Interaction of Spermatid-specific Protein TP2 with Nucleic Acids, in Vitro

A COMPARATIVE STUDY WITH TP1*

Rajasekaran Baskaran and Manchanahalli R. Satyanarayana Rao‡

From the Department of Biochemistry and Center for Genetic Engineering, Indian Institute of Science, Bangalore 560 012, India

TP2 was purified from rat testes employing a gentle method involving differential salt extraction of the sonication-resistant spermatid nuclei. The nucleic acid binding properties of TP2 were studied by fluorescence quenching, thermal denaturation, circular dichroism techniques and compared with those of TP1 (Singh, J., and Rao, M. R. S. (1987) *J. Biol. Chem.* 262, 734–740). The tyrosine fluorescence of TP2 was quenched upon binding to double-stranded and denatured DNA and poly(rA). The apparent association constants for binding of TP2 to these nucleic acids were calculated from the fluorescence quenching data, obtained at 50 mM NaCl, and found to be 1.63×10^5 M^{-1}, 6.5×10^5 M^{-1}, and 7.3×10^5 M^{-1}, respectively. Thermal denaturation studies of calf thymus DNA and its complexes with TP2 showed that at 1 mM NaCl, TP2 shifted the T_m from 53 °C to 62–67 °C, while at 50 mM NaCl, the T_m was shifted from 72 to 78 °C suggesting that TP2 is a DNA stabilizing protein. Circular dichroism studies of TP1·DNA and TP2·DNA complexes have revealed that TP2 has a better DNA condensing property than TP1. Furthermore, in contrast to TP1, TP2 does not destabilize *in vitro* the compactness of liver nucleosome core particles. The DNA binding properties of TP1 and TP2 have been discussed in relation to the significance of their transient appearance during mammalian spermiogenesis.

The structure of chromatin undergoes extensive alteration during mammalian spermatogenesis (1–3). Several testes-specific histone subtypes are synthesized and replace their somatic counterparts during premeiotic and meiotic stages of germ cell development (4). The chromatin of round spermatids (stages 1 to 12) contains the somatic histones (H1a, H1bde, H1c, H2A, X2 (H2A variant), H2B, H3, and H4) as well as testes-specific histone variants (H1t, TH2A, TH2B, and TH3; Ref. 4). During the later phases of spermiogenesis, the nucleosome type of chromatin organization is transformed into smooth chromatin fibers (stages 15 to 17; Refs. 5–7). Before this transformation, there is a transient phase (stages 12 and 15) wherein the histones and their variants are replaced by the two testes-specific transition proteins, TP1 and TP2 (3, 8). The replacement of histones by these two proteins

also coincides with the cessation of transcriptional activity and initiation of chromatin condensation (5, 9). Finally, these proteins are, in turn, replaced by S1 (equivalent to fish protamine) and TP3 during stages 16 to 19 (5, 10). Protein S1 is the only basic nuclear protein present in the mature spermatozoa of epididymis.

The mechanism of transformation of nucleosomal type of chromatin into nucleoprotamine fiber is rather poorly understood. The mechanism cannot be a universal one as the nucleoprotein transition during the later phases of spermiogenesis varies among different species of animals (4). With the exception of mammals, a direct transition from a nucleosome type of chromatin organization into a nucleoprotamine fiber operates in most of the species. It has been shown recently that the tyrosine protamines from rooster (galline), squid, and cuttlefish can disassemble nucleosomes, *in vitro*, at near physiological concentrations of protamine/nucleotide ratio (11). On the other hand, the fish protamines (iridine and salmine) can disassemble nucleosomes, only at a high protamine/nucleotide ratio, that too, at a very slow rate. In this context, it is important to understand the physiological significance of TP1 and TP2 appearing transiently, in order to understand the transformation process in mammals. One of the approaches would be to study the nature of nucleoprotein organization of elongating spermatids at which stage TP1 and TP2 are present in substantial amounts. However, it has been difficult to probe the chromatin structure of elongating spermatids because of its inaccessibility to probes like micrococcal nuclease and DNase I (12). Hence, we have taken an alternate approach to understand this phenomenon by studying the nature of the interaction of these two proteins, TP1 and TP2, with various nucleic acids, *in vitro*, which should yield valuable information from which one can deduce their biological significance. In this respect, we have reported earlier that TP1 behaves like a DNA melting protein, mediated probably through the intercalation of its 2 tyrosine residues between the nucleic acid bases (13). More recently, we have shown that TP1 destabilizes the compact nucleosome core particles, *in vitro* (14). From these studies, we had speculated that TP1 may be involved in the replacement of histones at the time of its appearance during spermiogenesis.

The transition protein TP2 was first isolated by Grimes *et al.* (15) from spermatid nuclei and shown to have a molecular weight of 13,000 containing high percentages of serine, proline, arginine, and lysine residues. Recently, the cDNA-derived amino acid sequences of mouse (16) and rat (17) TP2 have been reported. We have now studied the nature of interaction of TP2 with nucleic acids, *in vitro*, and have compared these results with the DNA binding properties of TP1 reported by us earlier (13). Based on these results, the

possible significance of the transient appearance of TP1 and TP2 during mammalian spermiogenesis has been discussed.

EXPERIMENTAL PROCEDURES

All the reagents employed in the present study were of analytical grade. Poly(rA), Sephadex G-100, calf thymus DNA, and phenyl-methylsulfonyl fluoride were purchased from Sigma. Male albino rats of Wistar strain (60–70 days old) were used in all the studies.

Isolation of Sonication-resistant Spermatid Nuclei (SRS)[1] and Purification of TP1 and TP2—The SRS nuclei were purified from rat testes as described by Platz *et al.* (18). The original method of purification of TP1 and TP2 from these nuclei involves differential precipitation of acid-soluble proteins with trichloroacetic acid. In order to avoid exposure of the proteins to high concentrations of trichloroacetic acid, we have developed a milder method for the purification of these proteins based on our earlier observation that both TP1 and TP2 can be extracted from SRS nuclei between 0.4 M and 1 M NaCl (12). Briefly, about 50 g of decapsulated testes obtained from male adult rats (I.I.Sc. strain, 200 g body weight) were processed per batch. The SRS nuclei were prepared as described by Platz *et al.* (18). They were initially extracted with 25 ml of 0.4 M NaCl, 10 mM Tris-HCl, pH 7.4, by keeping the mixture in ice for 30 min. The pellet was re-extracted with 25 ml of 1 M NaCl, 10 mM Tris-HCl, pH 7.4. The supernatant obtained after centrifugation at 10,000 × g for 10 min was dialyzed overnight against double-distilled water containing 0.1 mM phenylmethylsulfonyl fluoride. The dialysate was lyophilized, and the dried powder was dissolved in 2 ml of 7.5 M urea, 2% β-mercaptoethanol, 5% acetic acid and loaded on a Sephadex G-100 column (120 × 2.2 cm; bed volume 400 ml) which was equilibrated with 1 mM HCl. Fractions of 4 ml were collected and monitored for absorbance at 230 nm. The elution profile shown in Fig. 1A reveals three peaks, and the proteins in each peak were identified as TH1 (H1a + H1t) and H1 (peak 1), TP2 (peak 2), and TP1 (peak 3), respectively, by electrophoresis on an acid-urea-15% polyacrylamide gel (Fig. 1B; Ref. 19).

Purification of Histone H1 from Rat Liver—Histone H1 was purified from rat liver as described by us earlier, and its purity was confirmed by electrophoresis on an acid-urea-15% polyacrylamide gel (19).

Fluorescence Measurements—Nucleosome core particles containing 146 ± 4 base pairs of DNA were isolated from the micrococcal nuclease digest of rat liver nuclei prepared as described by us earlier (20). The DNA was isolated from nucleosome core particles by proteinase K treatment, phenol:chloroform extraction, and subsequent ethanol precipitation. This DNA was used as a source of native double-stranded DNA. Denatured DNA was prepared by heating core particle DNA at 90 °C for 15 min and rapidly cooling into ice.

The fluorescence spectra of TP2 and its complex with nucleic acids were recorded in the Hitachi fluorescence 650-60 spectrophotometer. The tyrosine fluorescence spectrum for free TP2 in solution (1 mM sodium cacodylate, pH 7.4, 0.1 mM Na$_2$EDTA, 1–50 mM NaCl) was recorded in a cuvette of 1-cm path length after exciting at 276 nm. The fluorescence emission maximum was at 305 nm. The slit widths for excitation and emission beams were 5 nm, and the fluorescence spectrophotometer was operated in the energy mode. For TP2-nucleic acid complexes, the spectra were recorded for a fixed amount of TP2 with increasing concentrations of nucleic acids in 1 mM sodium cacodylate, pH 7.4, 0.1 mM Na$_2$EDTA, 50 mM NaCl. The final concentrations of the nucleic acids were calculated using the following molar extinction coefficients (per mol of phosphate at 260 nm and at 25 °C) and expressed as molar concentrations of bases: double-stranded DNA, 6500; denatured DNA, 9400; and poly(rA) on fluorescence was applied by using N-acetyl-L-tyrosinamide at a concentration that had the same relative fluorescence intensity as protein TP2 solution.

The fluorescence quenching data were plotted according to the method of Kelly *et al.* (21). The following equation was used for calculating the binding constant, K_a:

$$\frac{1}{\Delta F} = \frac{1}{K[N]\Delta F_\infty} + \frac{1}{\Delta F_\infty}$$

wherein ΔF = decrease in fluorescence intensity emission maximum in the presence of $[N]$ concentrations of nucleic acid and ΔF_∞ = decrease in the fluorescence intensity at infinite ligand concentration.

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[1] The abbreviation used is: SRS, sonication-resistant spermatid.

A straight line is obtained when $1/\Delta F$ is plotted against $1/[N]$ for which the slope and the intercept will be $1/K\Delta F_\infty$ and $1/\Delta F_\infty$, respectively.

Thermal Melting of DNA and DNA·TP2 Complex—Thermal denaturation of calf thymus DNA and its complexes with TP2 at low (1 mM NaCl) and at high (50 mM NaCl) ionic strengths were recorded on a Beckman DU-8 spectrophotometer. Calf thymus DNA was sheared by sonication, extracted with phenol:chloroform (1:1), and precipitated with ethanol in order to remove any contaminating impurities. The concentrations of DNA used were in the range of 0.8 to 1.2 A_{260} units/ml. The heating rate was set to 2.5 min for every degree rise in the temperature. Absorbance was recorded at 1 °C intervals in the temperature range of 30–90 °C. All the melting studies were carried out in a buffer containing 1 mM sodium cacodylate, pH 7.4, 0.1 mM Na$_2$EDTA. The TP2 concentrations were below the range at which it resulted in the precipitation of nucleoprotein complexes. There was no change in the A_{320}/A_{260} ratio during the course of the experiment. A derivative plot $\Delta H/\Delta T$ for the hyperchromicity was obtained by the use of the 3-point average method.

Circular Dichroism Studies—Circular dichroism spectra were recorded at 22 °C in a JASCO J-20 spectropolarimeter attached to a JASCO DP 500 N data processor. Quartz cuvette of 5-mm path length was used, and samples were scanned at a rate of 5–10 nm/min with a slit width of 0.5 nm.

Effect of NaCl and DNA Concentrations on the Aggregation Properties of TP1 and TP2 Nucleoprotein Complexes—To determine the feasibility of calculating the binding constants at higher salt concentrations, we examined the solubility properties of TP1- and TP2-DNA complexes at higher salt concentrations as well as at different input DNA concentrations. For this purpose, the nucleoprotein complexes were taken in 1 mM sodium cacodylate, pH 7.4, and 0.1 mM Na$_2$EDTA (protein/DNA = 3.5). After adding increasing concentrations of NaCl, the absorbance at 400 nm was recorded. To study the effect of DNA concentrations, the proteins were taken in 1 mM sodium cacodylate, pH 7.4, 0.1 mM Na$_2$EDTA, 50 mM NaCl, and, after adding increasing concentrations of double-stranded nucleosome core particle DNA, the absorbance at 400 nm was recorded.

Analytical Methods—The concentrations of TP1 and TP2 and histone H1 were determined by the turbidimetric method of Platz *et al.* (18).

RESULTS

Purification of TP2—The earlier method described by Platz *et al.* (18) for the purification of TP2 from SRS nuclei involves the use of trichloroacetic acid as high as 20%. We have routinely observed that exposure of the protein to such high concentrations of trichloroacetic acid resulted in a protein preparation which could not be dissolved completely in water. Therefore, an alternate method was developed to purify TP1 and TP2. This procedure involves differential salt extraction of SRS nuclei (12). When the SRS nuclei were initially extracted with 0.4 M NaCl, very small amounts of histone TH1(H1a + H1t), TP1, TP2, and a majority of the non-histone proteins could be solubilized from these nuclei. Subsequent extraction of the residual nuclei with 1 M NaCl resulted in a quantitative extraction of TH1, TP1, and TP2. Proteins TP3 and S1 were absent in the 1 M NaCl extract and could be solubilized from the pellet only with 0.25 N HCl. The proteins that were extracted from SRS nuclei between 0.4 M and 1 M NaCl were further fractionated on a Sephadex G-100 column as described under "Experimental Procedures." The purity of the proteins separated on the Sephadex G-100 column is evident in Fig. 1.

Fluorescence Spectrum of TP2—The amino acid composition of rat TP2 as determined by Grimes *et al.* (15) showed the presence of tyrosine among aromatic amino acids. The recently reported cDNA-derived amino acid sequence of rat TP2 (17) reveals the presence of 2 tyrosine residues at positions 69 and 115. The second tyrosine being the penultimate residue at the carboxyl terminus is flanked by basic amino acids arginine and lysine (Fig. 2). Hence, interaction of TP2 with nucleic acids can be expected to involve tyrosine residues
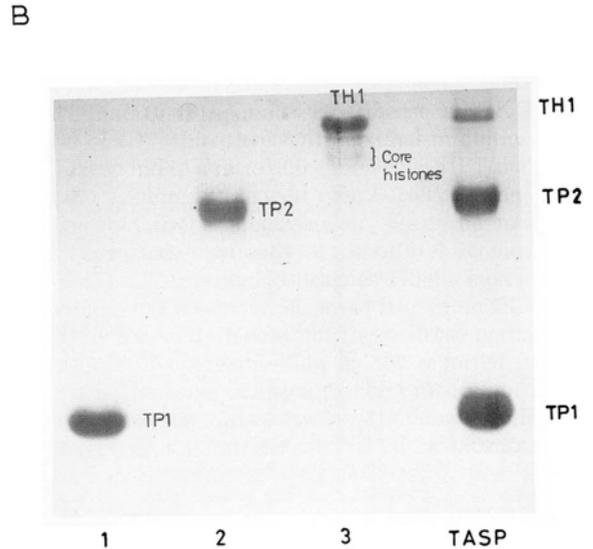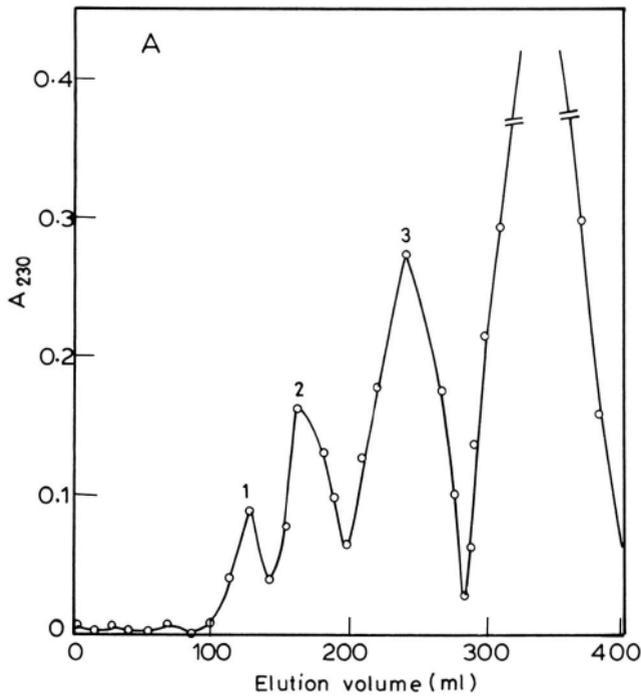
FIG. 1. *A*, gel filtration of salt-extracted proteins from SRS nuclei on Sephadex G-100. Approximately 10 mg of protein extracted from SRS nuclei between 0.4 and 1.0 M NaCl were dissolved in 2 ml of 7.5 M urea, 2% β-mercaptoethanol, 5% acetic acid and loaded on a Sephadex G-100 column (120 × 2.2 cm; bed volume, 400 ml) which was equilibrated with 1 mM HCl. Fractions of 4 ml were collected and monitored for absorbance at 230 nm. *B*, polyacrylamide gel electrophoretic pattern of the proteins fractionated on Sephadex G-100 column. Approximately 20 μg of the proteins from peaks 1 (230–260 ml), 2 (155–180 ml), and 3 (120–130 ml) were dissolved in sample buffer and analyzed on a 15% polyacrylamide/acid urea gel according to the method of Panyim and Chalkley (19). *Lanes 1, 2,* and *3* correspond to protein peaks 1, 2, and 3, while *TASP* is total acid-soluble proteins of SRS nuclei.
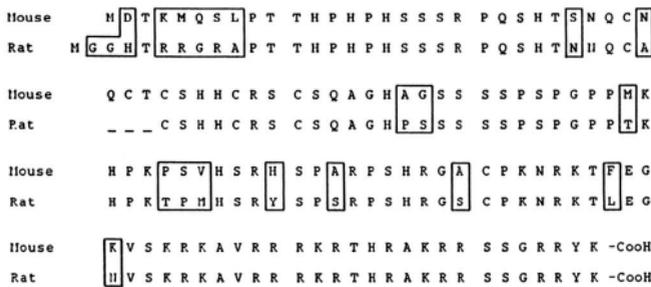
FIG. 2. **cDNA-derived amino acid sequences of mouse (16) and rat (17) TP2.** The *boxes* represent the regions of heterology between the two sequences. The two sequences have been aligned to obtain maximum homology.

FIG. 3. **Effect of NaCl on the fluorescence of protein TP2.** Fluorescence of TP2 was recorded in the presence of increasing concentrations of NaCl in a buffer containing 1 mM sodium cacodylate, pH 7.4, and 0.1 mM Na$_2$. The excitation was at 276 nm.

possibly as an indirect consequence of *a priori* binding of the highly basic carboxyl stretches of TP2 to DNA. Therefore, we have used the fluorescence of tyrosine residues of TP2 to study its interaction with nucleic acids. This was also facilitated by the absence of the other two aromatic amino acids which would have interfered with the quantum yield of tyrosine fluorescence.

The fluorescence emission spectrum of a TP2 solution in 1 mM NaCl, when excited at 276 nm, was observed at 305 nm which is characteristic of tyrosine spectra (Fig. 3). At 150 mM NaCl, there was approximately a 10% decrease in the fluorescence yield of TP2. It is possible that some secondary structure is induced in the protein so as to bury the tyrosine residues. This decrease in fluorescence is only marginal, and hence rules out the possibility of a charge effect of the nucleic acids on the fluorescence yield of TP2 in our binding studies.

Binding of Nucleic Acids to TP2 Quenches Its Fluores-

cence—Fig. 4 shows the fluorescence emission spectra of TP2 in the absence and in the presence of native and denatured DNA and poly(rA). These experiments were carried out under the same conditions as we employed in our previous studies with TP1. It can be seen from the figure that the relative fluorescence intensity at 305 nm was quenched upon binding to all three types of nucleic acids. The decrease in the fluo-
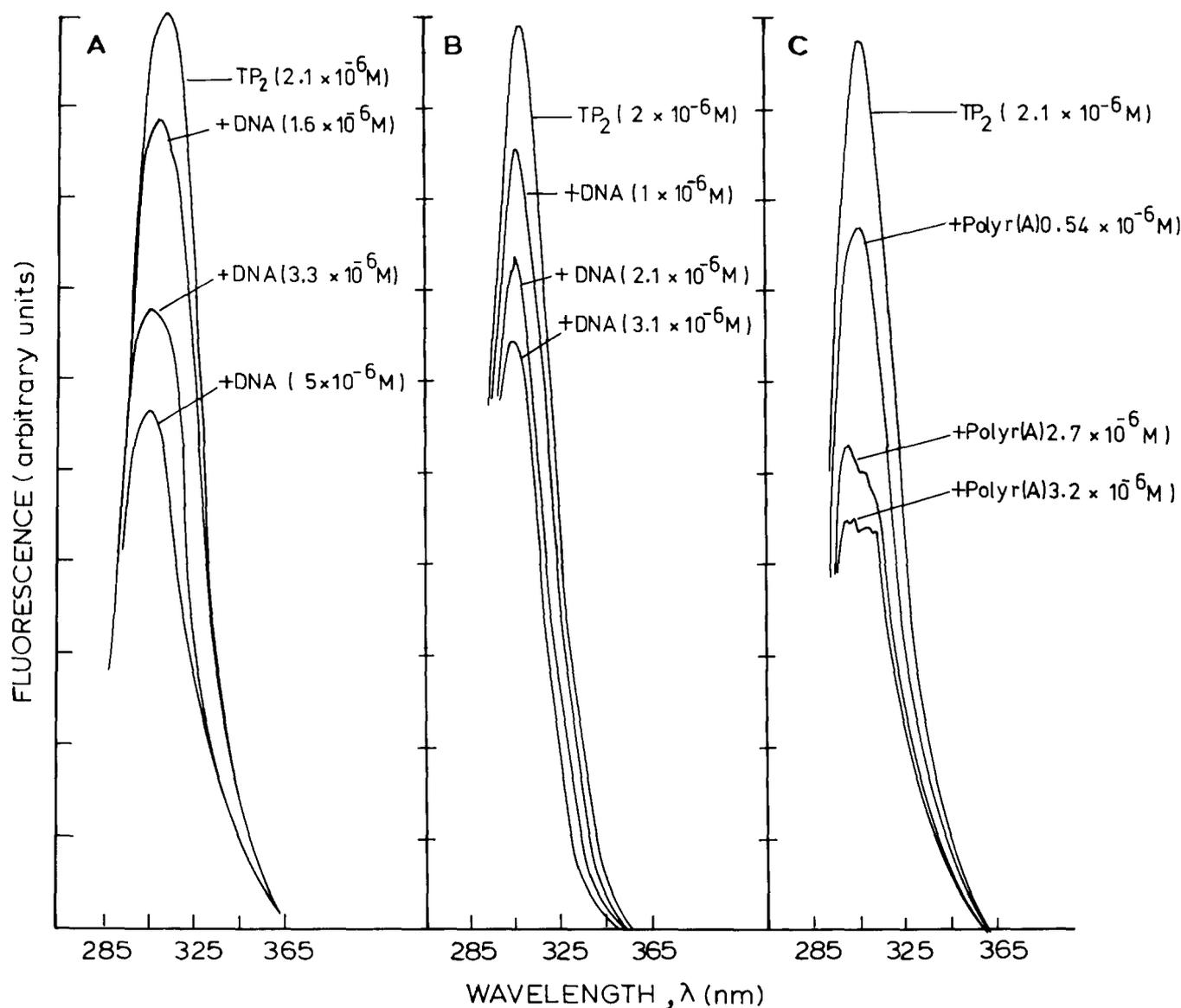
FIG. 4. **Fluorescence quenching of TP2 by nucleic acids.** Fluorescence of TP2 was recorded in the absence and presence of increasing concentrations of native DNA (A), denatured DNA (B), and poly(rA) (C).

rescence intensity showed a linear relationship with the increasing concentrations of nucleic acids added (Fig. 5, A, B, and C). Addition of still higher concentrations of nucleic acids resulted in the precipitation of nucleoprotein complexes. As shown in Fig. 2, rat TP2 has 2 tyrosine residues among which only the residue at 115 is flanked on either side by basic amino acids. Hence, theoretically, one would expect only this tyrosine to be involved in intercalation with nucleic acid bases in which case a saturation in quenching should be observed at 50%. From the data shown in Fig. 5, it can be seen that the quenching reached a near plateau at 50% with double-stranded and denatured DNA, and the saturation was not observed even at 60–70% in the case of poly(rA). At present, we can not explain why the quenching does not reach a plateau near 50% with poly(rA). The fluorescence quenching data were replotted according to the methods of Kelley *et al.* (21) as described under "Experimental Procedures." The double reciprocal plots of 1/percent quenching *versus* 1/nucleic acid concentrations are shown in Fig. 5, D, E, and F. From these curves, the apparent association constant, K_a, for double-stranded and denatured DNA and poly(rA) were calculated

and found to be 1.63×10^5 M^{-1}, 6.5×10^5 M^{-1}, and 7.3×10^5 M^{-1}, respectively (Table I). These binding constants are only rough estimates because of the insoluble nature of nucleoprotein complexes at nearly equimolar concentrations of input nucleic acids. However, these experiments were repeated at least three times, and the calculated apparent association constants are highly reproducible.

All the binding studies reported above for TP2 and reported earlier for TP1 were carried out at 50 mM NaCl concentrations. Our attempts to determine the K_a values for both TP1 and TP2 at 100 mM and at 150 mM NaCl concentrations were hampered by the fact that the nucleoprotein complexes precipitated out of solution as evident in Fig. 6, A and B. Fig. 6A shows that increasing the salt concentrations up to 160 mM NaCl resulted in a progressive increase in the absorbance at 400 nm. Fig. 6B shows the effect of input double-stranded DNA concentrations on the aggregation properties of TP1· and TP2·DNA complexes. When the binding experiments were repeated at 100 mM NaCl and at 150 mM NaCl, the nucleoprotein complexes started precipitating even at nucleotide to protein ratios of 0.2 and 0.5. Hence, we could not
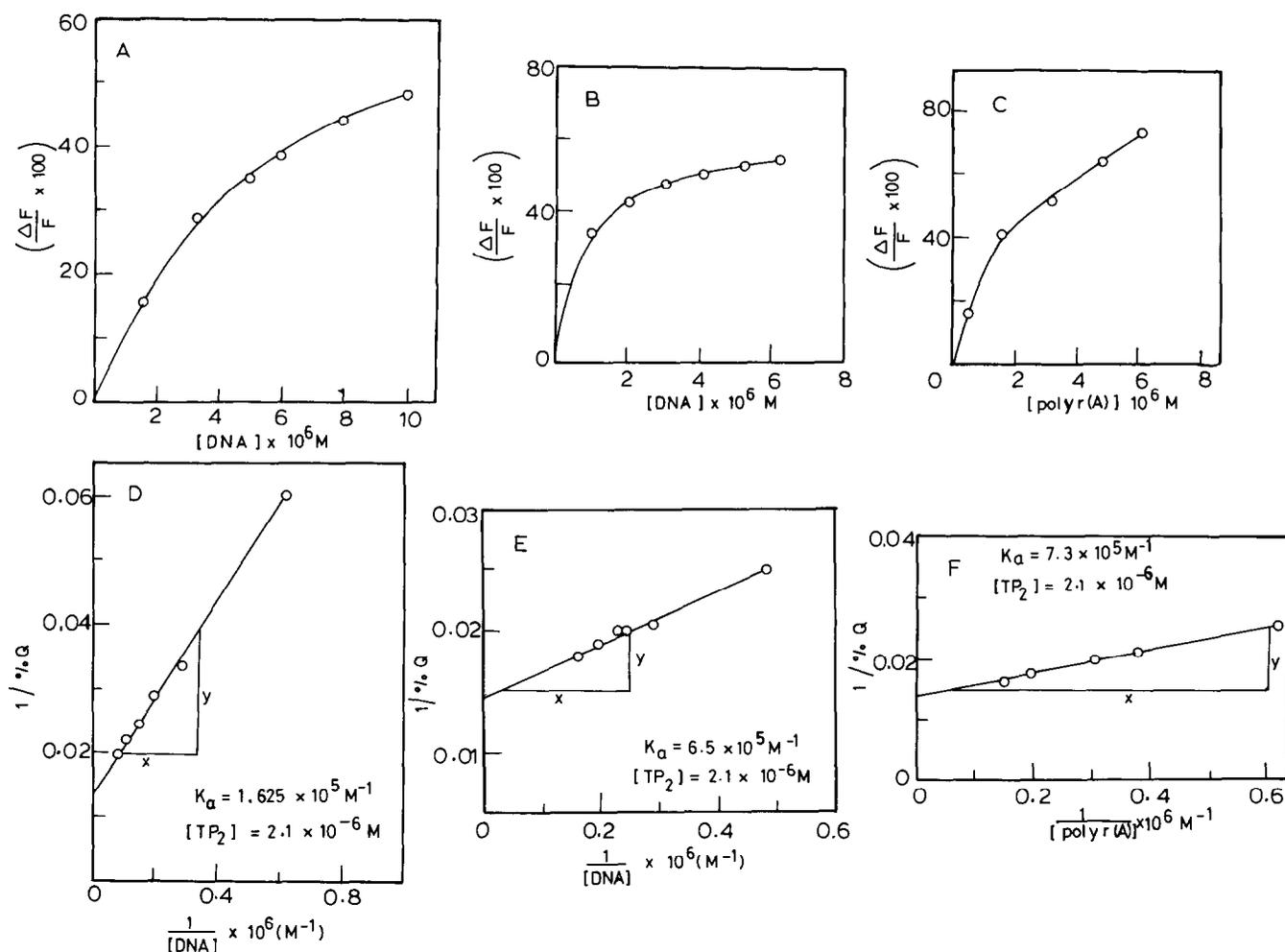
FIG. 5. **Dependence of fluorescence quenching on the concentrations of nucleic acids (A–C) and determination of K_a (D–F) of TP2 to double-stranded DNA (A, D), denatured DNA (B, E), and poly(rA) (C, F).**

TABLE I

Comparison of the association constants of TP1 and TP2 with nucleic acids

Nucleic acid	TP1[a]	TP2[b]
	M^{-1}	
Double-stranded DNA	4.4×10^3	1.63×10^5
Single-stranded DNA	2.86×10^4	6.5×10^5
Poly(rA)	8.5×10^4	7.3×10^5

[a] Data are from Singh and Rao (13).
[b] Data are from the present study.

obtain meaningful fluorescence quenching data to calculate the K_a values for TP1 and TP2 binding to various nucleic acids at 100 mM and at 150 mM NaCl concentrations.

A comparison of the association constants for TP1 (from Ref. 13) and TP2 (from the present study) for the three types of nucleic acids presented in Table I reveals two interesting features. 1) The overall affinity of TP2 to nucleic acids is approximately 40 times, 22 times, and 8 times higher for double-stranded DNA, denatured DNA, and poly(rA), respectively, than the affinity of TP1 to nucleic acids. 2) The preference for the binding of TP1 to denatured DNA and poly(rA) over duplex DNA is approximately 6.5-fold and 19.3-fold, respectively. On the other hand, TP2 binds to denatured DNA and poly(rA) with a preference of 4- and 4.5-fold, respectively, over duplex DNA.

Effect of TP2 on the Thermal Melting of Calf Thymus DNA—The effect of binding of TP2 at two different concen-
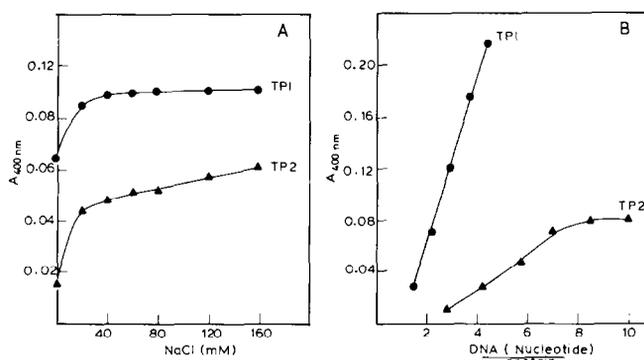
FIG. 6. **Effect of NaCl (A) and DNA (B) concentrations on the aggregation property of TP1 and TP2 nucleoprotein complexes.** For studying the effect of NaCl concentration, nucleoprotein complexes were prepared at a nucleotide/protein ratio of 3.5 in 1 mM cacodylate, pH 7.4, containing 0.1 mM Na₂EDTA, and the absorbance at 400 nm was recorded after adding increasing concentrations of NaCl. In the experiment described in *B*, proteins TP1 and TP2 were taken in 1 mM sodium cacodylate, pH 7.4, containing 0.1 mM Na₂EDTA and 50 mM NaCl, and the absorbance was recorded at 400 nm after adding increasing concentrations of double-stranded nucleosome core DNA.

trations (protein/DNA weight ratios of 1:10 and 1:5) on the thermal denaturation of native calf thymus DNA were studied at low (1 mM Na⁺; Fig. 7, *A* and *B*) and at high (50 mM Na⁺; Fig. 7, *C* and *D*) ionic strengths. At low ionic strength, the

binding of TP2 to DNA at a weight ratio of 1:10 resulted in a shift of the T_m from 53 to 63 °C. At a TP2 ratio of 1:5, the T_m was shifted to 66.9 °C. On the other hand, at 50 mM Na⁺ concentrations, the T_m of DNA was shifted to 78 °C at both the weight ratios of TP2 to DNA. There was a marginal (approximately 4 to 5%) decrease in the overall percent hyperchromicity of DNA when complexed with TP2 at both the salt concentrations. The various parameters deduced from the thermal denaturation studies are given in Table II. This table also includes the parameters obtained earlier by Singh and Rao (13) for the binding of TP1 and histone H1 calf thymus native duplex DNA. A careful comparison of these parameters reveals that TP2 stabilizes the DNA both at low (1 mM Na⁺) and high (50 mM Na⁺) ionic strengths. In fact, the stabilizing property of TP2 is even higher than that of histone H1. On the other hand, TP1 decreases the T_m by 6 °C at high (50 mM Na⁺) ionic strength while at low (1 mM Na⁺) ionic strength the T_m increases by 3 °C. Thus, it is clear from these results that the two proteins, TP1 and TP2, differ to a large extent

with respect to their DNA stabilizing properties.

Effect of TP1 and TP2 on Circular Dichroism Spectra of DNA—The circular dichroism spectrum of pBR322 DNA linearized with *Eco*RI exhibits the conservative spectrum typical of B type DNA with a positive band at 275 nm with a molar ellipticity of +8360 degrees cm² dmol⁻¹ and a negative band at 246 nm with an ellipticity of −7480 degrees cm² dmol⁻¹ (Fig. 8A). The ability of TP2 in distorting the B type spectrum of DNA was studied by complexing the DNA with TP2 by directly mixing in the presence of 0.05 M NaCl and recording the spectrum. It is clear from Fig. 8A that TP2 decreased the positive ellipticity at 275 nm and increased the negative ellipticity at 246 nm while at the same time the bands showed a red shift. The ellipticity values obtained at two different TP2 concentrations are listed in Table III. At a DNA:TP2 molar ratio of 1:0.5, ellipticity values at 275 nm decreased from 8360 to 5720, and, at 246 nm, the value increased from −7480 to −7920. At a molar ratio of 1:1, the positive value further decreased to 2640 degrees cm² dmol⁻¹, and the negative value increased to −8080 degrees cm² dmol⁻¹.

Such a distortion in the B type spectrum of DNA by TP2 could be interpreted as due to condensation of the DNA fibers into ordered aggregates. The aggregation may be brought about by holding together of the adjacent DNA strands or adjacent folds along a single strand. The results, therefore, obtained with TP2·DNA complexes indicate that TP2 brings about an effective condensation or aggregation of DNA fibers.

The effect of TP1, on the other hand, on the circular dichroism spectrum of DNA has revealed contrasting differences (Fig. 8B). Addition of TP1 caused a marginal increase in the positive band at 275 nm at both the molar ratios of 1:0.5 and 1:1 (DNA to TP1). The negative ellipticity at 246 nm is also decreased to a lesser extent (Table III). However, when the molar ratio of DNA to TP1 was increased to 1:1.5, there was a considerable distortion in the B type spectrum of the DNA.

At a TP1/DNA ratio of 2:1, there was a precipitation of the

TABLE II

Thermal denaturation parameters of TP1, TP2, and histone H1 complexes with calf thymus DNA

	Low salt (1 mM)		High salt (50 mM)	
	DNA/protein weight ratio	T_m values	DNA/protein weight ratio	T_m values
		°C		°C
DNA		53		74
DNA + histone H1[a]	3.5:1	53	3.5:1	74
DNA + histone H1	2.5:1	54	2.5:1	74
DNA		53		76
DNA + TP2[b]	10:1	62	10:1	78
DNA + TP2	5:1	66.9	5:1	78
DNA		51		77
DNA + TP1[a]	9.6:1	52	9.6:1	71
DNA + TP1	3.0:1	54	3.0:1	71

[a] Data are from Singh and Rao (13).
[b] Data are from the present study.

FIG. 7. **Effect of binding of TP2 on the thermal melting of calf thymus DNA at low ionic strength (1 mM NaCl, 1 mM sodium cacodylate, pH 7.4, 0.1 mM Na₂EDTA) and at high ionic strength (50 mM NaCl, 1 mM sodium cacodylate, pH 7.4, 0.1 mM Na₂EDTA).** *A* and *B*, hyperchromicity profile; *C* and *D*, first derivative profile DNA + TP2 (*1*), DNA to TP2 weight ratio of 10:1, DNA + TP2 (*2*), DNA to TP2 weight ratio of 5:1. The low salt curves were drawn with the help of a computer while the values obtained with high salt were plotted manually.
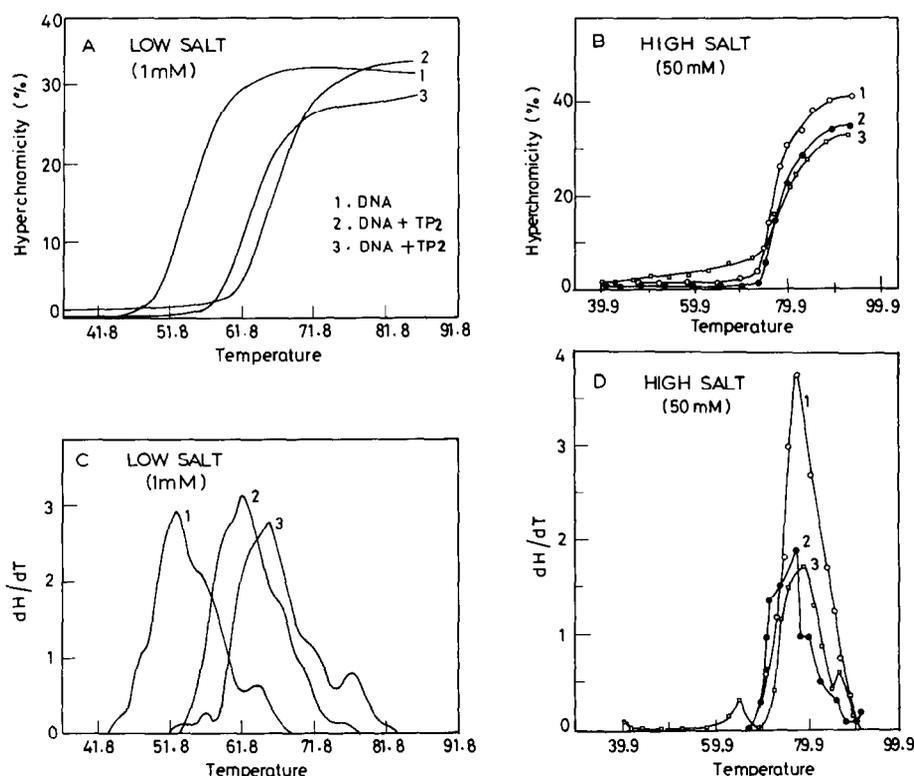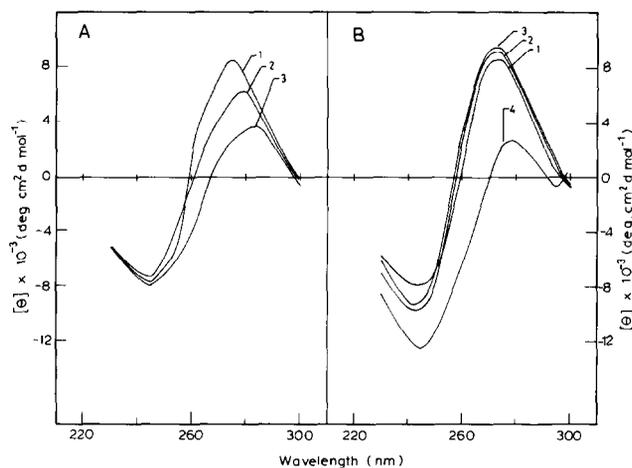
FIG. 8. **Effect of TP1 and TP2 on the circular dichroism spectrum of pBR322 (*Eco*RI linearized) DNA.** TP1 and TP2 were directly mixed with linear pBR322 DNA in the presence of 50 mM NaCl as described under "Experimental Procedures," and the spectra were recorded in the JASCO J20 spectropolarimeter. *A*, TP2; *B*, TP1. *A*: *1*, DNA alone; *2*, DNA + TP2 (1:0.5 molar ratio); *3*, DNA + TP2 (1:1). *B*: *1*, DNA alone; *2*, DNA + TP1 (1:0.5 molar ratio); *3*, DNA + TP1 (1:1), and *4*, DNA + TP1 (1:1.5).

TABLE III

Summary of the data obtained from circular dichroism studies of TP2- and TP1-DNA complexes

Sample	Mean residual ellipticity values			
	295 nm	285 nm	275 nm	246 nm
	degrees cm² dmol⁻¹			
DNA			+8,360	−7,480
DNA + TP2 (1:0.5)[a]			+5,720	−7,920
DNA + TP2 (1:1)			+2,640	−8,080
DNA			+8,600	−9,800
DNA + TP1 (1:0.5)			+9,000	−9,000
DNA + TP1 (1:1)			+9,200	−8,200
DNA + TP1 (1:1.5)			+2,400	−12,600
Rat liver nucleosome core particle	−396	+1,230	+1,200	
Core particle DNA (in 0.4% sodium dodecyl sulfate)	−132	+528	+3,500	
Core particle + TP2 (1:0.5)	−266	+920	+1,188	
Core particle + TP2 (1:1)	−146	+632	+660	

[a] The ratios of DNA to protein are molar ratios.

nucleoprotein complexes. These results, therefore, suggest that at lower concentrations of TP1, the effect on the DNA structure is very minimal, while at higher concentrations, it brings about an effective condensation of the DNA similar to TP2. It is quite likely that above a molar ratio of 1:1, TP1 may interact cooperatively with DNA in bringing about condensation of DNA fibers.

Effect of TP2 on Circular Dichroism Spectrum of Nucleosomal Core Particles—During the process of replacement of nucleosomal core histones by proteins TP1 and TP2, these transition proteins should interact with chromatin before the subsequent steps of replacement can ensue. Therefore, it is of interest to see the nature of interaction of these purified proteins with the nucleosome core particles. We have previously shown that addition of TP1 to nucleosome core particles, *in vitro*, causes destabilization of its compact structure as revealed by circular dichroism studies (14). We have now carried out similar studies with purified TP2. The circular dichroism spectra of rat liver nucleosome core particles in the

absence and in the presence of two different concentrations of TP2 are shown in Fig. 9, and the ellipticity values are listed in Table III. The spectrum of rat liver nucleosome core particles is very similar to the one reported by us earlier showing two positive bands at 285 nm and 275 nm with ellipticities of +1320 degrees cm² dmol⁻¹ and +1200 degrees cm² dmol⁻¹, respectively, and a negative band at 295 nm with molar ellipticity of −396 degrees cm² dmol⁻¹.

Addition of TP2 (at a molar ratio of DNA to protein of 1:0.5) resulted in a decrease in the ellipticity value of $[\Theta]_{285}$ and $[\Theta]_{275}$. The values were further decreased at a TP2:DNA ratio of 1:1 (Table III). These results, therefore, suggest that TP2 brings about a further compaction of nucleosome core particle. This observation is quite in contrast to the effect of TP1 on the nucleosome core particles (14).

DISCUSSION

The mechanism of transition from a nucleohistone type of chromatin to a nucleoprotamine fiber is one of the most poorly understood aspects of spermiogenesis. In most of the mammals, the chromatin undergoes an intermediate stage wherein most of histones are replaced by two testes-specific transition proteins TP1 and TP2, which in turn, are later replaced by the protamines (3, 8). This is in contrast to other species like fish and birds wherein protamines directly replace the histones (4). The present study is a continuation of our efforts to understand the nature of interaction of TP1 and TP2 with nucleic acids, *in vitro*, in order to gain an insight into the significance of their appearance during mammalian spermiogenesis. In this respect, we have shown earlier that TP1 behaves like a DNA melting protein based on fluorescence quenching, UV absorption, and thermal denaturation studies (13). By analogy with studies of Helene and co-workers (22) on tripeptides containing aromatic amino acids flanked by basic amino acids, we had speculated that the 2 tyrosine residues of TP1 having similar topology might intercalate between the nucleic acid bases resulting in local melting of the DNA duplex. Subsequently, we have further shown that addition of TP1, *in vitro*, to rat liver nucleosome core particles decreases the compactness of DNA around the histone octamer (14). We had, therefore, proposed that such a destabili-
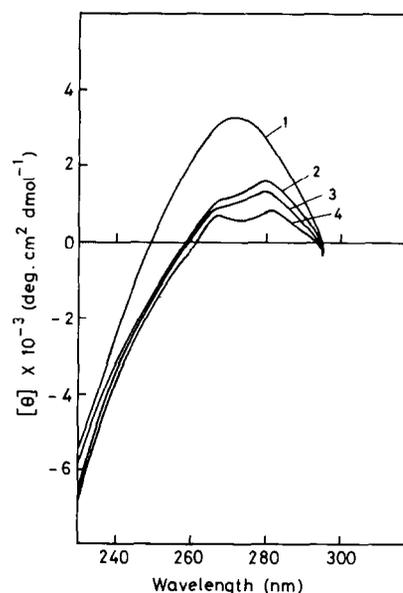
FIG. 9. **Effect of TP2 binding on the circular dichroism spectrum of rat liver nucleosome core particle.** *1*, core particle + 0.4% sodium dodecyl sulfate; *2*, core particle; *3*, core particle + TP2 (1:0.5 molar ratio); *4*, core particle + TP2 (1:1).

Nucleic Acid Binding Properties of TP2

zation process may mediate the transformation of nucleosome type of chromatin structure into a nucleoprotamine fiber during spermiogenesis.

TP2 is the second major transition protein of 13 kilodaltons that appears along with TP1. Recently, the cDNA-derived amino acid sequences of TP2 from mouse (16) and rat (17) have been reported (Fig. 2). The homology between rat and mouse TP2 sequence is nearly 85%. While Cole and Kistler (23) have reported the amino acid at the 27th position of rat TP2 from the carboxyl terminus to be glutamate, it is aspargine as deduced from its cDNA sequence (17). An interesting difference between the mouse and rat TP2-derived amino acid sequences is that while there are 2 tyrosine residues at positions 69 and 115 in rat TP2, there is only 1 tyrosine at position 116 in mouse TP2. Among the 2 tyrosines of rat TP2, the one at position 115 is flanked both sides by basic amino acids while the one at position 69 has a basic amino acid on only one side. Based on this cDNA-derived amino acid sequence, we would like to interpret that the fluorescence of tyrosine at position 69 is probably not quenched upon binding to nucleic acid, and hence we have observed a near plateau in the quenching at near 50% at least in the case of double-stranded and denatured DNA (Fig. 5, *A* and *B*).

A comparison of the apparent association constant, K_a, for the binding of TP2 to various nucleic acids (Table I) shows that TP2 does exhibit a preference for binding to single-stranded nucleic acids (6.5×10^5 M^{-1} and 7.3×10^5 M^{-1} for denatured DNA and poly(rA), respectively, as against 1.63×10^5 M^{-1} for double-stranded DNA). When these binding constants are in turn compared to those obtained for binding of TP1 to the same nucleic acids (Table I; the binding studies for TP1 to nucleic acids have been repeated with TP1, isolated by the salt extraction procedure described here, and been found to be nearly identical with those reported earlier by us (13)), two important differences become apparent. The magnitude of preference of TP1 to single-stranded nucleic acids (6.5-fold for denatured DNA and 19.3-fold for poly(rA) is slightly higher than that of TP2 to single-stranded nucleic acids (4-fold for denatured DNA and 4.5-fold for poly(rA)). It may be pertinent here that, while TP1 has 2 tyrosine residues flanked by basic amino acids, TP2 has only 1 tyrosine residue with such a topology. We would also like to stress here that although both TP1 and TP2 show preference of binding to single-stranded DNA, over double-stranded DNA, the magnitude of preference is not as high as that observed for T4 gene 32 (10^3-fold) protein, a prototype for DNA melting protein (24). Furthermore, we have to keep in mind that the binding constants for TP1 and TP2 were calculated at 50 mM NaCl concentration since at near physiological ionic strength the nucleoprotein complexes precipitated out of the solution. The second important difference between TP1 and TP2 is that TP2 has a much higher overall affinity toward nucleic acids than TP1 (K_a is in the range of 10^5 M^{-1} for TP2 and for TP1 it is 10^3–10^4 M^{-1}).

In addition to having a slightly higher preference of binding to single-stranded nucleic acids, TP2 shows a greater DNA stabilizing and condensing property as revealed by thermal denaturation and circular dichroism studies. We had reported earlier that at 1 mM NaCl, TP1 brought about a slight stabilization of calf thymus DNA against thermal denaturation, while at 50 mM NaCl, it brought about destabilization of the DNA (13). The results presented in this paper on TP2 show that both at 1 mM and 50 mM NaCl concentrations, TP2 brought about stabilization of DNA toward thermal denaturation. In fact, at 1 mM NaCl, TP2 was even more efficient in stabilizing the DNA than histone H1 (Table II).

The contrasting effects of TP1 and TP2 on the DNA were even more striking in circular dichroism studies. At the same relative molar ratios of protein to DNA, TP2 has a better DNA condensing property than TP1 (Table III). Such a contrasting difference could also be noticed in studies on the effect of TP1 and TP2 on the circular dichroism spectrum of rat liver nucleosome core particles. While TP1 brought about relaxation of the compact core particles (14), TP2 brought about a further compaction of the DNA around the histone octamer (Fig. 9).

Kleene and Flynn (16), from their cDNA-derived amino acid sequence of mouse TP2, suggested that the protein molecule can be divided into two domains. The basic amino acids are clustered in the one-third of the molecule toward the carboxyl terminus, while serine, threonine, proline, cysteine, and histidine are restricted to the NH_2-terminal two-thirds of the molecule. Recently, we have shown that both the NH_2-terminal two-thirds of mouse (16) and rat (17) TP2 sequence can be folded to generate zinc finger domains.[2] Further, we have also demonstrated that TP2 is a zinc metalloprotein as evidenced by the atomic absorption spectroscopic studies of purified rat TP2.

Thus, it remains to be seen as to which part of the TP2 molecule is responsible for the DNA stabilizing and condensing property and also whether these DNA binding properties are manifested *in vivo*.

The present paper primarily deals with the physicochemical aspects of the DNA binding properties of TP1 and TP2. An important question that arises in the present context is the fate of chromatin-bound histones upon interaction with TP1 or TP2 or in combination. The histone displacement process is not a simple process involving electrostatic competition between proteins of different basicity since histone H1, which is the least basic histone of all the histone subtypes, is still found in elongating spermatids even though the core histones have disappeared (25, 26). Recently, several other factors like topoisomerase II activity (27), histone hyperacetylation (11), ionic environment (28), ubiquitination (29), and ADP-ribosylation (30) have been reported to influence the histone transition process. Hyperacetylation of histones has also been reported in the chromatin of mammalian round spermatids (31). It will be interesting to see, therefore, the fate of histones of the round spermatid chromatin upon interaction with TP1 and TP2. These studies are now in progress and will be communicated elsewhere.

The comparative DNA binding properties of TP1 and TP2 must also be viewed in the context of their sequel of appearance in the chromatin and the important biological events that take place during the time of their appearance. The three most important events are: (*a*) initiation of chromatin condensation, (*b*) cessation of transcription, and (*c*) transformation of nucleosome type of chromatin into nucleoprotamine fiber. Moreover, Heideran *et al.* (32), from their immunological studies, have suggested that TP2 may precede TP1 in its appearance on the chromatin. It is possible that TP1, with its more even distribution of its basic amino acid residues along the length of the molecule, may interact with the chromatin randomly facilitating local destabilization of nucleosome core particles. On the other hand, TP2, with its zinc finger structure,[2] may interact with the DNA in a sequence-specific manner which in turn can influence the other two major events, namely, cessation of transcription process and initiation of chromatin condensation.

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[2] R. Baskaran and M. R. S. Rao, unpublished communication.

REFERENCES

1. Balhorn, R. (1982) *J. Cell Biol.* **93**, 298–305
2. Grimes, S. R., Jr. (1982) *Comp. Biochem. Physiol.* **83**, 495–500
3. Meistrich, M. L., Reid, B. O., and Barcellona, W. J. (1976) *Exp. Cell Res.* **99**, 72–78
4. Meistrich, M. L. (1989) in *Histones and Other Basic Nucleic Proteins* (Hnilica, L. S., Stein, G. S., and Stein, J. L., eds) pp. 165–182, CRC Press, Boca Raton, FL
5. Kierszenbaum, A. L., and Tres, L. L. (1975) *J. Cell Biol.* **65**, 258–270
6. Loir, M., and Lanneau, M. (1978) *Exp. Cell Res* **115**, 231–243
7. Loir, M., and Lanneau, M. (1984) *J. Ultrastruct. Res.* **67**, 305–325
8. Grimes, S. R., Jr., Meistrich, M. L., Platz, R. D., and Hnilica, L. S. (1977) *Exp. Cell Res.* **110**, 31–39
9. Loir, M., and Courtene, J. L. (1979) *J. Ultrastruct. Res.* **67**, 305–325
10. Kumaroo, K. K., Jahnke, G., and Irvin, J. S. (1975) *Arch. Biochem. Biophys.* **168**, 413–424
11. Oliva, R., Bazett-Jones, D., Mezquita, C., and Dixon, G. H. (1987) *J. Biol. Chem.* **262**, 17016–17025
12. Singh, J., and Rao, M. R. S. (1987) *Indian J. Biochem. Biophys.* **24**, 181–188
13. Singh, J., and Rao, M. R. S. (1987) *J. Biol. Chem.* **262**, 734–740
14. Singh, J., and Rao, M. R. S. (1988) *Biochem. Int.* **17**, 701–710
15. Grimes, S. R., Jr., Platz, R. D., Meistrich, M. L., and Hnilica, L. S. (1975) *Biochem. Biophys. Res. Commun.* **67**, 182–189
16. Kleene, K. C., and Flynn, J. F. (1987) *J. Biol. Chem.* **262**, 17272–17277
17. Luerssen, H., Hoyer-Fender, S., and Engel, W. (1989) *Nucleic Acids Res.* **17**, 3585
18. Platz, R. O., Meistrich, M. L., and Grimes, S. R., Jr. (1977) *Methods Cell Biol.* **16**, 297–316
19. Panyim, S., and Chalkely, R. (1969) *Arch. Biochem. Biophys.* **130**, 337–346
20. Rao, B. J., Brahmachari, S. K., and Rao, M. R. S. (1983) *J. Biol. Chem.* **258**, 13478–13486
21. Kelly, R. C., Jensen, D. E., and Von Hippel, P. H. (1976) *J. Biol. Chem.* **251**, 7240–7247
22. Helene, C., and Lancelot, G. (1982) *Prog. Biophys. Mol. Biol.* **39**, 11–68
23. Cole, K. D., and Kistler, W. S. (1987) *Biochem. Biophys. Res. Commun.* **147**, 437–442
24. Jensen, D. E., Kelly, R. C., and Von Hippel, P. H. (1976) *J. Biol. Chem.* **251**, 7215–7228
25. Marushige, Y., and Marushige, K. (1975) *J. Biol. Chem.* **250**, 39–45
26. Christenson, E., and Dixon, G. H. (1982) *Dev. Biol.* **93**, 404–415
27. Roca, J., and Mezquita, C. (1989) *EMBO J.* **8**, 1855–1860
28. Oliva, R., Vidal, S., and Mezquita, C. (1982) *Biochem. J.* **203**, 269–273
29. Agell, N., Chiva, M., and Mezquita, C. (1983) *FEBS Lett.* **155**, 209–212
30. Corominas, M., and Mezquita, C. (1985) *J. Biol. Chem.* **260**, 16269–16273
31. Grimes, S. R., Jr., and Henderson, N. (1983) *Arch. Biochem. Biophys.* **221**, 108–116
32. Heidaran, M. A., Showman, R. M., and Kistler, W. S. (1988) *J. Cell Biol.* **106**, 1427–1433