Transition protein 4 from boar late spermatid nuclei is a topological factor that stimulates DNA-relaxing activity of topoisomerase I

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Abstract Transition protein 4 (TP4) from boar late spermatid nuclei, having higher affinity for double-stranded DNA and a local melting activity of DNA, stimulated SV40 DNA-relaxing activity of eukaryotic topoisomerase I at TP4/DNA molar ratios of 6.6–11. A TP4-spermidine mixture stimulated the activity of topoisomerase I much more than spermidine alone, but no more than TP4 alone, and poly-L-arginine did not. These results suggest that TP4 contributes to the chromatin reorganization in the late spermatid nuclei from nucleosomal-type structure with negatively supercoiled DNA to nucleoprotamine structure with no supercoiled DNA.

Key words: Transition protein; Topoisomerase; Spermatid nucleus

1. Introduction

Mammalian spermiogenesis, during which round spermatids differentiate to elongated mature spermatozoa, is accompanied by extensive transformations in chromatin structure and function. Nucleosomal histones are transiently replaced by small basic proteins called transition proteins (TP1–4), and finally, by protamines [1–4]. At that time, reorganization of the nucleosomal-type chromatin into a highly condensed chromatin fiber and cessation of DNA transcription occur [5,6]. Rat TP1, having higher affinity for single-stranded DNA, induces local melting of DNA [7,8]. Rat TP2 with two possible zinc finger motifs prefers and stabilizes a GC-rich sequence zinc-dependently [9–11]. The transition in nuclear proteins requires changes of activities of topology-modifying enzymes such as topoisomerase II [12]. The testis-specific high-mobility-group protein from mouse late spermatids has been reported to be a DNA-packaging factor that can modulate the activity of topoisomerase I [13]. Recently, we have developed methods for isolating intact boar TP1–4, and have reported that boar TP1 and TP3, having higher affinity for single-stranded DNA, and boar TP4, having higher affinity for double-stranded DNA, are DNA-melting proteins, mediated through the stacking of Tyr-32, Trp-18 and Trp-126 with nucleic acid bases, respectively [14–19]. In this paper, we describe the nature of interaction of boar TP4 with SV40 DNA in the presence of topoisomerases, indicating that TP4 is a topological factor that stimulates DNA-relaxing activity of eukaryotic topoisomerase I.

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Abbreviations: TP, transition protein; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate

2. Materials and methods

SV40 DNA (form I, 5243 bp) and topoisomerase I from calf thymus (14 000 U/mg, M_r 65 000) were obtained from Gibco BRL. Human topoisomerase II (p170 form) was from TopoGEN Inc. Spermidine and poly-L-arginine (M_r 5000–15 000) were from Sigma.

2.1. Isolation of boar TP4

Boar TP4 was isolated from boar late spermatid nuclei, and renatured by our methods [15].

2.2. Effects of TP4, spermidine and poly-L-arginine on topoisomerase-catalyzed relaxation of DNA

Supercoiled SV40 DNA (form I, 1 μg) was incubated with 1.2–5 U of topoisomerase I in 50 μl of a reaction buffer (150 mM NaCl/10 mM $MgCl_2$/0.2 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride/0.44 μM bovine serum albumin (BSA)/10 mM Tris-HCl, pH 7.5) in the presence or absence of TP4, spermidine and poly-L-arginine at 37°C for 1.5–5.5 h. With 0.04–2 U of topoisomerase II, SV40 DNA (form I, 1 μg) was incubated in 30 μl of a reaction buffer (120 mM KCl/10 mM $MgCl_2$/0.5 mM ATP/0.5 mM dithiothreitol/0.44 μM BSA/50 mM Tris-HCl, pH 8.0) in the presence or absence of TP4 at 37°C for 1.5 h. The reaction was stopped by the addition of 10 μl of 5% sodium dodecyl sulfate (SDS) aqueous solution. DNA was extracted with SDS/phenol and then chloroform, followed by ethanol precipitation.

2.3. Agarose gel electrophoresis

Gels were prepared and run in the buffer system of Shure and Vinograd [20]. The DNA samples were analyzed on a 1% agarose gel in 30 mM sodium phosphate/36 mM Tris/1 mM EDTA, pH 8.0, at 2.9 V/cm for 22 h. The gel was stained with 1 μg/ml of ethidium bromide for 1 h and photographed under ultraviolet light. For two-dimensional agarose gel electrophoresis, supercoiled DNA was electrophoresed in the first dimension in the standard conditions as described above. The gel was soaked in the electrophoresis buffer containing 0.02 μg/ml of ethidium bromide for 1.5 h and re-electrophoresed in the orthogonal direction in the same buffer at 2.9 V/cm for 24 h.

3. Results and discussion

The effect of TP4 on topoisomerase-catalyzed relaxation of supercoiled SV40 DNA (form I) was analyzed by treating the DNA and the DNA-TP4 mixture with topoisomerases, deproteinizing the DNA and electrophoresing it to separate by degree of superhelicity. In the presence of topoisomerase I to relax DNA fully (5 U/μg DNA), no effect of TP4 on topoisomerase I-catalyzed relaxation of DNA was detected under the conditions used (Fig. 1a). However, in the presence of topoisomerase I to relax DNA partially (2 U/μg DNA), TP4 decreased the superhelicity of DNA depending on TP4/DNA weight ratios (Fig. 1b). The two experimental conditions used were: condition 1: increasing amounts of TP4 were added to the DNA preincubated with topoisomerase I for 1.5 h, and incubation was continued for an additional 4 h (Fig. 1b, lanes 4–9); condition 2: DNA, topoisomerase I and increasing amounts of TP4 were first mixed and the mixture was incu-

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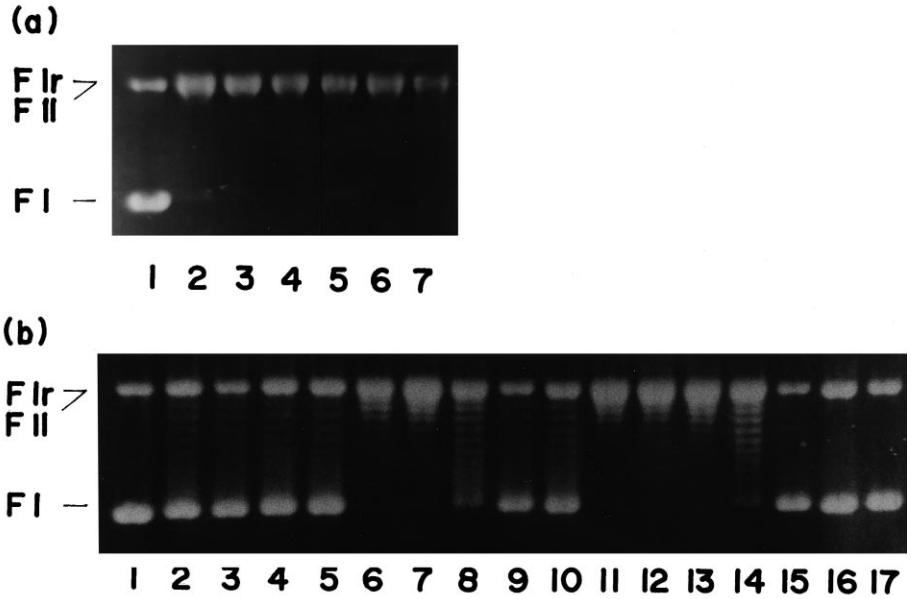

Fig. 1. Effect of increasing amounts of TP4 on the superhelicity of closed circular DNA. a: Lane 1: marker DNA; lanes 2 and 3: DNA (1 μg) incubated with 5 U of topoisomerase I for 1.5 h and 5.5 h, respectively, at 37°C. Lanes 4–7: DNA (1 μg) was preincubated with topoisomerase I (5 U) for 1.5 h at 37°C. Increasing amounts of TP4 were then added to the DNA solution, and incubation was continued for an additional 4 h at 37°C. The TP4/DNA weight ratios were 0.2, 0.4, 0.8 and 1.2, respectively. b: Lane 1: marker DNA; lanes 2 and 3: DNA (1 μg) incubated with 2 U of topoisomerase I for 1.5 h and 5.5 h, respectively, at 37°C. Lanes 4–9: DNA (1 μg) was preincubated with topoisomerase I (2 U) for 1.5 h at 37°C. Increasing amounts of TP4 were then added to the DNA solution, and incubation was continued for an additional 4 h at 37°C. The TP4/DNA weight ratios were 0.005, 0.01, 0.03, 0.05, 0.5 and 1.2, respectively. Lanes 10–15: DNA (1 μg) and TP4 were incubated with topoisomerase I (2 U) for 5.5 h at 37°C. The TP4/DNA weight ratios were the same as in lanes 4–9, respectively. Lanes 16–17: DNA (1 μg) and TP4 were incubated without topoisomerase I at TP4/DNA weight ratios of 0.05 and 0.5, respectively, for 5.5 h at 37°C. FI, FIr and FII represent supercoiled, relaxed and nicked DNA, respectively.

bated for 5.5 h (Fig. 1b, lanes 10–15). Under each condition, TP4 greatly decreased the superhelicity of DNA at TP4/DNA weight ratios of 0.03 and 0.05 (Fig. 1b, lanes 6–7 and 12–13). This effect of TP4 on the superhelicity of DNA decreased at larger weight ratios than 0.05 (Fig. 1b, lanes 8–9 and 14–15). The rate of relaxation of DNA treated with topoisomerase I for 1.5 h was almost the same as that treated with the enzyme for 5.5 h (Fig. 1a,b, lanes 2–3), suggesting that the relaxation of DNA was almost saturated after 1.5-h incubation. In the absence of topoisomerase I, no effect of TP4 on the superhelicity of DNA was detected under the conditions used (Fig. 1b, lanes 16 and 17). In order to distinguish the positive and negative superhelical conformations of DNA topoisomers induced by TP4, the DNA topoisomers obtained under condition 1 including 1.5-h preincubation with topoisomerase I were analyzed on two-dimensional agarose gel electrophoresis (Fig. 2). The supercoiled DNA topoisomers migrated more slowly in the second dimension, indicating that they were negatively supercoiled DNA [21]. The DNA topoisomers obtained under condition 2 without 1.5-h preincubation with topoisomerase I (the same topoisomers as lanes 13, 14 and 15 in Fig. 1b) showed the same results on two-dimensional agarose gel electrophoresis as described above (data not shown). When the mixture of DNA, topoisomerase I and TP4 was incubated for 1.5–5.5 h under condition 2, the effect of TP4 on the topoisomerase I-catalyzed relaxation of DNA was almost the same for the 1.5-h and 5.5-h incubations (data not shown), suggesting that the relaxation of DNA was almost completed after 1.5-h incubation. Consequently, addition of TP4 to the DNA preincubated with topoisomerase I for 1.5 h at TP4/DNA weight ratios of 0.03 and 0.05 resulted practically in the same decrease in superhelicity of DNA as that obtained after 1.5-h incubation of the mixture of DNA, topoisomerase I and TP4. These results indicate that the activity of topoisomerase I was not lost after 1.5-h incubation, and that TP4 stimulates the topoisomerase I-catalyzed relaxation of DNA at TP4/DNA weight ratios of 0.03 and 0.05 independently of the 1.5-h preincubation with topoisomerase I, that is, independently of the superhelicity of DNA.

Spermidine is known to stimulate the activity of topoisomerase I [22]. Accordingly, we investigated the effect of a TP4-spermidine mixture and poly-L-arginine on the topoisomerase I-catalyzed relaxation of DNA using 1.5 h as the incubation time. DNA and increasing amounts of spermidine were incu-

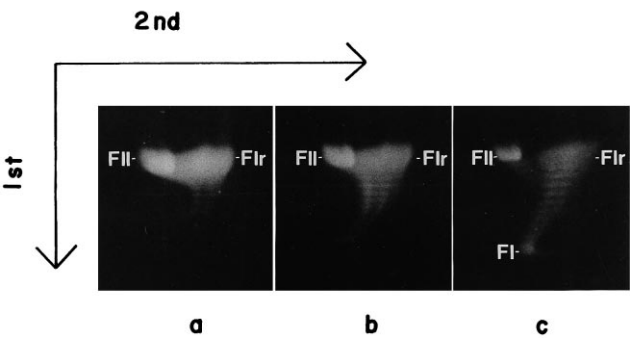

Fig. 2. Two-dimensional agarose gel electrophoretic pattern of the same DNA as lane 7 (a, TP4/DNA weight ratio of 0.05), lane 8 (b, TP4/DNA weight ratio of 0.5) and lane 9 (c, TP4/DNA weight ratio of 1.2) in Fig. 1b, respectively. FI, FIr and FII represent supercoiled, relaxed and nicked DNA, respectively.

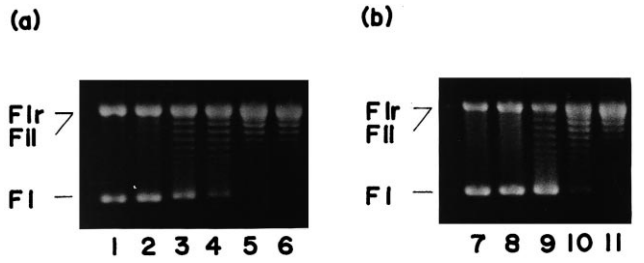

Fig. 3. Effect of spermidine and TP4 on topoisomerase I-catalyzed relaxation of DNA. a: Lane 1: marker DNA; lanes 2–6: DNA (1 μg) and increasing amounts of spermidine were incubated with 2 U of topoisomerase I for 1.5 h at 37°C. The spermidine/DNA weight ratios were 0, 2.9, 5.8, 12 and 23, respectively. b: Lane 7: marker DNA; lane 8: DNA (1 μg) was incubated with 1.2 U of topoisomerase I for 1.5 h at 37°C; lane 9: spermidine and DNA were incubated with topoisomerase I at a spermidine/DNA weight ratio of 12 in the same manner as in lane 8; lane 10: spermidine, TP4 and DNA were incubated with topoisomerase I at a spermidine/TP4/DNA weight ratio of 12/0.04/1 in the same manner as in lane 8; lane 11: TP4 and DNA were incubated with topoisomerase I at a TP4/DNA weight ratio of 0.04 in the same manner as in lane 8. FI, FIr and FII represent supercoiled, relaxed and nicked DNA, respectively.

bated for 1.5 h at 37°C in the presence of topoisomerase I to relax DNA partially. The effect of spermidine on the relaxation of DNA was saturated at a spermidine/DNA weight ratio of 12 (Fig. 3a, lanes 5–6). The TP4-spermidine mixture stimulated the activity of topoisomerase I much more than spermidine alone, but no more than TP4 alone (Fig. 3b, lanes 9–11), indicating that TP4 and spermidine might stimulate the activity of topoisomerase I by similar mechanisms. However, the activation mechanism of eukaryotic topoisomerase I by spermidine is not understood yet [22]. Boar TP4 has 48 mol of positively charged group (His 1, Arg 22, and Lys 25) per mol at pH 7.5–8.0. Spermidine has 3 mol of positively charged group per mol at the neutral pH. Using 16 044 as the M_r of TP4, 145.25 as the M_r of spermidine, and 3 512 810 as the M_r of SV40 DNA, the molar ratio of the positively charged group of TP4 to the negatively charged group of phosphate of DNA is calculated to be 0.03–0.05, and the molar ratio of the positively charged group of spermidine to the negatively charged group of phosphate is calculated to be 83. However, poly-L-arginine did not stimulate the activity of topoisomerase I at poly-L-arginine/DNA weight ratios of 0.01–20 (data not shown). Accordingly, the effect of TP4 and spermidine on topoisomerase I-catalyzed relaxation of DNA is not due only to their positive charges.

Circular dichroism spectroscopy, thermal denaturation and fluorescence quenching studies suggest that TP4, having a higher affinity for double-stranded nucleosome core DNA, induces a local destabilization of DNA at a TP4/DNA molar ratio of 0.54 which possibly introduces a bending of DNA [16]. The TP4/SV40 DNA weight ratios of 0.03 and 0.05 at which TP4 stimulates the activity of topoisomerase I correspond to TP4/SV40 DNA molar ratios of 6.6 and 11, respectively. These ratios correspond to 0.3–0.5 mol TP4 per 218–238 bp length of SV40 DNA. Per this length of the DNA one nucleosome is formed in the presence of core histones [23]. The decreased stimulation effect of TP4 on the topoisomerase I-catalyzed relaxation of DNA at TP4/DNA weight ratios higher than 0.05 (Fig. 1b, lanes 8–9 and 14–15) is probably due to the DNA-destabilizing and stabilizing effects of TP4 at the higher TP4/DNA ratios [16]. Since single-strand cleavage of the *Drosophila* HSP70 heat shock gene by topoisomerase I in vivo is confined to its transcribed region [24], TP4 may assist topoisomerase I in cleaving one of the double-stranded DNAs by inducing a local melting of DNA. The exquisite sensitivity of rat elongating spermatids to nick translation in the absence of exogenous DNase I indicates the presence of endogenous nicks which may relieve torsional stress and aid chromatin rearrangement in spermiogenesis [25]. The testis-specific high-mobility-group protein from mouse late spermatids protects supercoiled DNA against the DNA-relaxing activity of topoisomerase I, and introduces negative supercoils into a relaxed DNA in a topoisomerase I-dependent manner. Both reactions are dependent on the phosphorylation by protein kinase C [13]. Although TP4 has one cAMP- and cGMP-dependent protein kinase phosphorylation site motif and at least four protein kinase C phosphorylation site motifs [16], phosphorylated residues of TP4 have not been found.

The mammalian topoisomerase I gene is expressed in all nucleated cells, and a 250-bp region upstream of its transcription start exhibits sequence features found in many housekeeping genes [26]. Mammalian topoisomerase II activity correlates with cell proliferation, and progressively decreases in cells induced to differentiate [26]. The finding that topoisomerase II is localized to rat elongating spermatid nuclei and meiotic chromosomes of pachytene spermatocytes supports the possible involvement of topoisomerase II in DNA alterations during spermatogenesis [27]. However, TP4 did not stimulate the activity of topoisomerase II under the conditions used to relax DNA partially or fully (data not shown).

Round spermatid nuclei have a nucleosomal-type chromatin structure with left-handed supercoiled DNA around histone octamers [6,28]. Sperm nuclei in which DNA interacts with protamines have been reported to form linear side-by-side arrays of chromatin and have no supercoiled DNA [28]. It is proposed that rat TP2 with two possible zinc finger structures interacts with a GC-rich sequence zinc-dependently, which can influence cessation of the transcription process and initiation of chromatin condensation [9–11], and that rat TP1 interacts with chromatin randomly, facilitating local destabilization of nucleosome core particles [9,10]. Boar TP1 (54 residues), exhibiting 93% sequence similarity with rat TP1, interacts with DNA in a similar manner to rat TP1 [17]. Boar TP3, composed of an N-terminal region (residues 1–19) with two characteristic tryptophan residues and a C-terminal region (residues 20–76) having a close resemblance to boar TP1, interacts with DNA in a somewhat related but different manner from boar TP1 [17]. Boar TP4, inducing a local melting of DNA and possibly introducing a bending of DNA [16], is a topological factor that stimulates the activity of topoisomerase I as described above. Accordingly, boar TP4 probably aids the modulation of the structure of the nucleosome core particle with negatively supercoiled DNA to facilitate chromatin reorganization to a nucleoprotamine structure with no supercoiled DNA.

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