules to search and dock onto the GC-rich CpG island sequences. Subsequent dephosphorylation triggers the initiation of chromatin con-densation (Fig. 4).

In conclusion, histone variants and transition proteins play a significant role in mammalian spermatogenesis. In order to have a deeper insight into the chromatin remodeling process, a more comprehensive study is necessary to evaluate the role of several histone modifications like acetylation and methylation and also the chromatin remodelling molecular complexes, which are gaining importance in recent years.
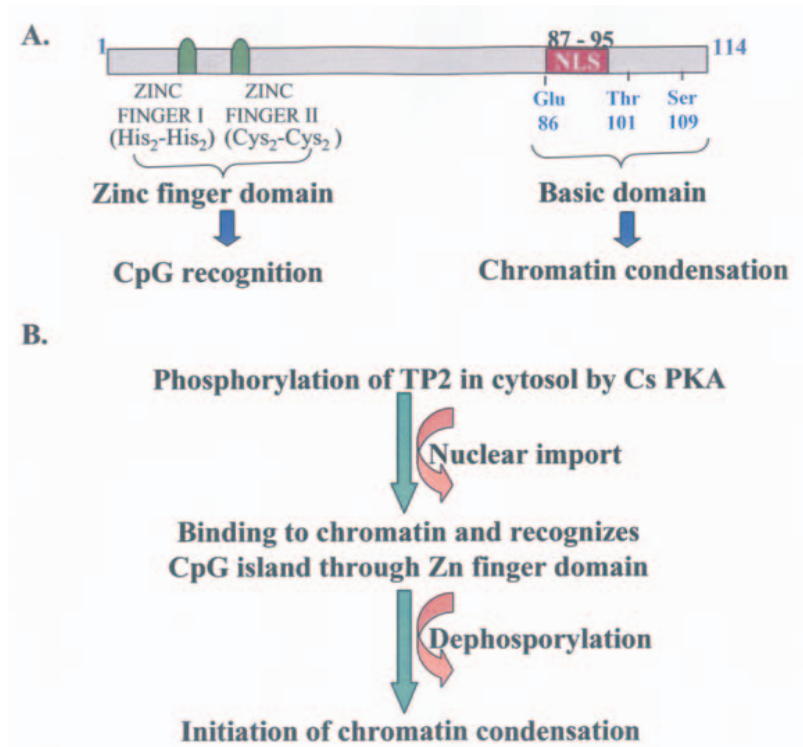
Fig. 4 A. Structural and functional domains of TP2: TP2 has two zinc fingers in the N-terminal region. The first finger involves His2-His2 finger while the second finger involves Cys2-Cys2 finger. These two zinc fingers are implicated to recognize CpG islands in the genome. The C-terminal one third of TP2 is basic in nature having NLS as well as CsPKA phosphorylation sites. B. Sequence of events leading to TP2 mediated chromatin condensation.

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