Condensation of DNA by Spermatid Basic Nuclear Proteins*

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Two transition proteins, TP1 and TP2, participate in the repackaging of the spermatid genome early in mammalian spermiogenesis, coincident with the first detectable changes in chromatin condensation. Using an optical trap and a two-channel flow cell to move single DNA molecules into buffer containing protein, we have measured the rates of DNA condensation and decondensation induced by the binding of Syrian hamster transition proteins TP1 and TP2 and protamines P1 and P2. The results show that both transition proteins condense free DNA, with rates similar to those of protamine 1 and 2. DNA molecules condensed with TP1 were significantly less stable than DNA condensed by protamine or by TP2. Experiments conducted with a peptide corresponding to the C-terminal 25 residues of TP2 showed that this domain is responsible for condensing DNA. Experiments conducted with two fragments of TP1 containing arginine and lysine residues demonstrated that DNA binding by TP1 must involve more than these basic sequences. Zinc facilitated the condensation of DNA by P2 but not by TP2. The dissociation rates of TP2 and P2 from DNA were not affected by the addition of zinc.

The structure of chromatin is changed dramatically during the final stages of spermiogenesis in mammals as the spermatid's genome is condensed and inactivated by the sequential binding of several basic nuclear proteins (1). Although the most dramatic change in condensation occurs in late-step spermatids (2) when the protamines displace transition proteins TP1 and TP2 (3) and coil the DNA into toroidal subunits (4), the replacement of histones by these two transition proteins several days earlier coincides with the first appearance of a detectable change in the condensation state of chromatin (5, 6). Studies in the rat have demonstrated that the deposition of the two transition proteins in spermatid chromatin occurs sequentially, with TP2 appearing first in step 10 spermatids. TP1 was observed to appear ~24 h later in step 12 spermatids (7).

Previous experiments have shown that TP2 binds preferentially to CG sequences, and the observation that TP2 appears early in spermatid chromatin (8) suggested that the function of TP2 may be to shut down transcription by binding to the CG islands that are associated with gene promoter domains. TP1, which appears a day later, has been reported to stimulate DNA repair of single-stranded breaks (9) and is suggested to function by binding to the breaks induced during the removal of the histones until they can be repaired (9, 10). Following the removal of the histones and the repair or ligation of the single-stranded breaks, protamines are synthesized and deposited on chromatin to complete the compaction of the chromatin and ensure the sperm genome remains inactive until it can be deposited inside an egg and reactivated.

Protamines, on the other hand, are highly charged, arginine-rich proteins that bind to DNA in a nonspecific manner. Previous in vivo and in vitro studies have been carried out to determine how DNA is condensed by protamines. The primary factor that induces compaction is thought to be the neutralization of the negative charge on the phosphodiester backbone of DNA, which is achieved when protamines bind (11, 12). The condensation of duplex DNA by protamine occurs in a unique fashion, one that involves the coiling of the sperm's DNA into toroidal subunits containing ~50 kb of DNA (4, 13, 14). Similar structures have been produced in vitro using a variety of polycations (15–17) including protamine (13, 18). At the completion of spermatid maturation, the mature sperm cell has been estimated to contain as many as 50,000 of these toroidal structures packed inside the nucleus (4).

Several studies performed in vitro have shown that both TP1 and TP2 bind to and condense DNA (8, 9, 19–22), but the mechanism of condensation (DNA collapse, intermolecular aggregation, toroid formation) has not been resolved. Previous experiments have suggested that TP2 is more effective in condensing DNA than TP1 (20). Analyses of the TP2 protein sequence (23) (Table I) and DNA-binding experiments conducted with fragments of the TP2 protein (21) have also provided evidence to suggest that the TP2 protein may have two structural domains, an amino-terminal sequence that provides the protein with a specificity for binding to CG islands (8) and a carboxyl-terminal arginine- and lysine-rich segment that enhances the protein's ability to condense DNA. TP1 contains three short arginine- and lysine-rich sequences of 4–5 residues each, which may participate in its binding to DNA (23).

Using a new technique that permits the analysis of the kinetics of DNA condensation without the complications of intermolecular aggregation, we have examined TP1- and TP2-mediated DNA condensation to determine whether these proteins are capable of inducing the condensation of single, histone-free DNA molecules in vitro. The results show that the condensation rates observed for TP1 and TP2 are similar to those obtained with protamines 1 and 2. Experiments were also conducted to assess the stability of the protamine and transition protein-DNA complexes and determine whether particular basic segments of the proteins bind and participate in DNA condensation. The effect of zinc on the condensation/dissociation process induced by these transition proteins and protamines was also investigated.
3D. Stigter, personal communication.

EXPERIMENTAL PROCEDURES

Isolation and Purification of Protamines and Transition Proteins—Hamster sperm were obtained by teasing the epididymides from sexually mature Syrian (Mesocricetus auratus) hamsters in Tris-saline and filtering the suspended sperm through 80-μm nylon gauze. The sperm basic nuclear proteins were extracted, and the two protamines were separated as described by Corzett et al. (24).

The transition proteins TP1 and TP2 were extracted from testicular sperm. Frozen Syrian hamster testes were thawed, homogenized in a Virtilis homogenizer at 5000 rpm for 3 min in 0.25 mM sucrose, 0.01 M Tris, pH 8, 2.5 mM magnesium chloride, and 1 mM phenylmethylsulfonfyl fluoride and filtered through three layers of cheese cloth. The nuclei were centrifuged at 4100 × g for 3 min at 4 °C. To remove contaminating somatic nuclei and early spermatids that were not sonication-resistant, the nuclear pellet was washed four times by sonicating it in distilled water with 1 mM phenylmethylsulfonfyl fluoride and centrifuging the suspension at 3000 × g for 3 min (3). The sonication-resistant nuclear pellet was resuspended in 0.25 N HCl, 1 mM phenylmethylsulfonfyl fluoride, and the proteins were extracted on ice for 1 h with occasional mixing and centrifuged at 10,500 × g for 10 min. The pellet was re-extracted for an additional hour, and the supernatants were pooled. Trichloroacetic acid was added to a final concentration of 3.5% for 1 h and then centrifuged at 4100 × g for 15 min to remove the protamines. Additional trichloroacetic acid was added to the supernatant to increase the concentration to 25% to precipitate the transition proteins. The precipitated proteins were sedimented by centrifugation, washed with acidified acetone, and air-dried.1

Protamines P1 and P2 and transition proteins TP1 and TP2 were separated and purified by high performance liquid chromatography (HPLC).2 The extracted proteins were reduced with 30 mM dithiothreitol and purified by high performance liquid chromatography; DTT, dithiothreitol.

Preparation of Stained DNA Molecules Attached to Beads—Lambda phage DNA (Invitrogen) was tagged with biotin and attached to 1-μm diameter streptavidin-coated polystyrene beads (Bangs Laboratories Inc, Fishers, IN) as described previously (31). The DNA attached to beads was digested in degassed 50% sucrose (w/v), 100 mM sodium bicarbonate pH 8, 30 mM DTT, and a sufficient quantity of YOYO-1 dye such that the dye molecule to DNA base pair ratio was less than or equal to 1:4. The same buffer, minus the YOYO-1 dye, was used for dissolving the protein samples. For experiments testing the effect of zinc (50 μM zinc chloride), DNA condensation/decondensation, the antioxidant in the buffer used for both DNA and protein was changed from 30 mM DTT (which binds zinc) to 30 mM cysteamine.

Design of Condensation/Decondensation Experiments—The experimental apparatus (shown in Fig. 1A) is identical to that used previously to examine the condensation of individual DNA molecules by salmine (31, 32). Separate solutions of DNA molecules attached to polystyrene beads and protein were introduced into a dual-port flow cell (Fig. 1A) at a flow velocity of ~50 μm/s. Beads containing individual DNA molecules were trapped using an infrared laser optical trap and transported into the protein solution, where the condensation of an extended, single DNA molecule (Figs. 1B and 2) could be observed via fluorescence microscopy. An argon-ion laser (λ = 488 nm) was used to illuminate the YOYO-1 stained DNA molecules, which could be observed for times as long as 10–15 min before photobleaching occurred. The same molecule could then be observed as it decondensed by pulling the molecule back to the DNA side of the flow cell, where there was an absence of peptide, and watching the DNA molecule re-extend.

The fluorescence image of each DNA molecule was detected using an image-intensified CCD camera, and a frame grabber was used to digitize the resultant images. The conversion factor for the observed DNA length in μm to kb (2.53 kb/μm) was determined by measuring the length of stained lambda phage DNA (48.5 kb) extended by flow. It was measured to be 19.2 ± 0.82 μm at a flow rate of 72 μm/s in 50% sucrose. Under these conditions the DNA was 95% extended.3

RESULTS

Previous experiments have shown that both salmine and bull protamine 1 induce the coiling and condensation of DNA molecules (31–33) into toroidal structures similar to those observed in native sperm chromatin (4). Single molecule fluorescence experiments (31) showed that condensation of DNA into a toroid appears as a distinctive fluorescent spot, starting at the free end of the molecule, which increased in brightness as more of the DNA was coiled into a toroid. The appearance of the fluorescent DNA molecules condensed by the proteins used in these experiments (Table I) were identical to those reported previously. Nevertheless it would be premature to assume that the DNA was, in fact, also condensed into toroids by TP1, TP2, and the synthesized peptides corresponding to their subdomains unless we verify this through further atomic force microscopy or electron microscopy of the condensed DNA molecules.

Determination of Protein Binding Rates—The rate of condensation of the DNA molecule has been previously shown to be linearly related to the concentration of the protein available for binding (31). Because the process of DNA condensation appears

| Amino acid sequences for hamster protamines and mouse transition proteins |
|-------------------------------|------------------|
| **TP2 (mouse)** | **Protamine 2 (Syrian hamster)** |
| MDTKMQSPLTTHFPHSSRSSQHSPSQCNQCTSHCRSSCQAOHSGSSSP | SGGPFKMKPSPSHSPPHSHRGSCPNNKTQFEGKVSRRKAVRRKKRTHRARRRTGRKR |
| **TP1 (mouse)** | **Protamine 1 (Syrian hamster)** |
| STSRLKTHGMRDKNNRHAPKGLKVRGKSGRRYKRVLKEDGRHNKSHLYSL | MKYRSMFSEIFRHGFPQGKEQREQQQQLSPVEDGRHRQHHRRCRCR |
| **Protamine 2 (Syrian hamster)** | | |
| MVRYRMRSPSERPHQGPGQEHGREEQGQGQGLSPERVEDYGRTHRGQHHHRRRCS | RKRKVEHRSEHRRCRRRRHCRHRSCRRRRHCRHRSCRRRRHR |

1 M. L. Meistrich (1998) personal communication.

2 The abbreviations used are: HPLC, high performance liquid chromatography; DTT, dithiothreitol.

3 D. Stigter, personal communication.
to be driven by the neutralization of the charge along the phosphodiester backbone as the basic nuclear protein or polycation binds (15, 16, 34, 35), the rate of protein binding to the DNA molecule can be determined by monitoring the rate of shortening of the DNA molecule. For each protein, a range of concentrations (\(10^{-4} - 10^{-3}\) M) was tested to determine whether the protein bound to the DNA molecule and condensed it. The condensation rate for 25–30 molecules was plotted as a function of protein concentration (Fig. 3). The data were fit to a straight line using a least-squares analysis, and the fitted slope was proportional to the binding rate constant, \(k_b\). Dividing by the DNA binding footprint (if known) of the protein gave the binding rate constant. Footprints are known for hamster Protamine 1 (10–11 bp) and Protamine 2 (15 bp) (36) but not TP1 or TP2. Control experiments were also conducted using bovine serum albumin (3 \(\mu\)M), a protein that does not bind to DNA. In these experiments the DNA molecule remained fully extended throughout the duration of the experiment, which lasted several minutes. At this same concentration, DNA condensation times for all other proteins reported in this paper were typically much less than 1 min.

Experiments performed with hamster protamine 1 and protamine 2 showed that both protamines condense individual DNA molecules \textit{in vitro}. These experiments, which were performed over a range of protein concentration (Table II), reveal that the two protamines appear to work equally well in condensing DNA. Protamine 2 condensation experiments, conducted with and without 50 \(\mu\)M zinc chloride, showed that the presence of the zinc increased the rate of protamine 2 binding to DNA nearly 3-fold.

Similar experiments conducted with hamster TP1 and TP2 showed that both transition proteins are capable of inducing DNA condensation. Both transition proteins condensed DNA at a rate similar to protamines 1 and 2. In contrast to the results obtained for protamine 2, the presence of 50 \(\mu\)M zinc chloride had no effect on the ability of TP2 to condense DNA.

\textbf{Determination of Protein Dissociation Rates}—The dissociation rates of the protamines and transition proteins were determined by pulling a DNA molecule into a low to moderate concentration of the protein (so it would not condense too rapidly), allowing it to condense to approximately one-half its length (to avoid nonspecific binding of the DNA-protein complexes to the streptavidin-coated bead), and then pulling the incompletely condensed DNA molecule back across the buffer interface, out of the protein solution and into the buffer stream containing only DNA. Using this approach, the upper limit of the dissociation rate of protein from the DNA molecule could then be determined by measuring the rate of re-extension of the DNA molecule (31). The mean and standard deviation of the dissociation rate constant, \(k_d\), for 10 DNA molecules is tabulated for each peptide in Table III. The dissociation constant, \(K_d\), was determined by dividing the protein dissociation rate constant, \(k_d\), by the binding rate constant, \(k_b\), or if the protein footprint was not known, the decondensation rate divided by the condensation rate. The dissociation constant standard deviation was determined by using the standard propagation of uncorrelated errors.

Off-rates determined for both hamster protamine 1 and prot-
amine 2 were observed to be similar (−0.4–0.5 molecules/s) to those previously determined for salmine (0.7 molecules/s). Zinc had no effect on the rate of dissociation of either protamine. The transition protein TP1, by comparison, dissociated from DNA much more rapidly than either protamine. TP1 was measured to have a decondensation rate (the footprint is unknown) that was 10 times that of protamine 1. In contrast, the dissociation rate of TP2 from DNA was determined to be comparable to that of protamine 1 and protamine 2. As observed for protamine 2, zinc concentrations as high as 50 μM had no effect on the dissociation rate of TP2.

**Binding and Dissociation of TP1 and TP2 Peptide Sequences**—Condensation and off-rate experiments were conducted with synthetic peptides, a 25-residue peptide corresponding to the carboxyl-terminal end of TP2 (SKRRK-YRRRKRTHRAKRTRGSKRRKY) and two arginine- and lysine-rich peptides present in TP1 (KRKYNKSVLKSRRKR and RRGKNNR). The rate of condensation and stability of the TP2-DNA complex and the TP2(25-mer)-DNA complex, as evidenced by their off-rates, were essentially identical. This was not observed for the TP1 peptides. Only one of the peptides (KRKYNKSVLKSRRKR) was observed to condense DNA, and the rate of dissociation of this peptide from DNA was observed to occur at a much higher rate than observed for the TP1 (Table III).

**DISCUSSION**

**DNA Condensation Induced by Protamines P1 and P2**—In the present study, we have examined and compared the DNA binding kinetics of the four basic nuclear proteins (transition proteins TP1 and TP2 and protamines P1 and P2) that lead to the repackaging of chromatin during the final stages of mammalian spermatogenesis. The kinetic experiments not only show that both protamine 1 and 2 condense DNA, but they also demonstrate that protamine 2 is just as effective in condensing DNA as protamine 1. The rates of condensation achieved with hamster protamines in this study were similar to those observed previously (31) for equivalent concentrations of salmine.

Binding experiments conducted in the presence of zinc show that zinc increases the rate of protamine 2 binding to DNA nearly 3-fold, whereas it did not alter the rate of dissociation of protamine 2 from the DNA-protamine 2 complex. The effect of zinc on the protamine 2 on-rate is consistent with previous studies that have shown zinc binds to protamine 2 in vitro and with the hypothesis that zinc binding to protamine 2 may induce a structural change in the protein, such as the formation of a zinc finger that facilitates its binding to DNA (37, 38). Although a zinc-induced conformational change in protamine 2 has not been demonstrated, zinc is bound to protamine 2 in vivo in sperm chromatin (39), and its presence appears to be important for sperm chromatin function. Several studies have correlated zinc deficiencies in the male with infertility (40, 41).

**Rate of Protamine Dissociation from DNA**—Our analyses of the off-rates of the two hamster protamines have provided valuable new information that may be relevant to our understanding of how these proteins interact with DNA and perform their function. The off-rates determined for both hamster protamines were similar to the rates measured previously for salmine using the same technique (31). Although hamster protamines 1 and 2 contain cysteine residues that might form inter-protamine disulfide bonds and slow down the dissociation process (salmine does not contain cysteine), the current experiments were conducted in the presence of sufficient reducing agent (30 mM DTT) to preclude disulfide bond formation. These experiments also show that the off-rate determined for hamster protamine 2 is not significantly different from that observed for protamine 1. This indicates that the previously reported finding that sperm containing substantial amounts of protamine 2 decondenses more rapidly in the oocyte after fertilization (42) must not be caused by differences in the affinities of the two protamines for DNA.

The absence of an effect of zinc on the off-rate of protamine 2 also suggests that once protamine 2 is bound to DNA, its affinity is not dictated by the presence of the zinc or its ability to maintain a zinc finger (or some other) conformation. Those factors that stabilize the interaction between protamine 2 and DNA once it is bound, such as the bonds formed between the
arginine residues and the DNA backbone phosphates, may not differ significantly in protamine 2-DNA complexes formed with and without zinc.

**TP1 and TP2 Condense DNA**—Baskaran and Rao (20) have previously reported that TP2 condenses DNA in vitro. In these earlier studies, results were obtained that suggested that transition protein-induced condensation must be quite different from that achieved with protamine. TP1 was also reported to be less effective in condensing DNA than TP2 (20). The results we obtained using single DNA molecules show that both transition proteins TP1 and TP2 can condense free DNA in vitro, but comparisons of the decondensation rates of the TP1-DNA and TP2-DNA complexes obtained from these studies demonstrate that TP2 binds more tightly to DNA. The dissociation constant determined for TP2 (0.82 nM) was found to be similar to that of hamster protamines P1 (0.25 nM) and P2 (0.54 nM). TP1 was observed to bind DNA 6-fold less tightly ($K_d = 5.0$ nM). The TP1 and TP2 dissociation constants, which were obtained under conditions that prevented DNA aggregation and precipitation, are orders of magnitude lower than those measured by Baskaran and Rao (20).

The results obtained in the decondensation studies also suggest that the dissociation rate of hamster TP2, protamine 1 and protamine 2 are determined primarily by the sequences (and number of positively charged amino acids) of their DNA binding domains. As the earlier studies showed (31, 32) the off-rates for salmine (which contains 21 arginine residues/molecule) and Arg9 (0.71 and 18,000 molecules/s, respectively) appear to be related to the number of arginine residues present in the molecule. The more arginine residues that are available to bind to the phosphodiester backbone of DNA, the lower the off-rate. Similar results were obtained in this study for both hamster protamines and TP2. Because TP2 binds to DNA with the same affinity as protamine, its seems likely that this protein may displace the histones directly by competing for DNA binding and initiating condensation. TP1, on the other hand, exhibits significantly weaker binding, which suggests that it may be less likely to displace histones simply by direct competition.

**Role of Transition Protein Subdomains**—Because both transition proteins condense DNA, these results suggest the arginine- and lysine-rich sequences in TP1 and TP2 participate in DNA binding in such a manner that they effectively neutralize the charge along the DNA backbone. TP2 appears to have two separate domains that contribute to its interaction with DNA. The amino-terminal three-quarters of the molecule has been reported to provide the specificity in the protein’s binding to CpG sequences (21, 43), possibly through the formation of two or more zinc finger domains (44), while the carboxy-terminal domain may play a major role in facilitating condensation (45). This hypothesis was confirmed for TP2 by condensation experiments conducted using only the carboxy-terminal 25-mer of TP2: SKKAVRRRRKTHRAKRRTSGRRYK. Experiments performed with this peptide revealed that both the rate of DNA condensation and rate of dissociation of the carboxy-terminal peptide from DNA were indistinguishable from rates observed for the intact TP2 protein. This observation adds additional support to Kundu and Rao’s hypothesis (21) that this sequence must be the DNA binding domain of TP2. It also shows that this sequence alone is sufficient to confer the DNA condensation properties of the protein. This result and our observation that the presence of zinc does not alter either the rate of DNA condensation induced by TP2 or the decondensation of the complex suggest that the remainder of the protein (the amino-terminal domain) may provide some sequence specificity in binding to CpG islands, but it cannot contribute significantly to the proteins binding affinity.

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**Relevance of Transition Protein Condensation of Free DNA**—Although these experiments clearly show that the transition proteins can condense histone-free DNA, the relevance of this observation is not entirely certain. It is clear from electron microscopy studies that the deposition of the transition proteins on DNA in early spermatids is correlated with a noticeable change in the condensation state of chromatin (6), but the chromatin does not appear to achieve a level of compaction comparable with that attained when the protamines replace TP1 and TP2. Although TP1 and TP2 can bind to and condense free DNA, these proteins may not be able to induce the condensation of DNA when they bind to chromatin. The presence of the histones and the nucleosomal organization present in early spermatids could easily alter how the transition proteins interact with and condense the DNA.

**Single Molecule Versus Ensemble Condensation Studies**—These results clearly demonstrate the utility of single molecule binding studies to assess the kinetics of DNA condensation and determine protein dissociation rates. Previous studies of the binding of the protamines and transition proteins have been complicated by the aggregation and precipitation that occurs when these proteins bind to bulk DNA in solution. Using a single-molecule approach, the condensation process can be identified (torus formation versus nucleosome or other form of compaction) and the kinetics of the process (rates of protein binding and dissociation) can be measured directly. The results obtained in this study not only confirm the previous observation that the binding of TP1 and TP2 to DNA are different (20), but they have also provided new information showing that both proteins are capable of condensing histone-free DNA. Whether this actually is likely to occur in the spermatid, where the DNA is complexed with histone, remains to be determined.

Although these experiments provide new information about the kinetics of DNA condensation by the protamines and transition proteins, it is critical that future studies of this kind be conducted under conditions that will allow the sequential complexation of DNA with a series of proteins, to assess how effective individual proteins are in displacing histones and reorganizing the structure of chromatin under conditions more closely resembling that encountered in vivo. This should be possible in the near future, once we complete the construction of a multiport flow cell (which will enable the DNA molecule to
be moved through multiple protein solutions) and the preparation of defined lengths of DNA complexed with histones or chromatin fibers attached to beads.

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