Stabilization of Intramolecular Triple/Single-Strand Structure by Cationic Peptides†

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ABSTRACT: For better comprehension of possible physiological roles of triple-helical DNA structures, it is important to understand if the proteins can stabilize intramolecular triplex (H-DNA). One plausible mode of stabilization is through the neutralization of electrostatic repulsion of negatively charged phosphates in the three DNA strands by positively charged arginine and lysine residues of a bound protein. To gain an insight into interactions between H-DNA and cationic protein domains, we examined the effect of Lys- and Arg-rich oligopeptides on the B-DNA to H-DNA transition. These oligopeptides as well as another type of polycation, spermine, shifted the equilibrium toward H-DNA. These polycations introduced little change in DNA superhelicity, so that an increase in torsional stress was not responsible for facilitated H-DNA formation. Competing influences of polycations and monovalent cations suggest a significant involvement of electrostatic interactions in H-DNA stabilization. The Arg-rich peptides are more effective in H-DNA stabilization than the Lys-rich ones. However, as inferred from experiments on intermolecular complexes, this is not due to a better stabilization of triple helix or destabilization of double helix. It is possible that Arg-rich peptides interact with the unpaired single strand in H-DNA and stabilize its unpaired conformation.

A homopyrimidine-homopurine (Py·Pu)[1] sequence of mirror symmetry can form an intramolecular structure (H-DNA) that consists of a triple helix and an unpaired single strand (*1*). To form this structure, DNA strands in half of the Py·Pu tract unwind, and an unpaired Py strand bends around a point of symmetry and hybridizes as a third strand to another half of the Py·Pu tract by forming Hoogsteen hydrogen bonds with Pu strand in the duplex. The unwound Pu strand remains unpaired (*1*). In a similar fashion, a variant of H-DNA (H′-DNA) is formed when half of the Pu strand forms Hoogsteen base pairs with the Py·Pu duplex and half of the Py strand remains unpaired (*2*).

The formation of H-DNA may be promoted by different factors that may partially substitute for each other. Unrestrained supercoiling in a topologically closed DNA molecule locally destabilizes double-stranded DNA and favors the formation of H-DNA (*3*−*6*). For H-DNA structures involving C·G·C$^+$ base triads with protonated cytosines, lowering the pH may enhance the effect of supercoiling. The triplex structure acquires protons from a moderately acidic pH medium (*3, 4*). The formation of H′-DNA is promoted by polyamines and divalent metal cations (*2, 7, 8*), which decrease the phosphate repulsion of three closely spaced DNA strands. Some divalent metal cations, such as zinc, coordinate to purine bases and may therefore enhance the Hoogsteen-like hydrogen bonds (*9, 10*). Finally, both H- and H′-DNA form more easily in longer Py·Pu sequences than in the shorter ones (*5, 11*−*13*).

According to a recent database analysis, H-DNA-forming sequences in the human genome occur as frequently as one in 50 000 bp (*14*). The formation of H-DNA has been suggested to play a role in a variety of biological processes such as transcription, replication, and recombination (*15*−*23*). H- (H′-) DNA structures have been detected in cells (*24*−*26*), and several factors are likely responsible for the structure formation and stability in vivo, although their relative contributions are not known. Appropriate superhelical tension could be provided by a local wave of supercoiling created by a transcribing RNA polymerase (*27*) or by transformation of restrained supercoiling into an unrestrained one upon nucleosome displacement (*28*). Little is known about possible H-DNA stabilization by supercoiling in eukaryotes, as the relevant data on supercoil distribution in eukaryotic genomes have only recently begun accumulating (*29*−*33*). A group of cellular polycations, polyamines (e.g., spermidine and spermine) (*34*−*36*), may provide another H-DNA-stabilizing contribution by reducing the repulsion of the three polyanionic DNA strands.

It is important to understand if H-DNA stabilization might be provided by protein interaction with the triple helix. At least one protein that preferentially binds to a triple-helical DNA has been reported (*37*). The triplex-binding proteins might be enriched in positively charged basic amino acid residues, thus providing the basis for their electrostatic interaction with triple helices that have a high negative charge density. For example, the NSEP-1 protein requires two basic fragments for binding to the Py·Pu sequence in c-*myc* promoter (*38*). A transcription repressor with seven lysines

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[1] Abbreviations: bp, base pair; nt, nucleotide; Pu, homopurine; Py, homopyrimidine; CAA, chloroacetaldehyde; DMS, dimethyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid; σ, superhelical density; EtBr, ethidium bromide.

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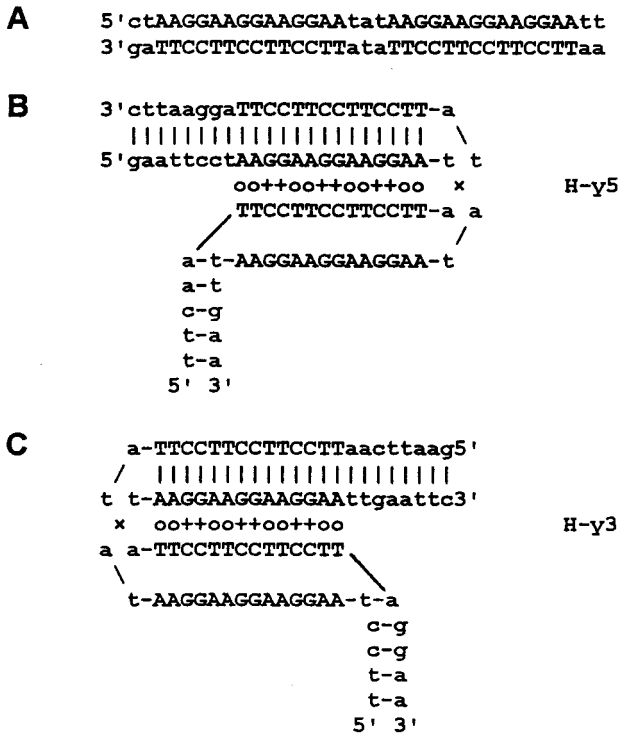

FIGURE 1: Triplex-forming sequence of plasmid pRDH7 and possible isomers of H-DNA. (A) Mirror-repeated Py·Pu sequence. Halves of Py and Pu strands capable of folding into an intramolecular triplex are shown in capital letters. (B) Hoogsteen hydrogen bonding of the 5′ half of Py strand with the duplex purines results in the H-y5 isomer, which predominates at low superhelical density. Hoogsteen hydrogen bonding in neutral T·A·T triads is shown with (o), whereas that in protonated $C\cdot G\cdot C^+$ triads is shown with (+). (C) Hoogsteen hydrogen bonding of the 3′ half of Py strand with the duplex purines results in the H-y3 isomer, which predominates at high superhelical density.

and seven arginines in its N-terminal domain binds to (G + C)-rich DNA sequences with a clear Py·Pu bias (*39*). Surface-localized cationic domains of proteins that potentially interact with the triple-helical nucleic acids might be approximated by basic oligopeptides. We have recently shown that basic peptides can stabilize an *intermolecular* triplex (*40*), presumably by reducing interstrand phosphate repulsion. Here we have studied the interactions of basic peptides with an *intramolecular* triplex (H-DNA). We show that the superhelical tension required for H-DNA formation may be reduced by the presence of lysine- and arginine-rich peptides.

MATERIALS AND METHODS

Materials. Plasmid pRDH7 is a derivative of plasmid pUC8 in which a Py·Pu mirror repeat $AA(GGAA)_3TAT(AAGG)_3AA$ has been cloned into the polylinker *Eco*RI site (Figure 1A). Oligopeptides Lys-Gly-Lys-Gly-Lys and $(Lys)_5$ were synthesized by Dr. S. Gurusiddappa (Institute of Biosciences and Technology, Texas A&M University) and were >90% homogeneous according to HPLC analysis. Peptides $(Lys)_3$, $(Arg)_3$ and $(Lys)_3$-Trp-$(Lys)_3$ were from Bachem Bioscience, and Lys-Arg-Thr-Leu-Arg-Arg was from American Peptide. Chloroacetaldehyde (Aldrich) was distilled twice and stored at −20 °C. *Pvu*II (New England Biolabs), Taq DNA polymerase (Gibco BRL), and Stoffel fragment of AmpliTaq DNA polymerase (Perkin-Elmer) were used according to the manufacturer's specifications.

Chemical Modifications. Topoisomers of particular superhelical densities were generated by incubating supercoiled plasmid with a topoisomerase extract from HeLa cells in the presence of varying concentrations of ethidium bromide (*41*). The resultant superhelical densities were estimated by analysis of topoisomer fractions in agarose gels run in the presence of chloroquine. In a standard chemical probing experiment, each sample contained 0.5 μg of plasmid topoisomer in 40 μL of 50 mM sodium acetate and 1 mM EDTA (pH 5.0) and peptides in concentrations indicated in the figure legends. After a 1-h incubation at 37 °C, samples were treated with 1.6 μL of 50% CAA at room temperature for 2.5 h, and then reactions were stopped by extraction with diethyl ether. Following two ethanol precipitations, modified DNAs were digested overnight with *Pvu*II, and the reaction mixtures were then extracted with phenol−chloroform. After two additional ethanol precipitations, samples were resuspended in 20 μL of TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) and 5 μL was used for primer extension analysis of modification products as described (*40*). Two 23 nt long primers that hybridized approximately 60 nt away from the Py·Pu tract to different strands were used in primer extension reactions with a Stoffel fragment of Taq DNA polymerase. Products of primer extension were resolved on a 7% denaturing polyacrylamide gel in TBE buffer (90 mM Tris−borate, pH 8.3, and 2.5 mM EDTA) with sequence markers obtained by dideoxy sequencing of the plasmid using Taq DNA polymerase. Gels were dried and exposed to X-ray film or a PhosphorImager plate for analysis of the radioactivity pattern using ImageQuant software (Molecular Dynamics). For each lane corresponding to a DNA topoisomer of particular superhelical density, intensities of the bands corresponding to CAA modification at the Py·Pu site (I_{CAA}) and total intensity of the bands in the lane (I_T) were determined. The B- to H-DNA transition curves were constructed by using the I_{CAA}/I_T ratios and assuming that these ratios for the relaxed DNA and DNA with the highest superhelical density correspond to 0% and 100% transition, respectively. Determination of intermolecular triplex stability in the presence of peptides and spermine by the dimethyl sulfate (DMS) assay was performed as described (*40*). Determination of a structural isomer of H-DNA by the psoralen photo-cross-linking assay (*26*) was based on a differential psoralen cross-linking to 5′-TA dinucleotides in duplex and H-DNA forms. Upon H-DNA formation, psoralen does not react with the TA in the center of the mirror repeat and, dependent on the particular H-DNA isomer, either one or another TA immediately adjacent to the Py·Pu sequence.

Analysis of the Effect of Peptides on DNA Supercoiling. Reaction mixtures (20 μL) contained 0.25 μg of plasmid DNA, 2 μL of HeLa topoisomerase extract, 10 mM Tris (pH 7.6), 50 mM NaCl, 1 mM EDTA, and selected peptides or spermine. After an overnight incubation at 37 °C, reaction mixtures were extracted with phenol and chloroform (three times each), and DNA was ethanol-precipitated three times. DNA topoisomers from the relaxation reaction were separated on a 1.5% agarose gel using 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA (pH 8.3), and 3 μg/mL chloroquine as a running buffer. After electrophoresis, gels were washed to remove the chloroquine, stained with ethidium bromide, and photographed. A Molecular Dynamics densitometer was

used to scan the negative and evaluate the distribution of topoisomers in the lanes corresponding to different reaction conditions.

Thermal Melting. Oligonucleotides (0.85 μM each) 5′-GGAATTCCTAAGGAAGGAAGGAATAT and 5′-ATAT-TCCTTCCTTCCTTAGGAATTCC that form a double-stranded fragment and 5′-TTCCTTCCTTCCTT that hybridizes as the third strand were mixed in 1 mL reaction volume, heated to 80 °C and then slowly cooled to room temperature overnight. Thermal melting was accomplished in a Varian Cary 3 spectrophotometer by heating samples from 0 to 80 °C at a rate of 0.5 °C/min. Computer-collected data (two data points/min) were processed to generate first-derivative melting profiles. The duplex and triplex melting points were determined from the first-derivative profiles with an estimated precision of 1 °C.

RESULTS

Experimental Design. Previously, we showed that several cationic peptides stabilized intermolecular triplex DNA (ref *40* and unpublished results). A stabilizing effect was detected as the peptide concentration increased from 10^{-5} to 10^{-3} M (*40*). To test the influence of peptides on the H-DNA formation, plasmid pRDH7 containing a mirror-repeated Py·Pu tract (Figure 1A) was used. Upon H-DNA formation this sequence folds into either the H-y3 isomer (Figure 1B), which is favorable at intermediate and high superhelical density, or the H-y5 isomer (Figure 1C), which may form at low superhelical density (*12*). Chloroacetaldehyde (CAA) was used as a convenient indicator of H-DNA formation in this plasmid since it reacts with unpaired adenines in the Pu strand when its counterpart Py strand folds back to form a triple helix (Figure 2). CAA also reacts with unpaired adenines at the tip of the triple helix and adenines and cytosines at the single strand−duplex junction.

Cationic Peptides Can Reduce the Level of Superhelical Tension Necessary for H-DNA Formation. CAA modification (Figure 2) shows that, in 50 mM sodium acetate, pH 5, with increasing superhelical tension, adenines in the 5′-half of Pu sequence become susceptible to CAA modification (lanes 4−8). This is consistent with local duplex unwinding and folding of the Py·Pu sequence into an H-y3 triplex, in which the 3′-half of Py sequence folds back and serves as the third strand, whereas its complementary strand, the 5′-half of Pu sequence, becomes unpaired. The formation of the H-y3 isomer was also confirmed by differential psoralen cross-linking to duplex and H-DNA forms (not shown). Quantitation of the CAA modification signal shows that with increasing superhelical density there is an increase in CAA reactivity, and the midtransition point corresponds to an average superhelical density $\sigma = -0.038$ (Figure 3), in good agreement with the two-dimensional (2D) gel electrophoresis data (not shown).

To determine if polycationic peptides could influence H-DNA formation or stability, triplex formation was analyzed in the presence of different concentrations of pentalysine. In the presence of 0.3 mM pentalysine, the midtransition point was at $\sigma = -0.030$ (Figure 3). The local DNA unwinding was first detected in the topoisomer fraction with $\sigma = -0.025$ (Figure 2, lane 11) compared with $\sigma = -0.033$ in the absence of peptide (Figure 2, lane 4). This corresponds

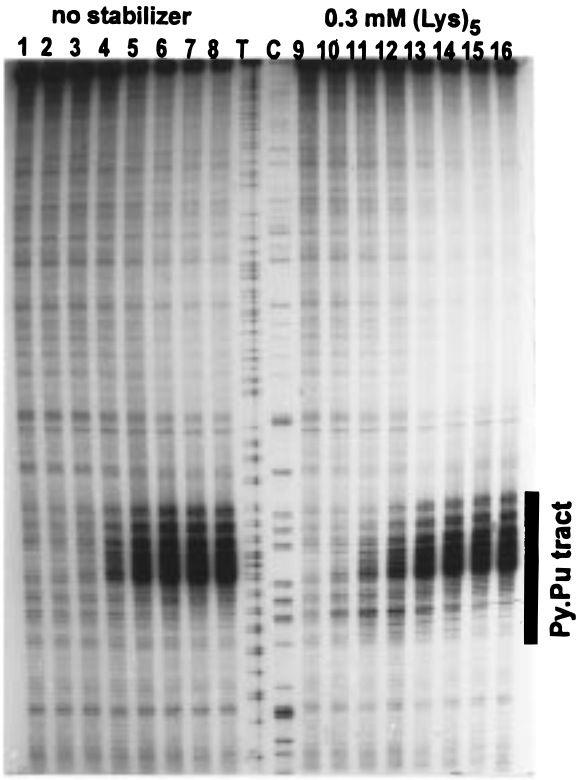

FIGURE 2: Supercoil-dependent H-DNA formation detected by reactivity of adenines in the Pu-rich strand toward chloroacetaldehyde. Topoisomers were incubated in 40 μL of 50 mM sodium acetate, pH 5.0, for 1 h at 37 °C in the absence of stabilizing additives (lanes 1−8) or in the presence of 0.3 mM $(Lys)_5$ (lanes 9−16). Following incubation, samples were analyzed by primer extension as described under Materials and Methods. Superhelical density increases from left to right as follows: lanes 1 and 9, $\sigma = 0$; lanes 2 and 10, $\sigma = -0.013$; lanes 3 and 11, $\sigma = -0.025$; lanes 4 and 12, $\sigma = -0.033$; lanes 5 and 13, $\sigma = -0.040$; lanes 6 and 14, $\sigma = 0.048$; lanes 7 and 15, $\sigma = -0.054$; and lanes 8 and 16, $\sigma = -0.059$. Lanes T and C show the sequence of a complementary Py-rich strand.

to an approximate 20% difference in σ, or two less supercoils required to induce H-DNA formation. A prominent feature of modification in the presence of peptide is that CAA reacts not only with adenines in the 5′-half of Pu sequence (lanes 12−16) but also with adenines in the 3′-half of Pu sequence (lanes 11−13). This likely reflects nucleation of the H-y5 isomer of H-DNA at low superhelical tension. In this isomer, a 5′-half of Py sequence folds back and serves as the third strand, and adenines in the 3′-half of Pu sequence become susceptible to CAA modification. Thus, at 0.3 mM pentalysine, the superhelical density required for detectable duplex unwinding was reduced to values low enough for the formation of a different H-DNA isomer. The 0.3 mM pentalysine concentration seems to be optimal for H-DNA stabilization, as at lower (0.1 mM) concentration H-DNA formation proceeded similar to that in the absence of the peptide, whereas at higher (1 mM) concentrations the CAA reactivity of DNA was intermediate between those for the 0.3 mM peptide and peptide-free solutions (Figure 3). It is possible that the H-DNA-stabilizing effect increases with pentalysine concentration similar to its effect on intermolecular triplex (*40*); however, at higher peptide concentrations a competing stabilization of the duplex DNA interferes with an efficient DNA unwinding and H-DNA formation.

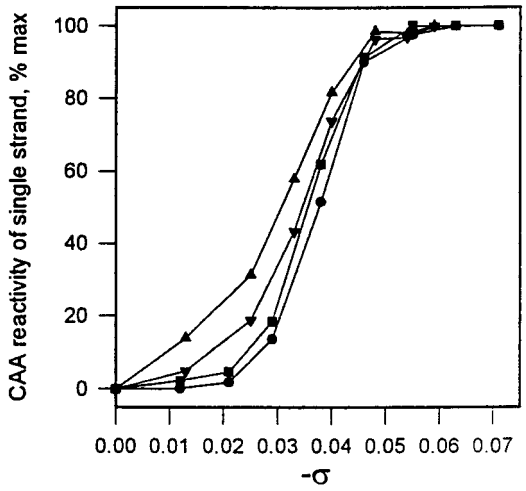

FIGURE 3: Effect of $(Lys)_5$ on H-DNA formation. H-DNA was formed and detected as described in the legend to Figure 2, except that the incubation mixtures contained no $(Lys)_5$ (●) or $(Lys)_5$ at 0.1 mM (■), 0.3 mM (▲), or 1 mM (▼). The B- to H-DNA transition curves were constructed using the I_{CAA}/I_T ratios [intensity of the bands corresponding to CAA modification at the Py·Pu site (I_{CAA}) divided by the total intensity of the bands in the lane (I_T)] and assuming that these ratios for the relaxed DNA and DNA with the highest superhelical density correspond to 0% and 100% transition, respectively.

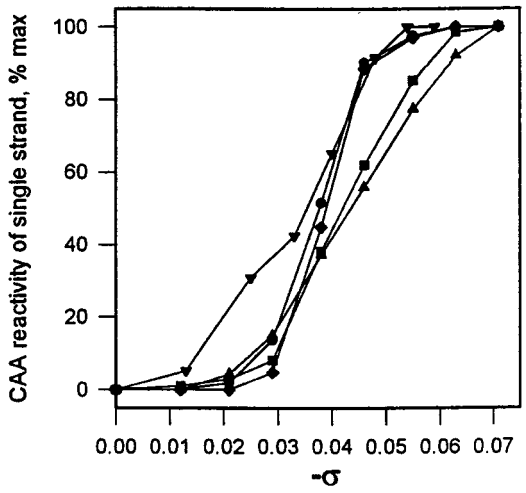

FIGURE 4: Effect of $(Lys)_3$ and $(Lys)_3$-Trp-$(Lys)_3$ on H-DNA formation. The incubation mixtures contained no peptides (●); $(Lys)_3$ at 0.3 mM (■) or 1 mM (▲); or $(Lys)_3$-Trp-$(Lys)_3$ at 0.3 mM (▼) or 1 mM (◆).

Trilysine, a peptide with fewer similarly spaced positive charges, did not promote H-DNA formation. At both 0.3 and 1 mM concentrations, it broadened the B- to H-DNA transition, and the midtransition point was shifted to higher superhelical density (Figure 4). A low triplex-stabilizing potency was also detected in experiments on intermolecular triplex stabilization where trilysine required severalfold larger concentrations than pentalysine to elicit a comparable effect (Figure 9A, see below). Thus, the positive charge density created at higher trilysine concentrations stabilizes the double-stranded DNA but is not sufficient for the efficient stabilization of an unwound structure containing triple-stranded conformation. When a bulky tryptophan residue was incorporated within a stretch of lysines, in $(Lys)_3$-Trp-$(Lys)_3$, the resulting H-DNA stabilization was similar to that of pentalysine: at 0.3 mM $(Lys)_3$-Trp-$(Lys)_3$, local duplex unwinding was detected at lower superhelical density than in a peptide-free solution (Figure 4).

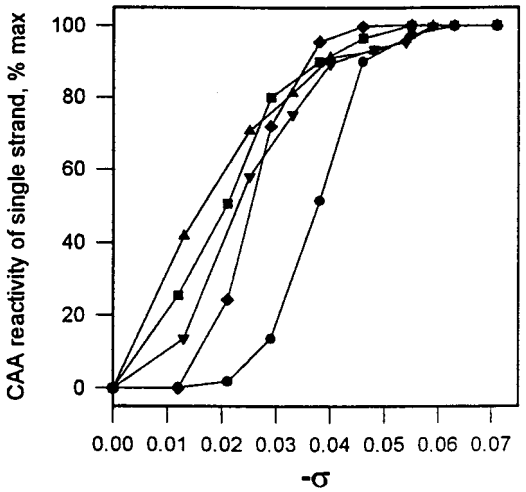

FIGURE 5: Effect of $(Arg)_3$ and Lys-Arg-Thr-Leu-Arg-Arg on H-DNA formation. The incubation mixtures contained no peptides (●); $(Arg)_3$ at 0.1 mM (■), 1 mM (▲), or 3 mM (◆); or Lys-Arg-Thr-Leu-Arg-Arg at 0.3 mM (▼).

Arginine-Rich Peptides. H-DNA stabilization by triarginine was tested in the 0.1−3 mM concentration range (Figure 5). The B- to H-DNA transition was significantly shifted to lower superhelical tension relative to that in the peptide-free solution. At 0.1 and 1 mM triarginine, DNA was susceptible to CAA modification throughout the entire superhelical tension range. Local DNA unwinding (consistent with the nucleation of H-y5 structure) was detected even in the topoisomer fraction where topoisomers were centered on relaxed molecules. At 3 mM triarginine, H-DNA formation was also significantly facilitated relative to that in the peptide-free solutions; however, only the H-y3 structure was detected. Another Arg-rich peptide, Lys-Arg-Thr-Leu-Arg-Arg, at 0.3 mM also significantly facilitated H-DNA formation (Figure 5). Thus, the Arg-rich peptides were more effective in H-DNA stabilization than the Lys-rich ones, and this effect might be due to the differences in interaction of arginine and lysine with DNA.

The data presented show that several cationic peptides promote H-DNA formation. At least two effects might be involved. First, peptide binding might increase the superhelical tension of DNA, similar to some peptide-like antibiotics (*42*, *43*), thereby facilitating its local unwinding and subsequent folding into H-DNA. Second, electrostatic interactions between cationic peptides and anionic DNA might favor the formation of H-DNA similar to the effect of peptides on intermolecular triplex formation (*40*). To gain an insight into which of the possible peptide−DNA interactions favors the formation of H-DNA, we tested (i) possible cation-induced changes in DNA superhelicity, (ii) effects of other cations on H-DNA stabilization, and (iii) intermolecular triplex and duplex stabilization as a function of polycation concentration.

At H-DNA Stabilizing Concentrations, Polycations Have Little Effect on DNA Supercoiling. To evaluate a contribution to H-DNA formation of a possible polycation-induced change in DNA superhelicity, topoisomerase was used to relax supercoiled DNA in the presence of spermine, pentalysine, and triarginine. Polycation binding to supercoiled

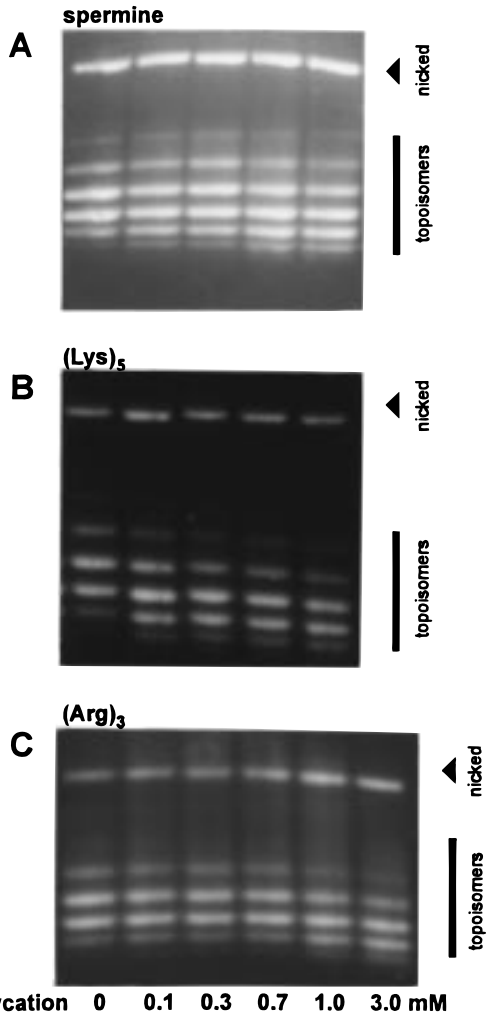

FIGURE 6: Effect of polycations on DNA supercoiling. Plasmid pRDH7 of native superhelical density was relaxed by topoisomerase activity in the presence of the indicated concentrations of polycations. After extensive phenol−chloroform treatment and ethanol precipitation in order to remove polycations, DNA samples were separated on a 1.5% agarose gel in the presence of 3 μg/mL chloroquine phosphate.

DNA molecules might result in a decrease in torsional stress due to binding-induced underwinding (similar to the well-known effect of ethidium bromide, EtBr). When relaxed by treatment with topoisomerase, such DNA molecules must be underwound due to the bound agent, and following the removal of the agent (polycation or EtBr) the proper helical twist is restored with a concomitant increase in negative supercoiling. Alternatively, polycation binding might result in an increase in negative torsional stress. In such a case, after relaxation, DNA molecules would have positive supercoils. When run in the presence of 3 μg/mL chloroquine, positively supercoiled molecules migrate faster than the negatively supercoiled ones. Figure 6 shows that DNA relaxed in the presence of polycations migrated slightly faster than DNA relaxed in the absence of polycations. Thus, binding of all polycations tested resulted in a slight increase in negative torsional stress. From densitometric analysis of topoisomer distributions, the maximum increases in the numbers of negative supercoils were 0.5 for 1 mM spermine, 0.8 for 1 mM pentalysine, and 0.4 for 1 mM triarginine. The corresponding increases in negative superhelical density were $\Delta\sigma = -0.002$, -0.003, and -0.0015. However, this

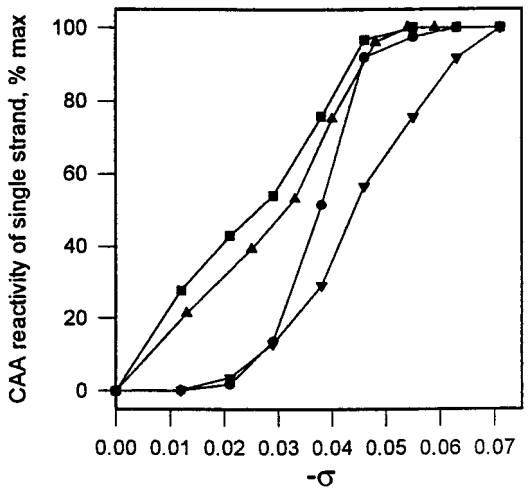

FIGURE 7: H-DNA formation in the presence of spermine. The incubation mixtures contained no spermine (●) or spermine at 0.1 mM (■), 0.3 mM (▲), or 1 mM (▼).

difference cannot account for the entire H-DNA stimulatory effect, as it is smaller than the shift of the H-DNA transition curves. For example, the change is as large as $\Delta\sigma = 0.021$ for 1 mM triarginine.

Other Polycations May Favor H-DNA Formation. The effect of spermine is somewhat similar to that of pentalysine: H-DNA formation is facilitated at 0.1 and 0.3 mM (Figure 7), and the transition becomes partially inhibited at 1 mM spermine. Note that, perhaps due to the higher positive charge density in spermine, it is effective at 0.1 mM, whereas pentalysine is not. Another difference is that spermine is more efficient than pentalysine at 50 mM Na^+ but is less efficient at 150 mM Na^+ (see below). Thus, details of spatial charge distributions in spermine and pentalysine are important for interaction with and stabilization of H-DNA. A comparison of polycations with four positive charges underscores the importance of the charge density necessary to elicit an H-DNA-inducing effect. Closely spaced charges in a small spermine molecule make it effective at H-DNA stabilization, whereas distributed charges in a more extended $(Lys)_3$ molecule do not seem to create sufficient neutralization of phosphates in triple helix so that a significant facilitation of H-DNA formation does not occur. Similarly, the charge density in Lys-Gly-Lys-Gly-Lys is insufficient for H-DNA stabilization (data not shown).

Increasing Na^+ Concentration Partially Inhibits H-DNA Formation. According to the 2D gel analysis (not shown) and psoralen cross-linking (Figure 8A), in a low ionic strength buffer (13.5 mM Tris−acetate, pH 5.0, and 1 mM Mg^{2+}) H-DNA formation occurred with the midtransition superhelical density $\sigma = -0.035$. CAA probing shows (Figure 8A,C) that increasing ionic strength makes H-DNA formation more difficult, resulting in the midtransition points of $\sigma = -0.038$ at 50 mM Na^+ and $\sigma = -0.045$ at 150 mM Na^+ (note that 150 mM Na^+ is a sum of 50 mM Na^+ from sodium acetate buffer and 100 mM Na^+ from added NaCl). The partially inhibitory effect of higher ionic strength was limited, as the H-DNA formed at approximately the same σ at 150 and 300 mM Na^+. When 0.3 mM spermine was added to a DNA mixture at 150 mM Na^+, the midtransition superhelical density decreased by about 10% (Figure 8B,C).

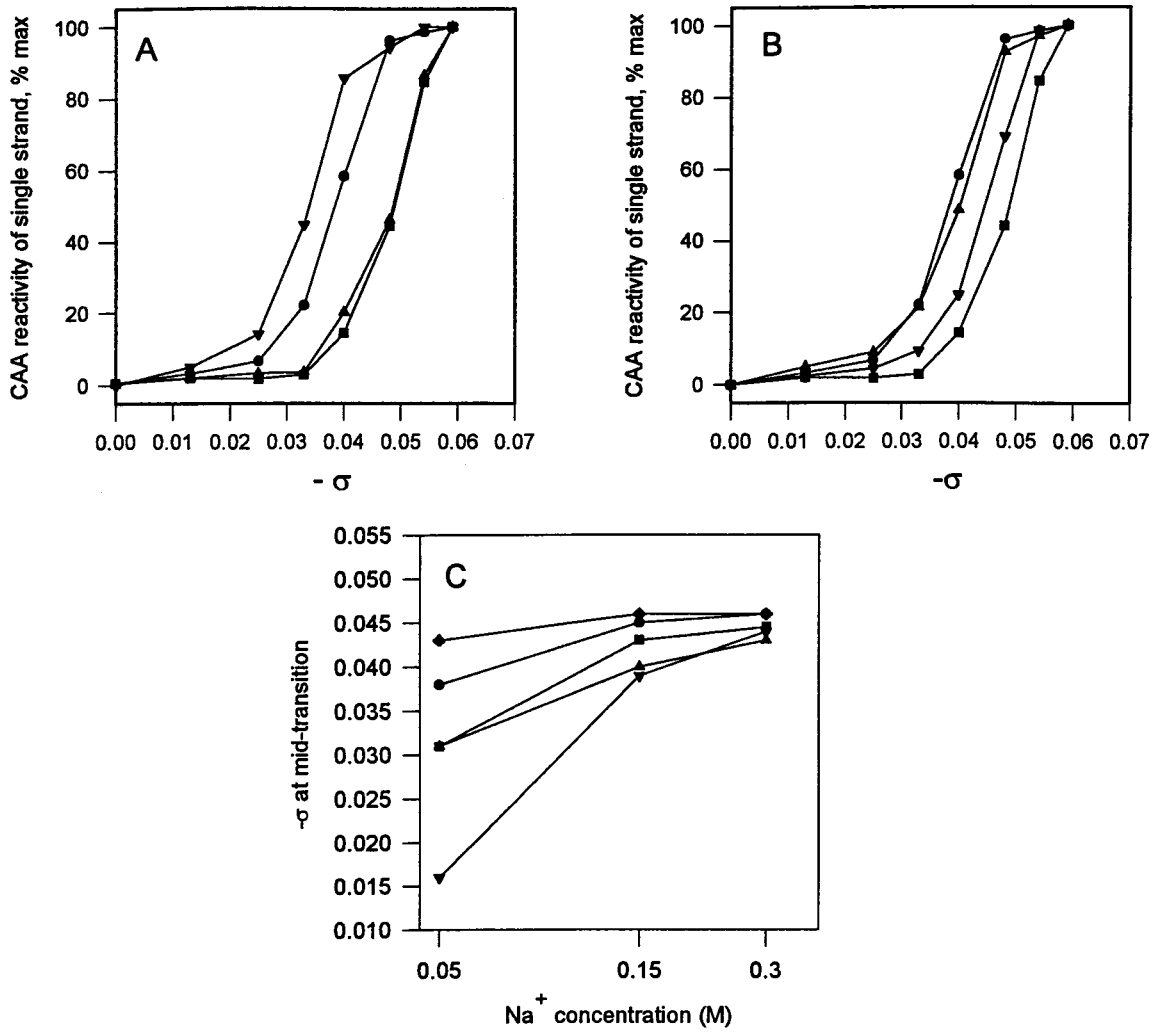

FIGURE 8: (A) H-DNA formation is partially inhibited at increasing salt concentrations. H-DNA was formed by incubating pRDH7 topoisomers for 1 h at 37 °C in 40 μL of 50 mM sodium acetate, pH 5.0, and NaCl at 0 mM (●), 100 mM (■), or 250 mM (▲) or and in 40 μL of 13.5 mM Tris acetate, pH 5.0, and 1 mM magnesium acetate (▼). H-DNA formation in sodium acetate buffer was monitored by CAA probing, whereas that in Tris acetate buffer was monitored by psoralen cross-linking assay. (B) Polycations relieve inhibition of H-DNA formation at increased salt concentration. H-DNA was formed by incubating pRDH7 topoisomers for 1 h at 37 °C in 40 μL of 50 mM sodium acetate, pH 5.0, and 100 mM NaCl (■); or in the same buffer plus 0.3 mM $(Lys)_5$ (▲) or spermine (▼). The H-DNA formation curve at 50 mM sodium acetate (●) is shown for reference. (C) Superhelical density required for H-DNA formation in the presence of polycations at various Na^+ concentrations. H-DNA was formed in 50 mM sodium acetate, pH 5.0, plus 0, 100, or 250 mM NaCl and no polycations (●), 0.3 mM spermine (■), 0.3 mM $(Lys)_5$ (▲), 1 mM $(Arg)_3$ (▼), and 1 mM $(Lys)_3$ (◆).

Similarly, the addition of 0.3 mM pentalysine or 1 mM $(Arg)_3$ shifted the transition curve to a lower σ by about 20% (Figure 8B,C). Since the ability of a counterion to bind and stabilize a nucleic acid complex increases with the charge on the counterion (*44*, *45*), spermine and peptides seem to stabilize H-DNA formation by displacing some of the monovalent cations that are more loosely bound to DNA. The H-DNA stabilizing efficiency [expressed as the superhelical density at the mid-transition point, σ_{50} (Figure 8C)] is ionic strength dependent, but for different polycations it decreases in magnitude at different rates as the salt concentration increases. At higher ionic strength where monovalent cations mostly account for the net counterion condensation on DNA, the σ_{50} is almost independent of the presence of polycations because they bind poorly to DNA (*51*).

H-DNA Stabilizing Potencies of Polycations Partially Correlate with Their Effects on Intermolecular Triplex Formation. In a previous paper (*40*) we showed that pentalysine, Lys-Ala-Lys-Ala-Lys, and Lys-Gly-Lys-Gly-Lys stabilize an intermolecular triplex, presumably by reducing electrostatic repulsion between the double-stranded DNA and an oligonucleotide. Here we compared the H-DNA stabilization effects of several polycations (Figures 3−5 and 7) with their effects on intermolecular triplex at the same Py·Pu target (Figure 9). Chemical probing (Figure 9A) shows that the H-DNA-stabilizing polycations, spermine and pentalysine, are also strong stabilizers of intermolecular triplex. Guanines in the Pu strand of the duplex target were protected from DMS modification at lower concentrations of spermine and pentalysine than trilysine and triarginine. Trilysine, which does not stabilize H-DNA, was also the weakest of the intermolecular triplex stabilizers tested. Triarginine had an intermediary stabilizing effect on intermolecular triplex formation. Similar results have been obtained in experiments on thermal stability of triple-stranded complexes (Figure 9B). At several concentrations of polycations tested, their ability to increase the melting temperature, that is, to stabilize the triplex, increases in the following order: $(Lys)_3 < (Arg)_3 <$

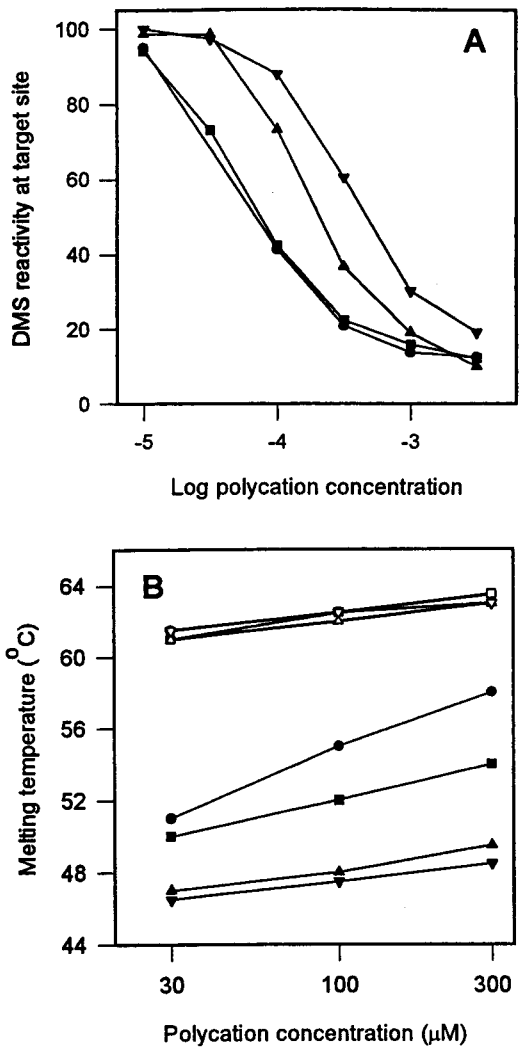

FIGURE 9: Polycation concentration dependence for the stabilization of the intermolecular Py·Pu·Py triplex. (A) Detection by the DMS modification assay. The triplex between the Py·Pu region of pRDH7 and pyrimidine triplex-forming oligonucleotide (TFO) was formed at a molar TFO/DNA ratio of 100 in 20 mM MES and 36.5 mM NaCl (pH 6.5). The reaction mixtures contained 10 μM, 30 μM, 100 μM, 300 μM, 1 mM, or 3 mM spermine (●), $(Arg)_3$ (▲), $(Lys)_3$ (▼), and $(Lys)_5$ (■). (B) Detection by the thermal melting assay. The triple-stranded complex was formed from oligonucleotides 5′-GGAATTCCTAAGGAAGGAAGGAATAT and 5′-ATATTCCT-TCCTTCCTTAGGAATTCC (double-stranded fragment) and 5′-TTCCTTCCTTCCTT (third strand) in 20 mM MES and 146.5 mM NaCl (pH 5.5). The differential melting curves were used to determine the half-transition points for the triplex (filled symbols) and duplex melting (open symbols) in the presence of 30 μM, 100 μM, or 300 μM spermine (●, ○) $(Arg)_3$ (▲, △), $(Lys)_3$ (▼, ▽), and $(Lys)_5$ (■, □).

$(Lys)_5$ < spermine (lower series of lines). On the other hand, the differences in the duplex-stabilizing ability of the same polycations are much less pronounced (upper series of lines), being within the limits of an estimated experimental error. Thus, $(Arg)_3$ was the best H-DNA stabilizer, but only an intermediary intermolecular triplex stabilizer. $(Arg)_3$ was not different from other polycations in stabilization of double-stranded DNA.

DISCUSSION

The formation of H-DNA as a function of superhelical density was tested in the presence of basic peptides and spermine as well as at varying salt concentrations. Our results show the following: that (i) In the presence of spermine and some Lys-rich peptides, the DNA superhelical tension required for local duplex unwinding and subsequent folding into alternative triple-stranded conformation is decreased. A similar reduction in the superhelical tension required for the transition may be obtained by lowering pH or by using sequences with longer Py·Pu tracts (*3*−*5*, *11*−*13*). (ii) Arg-containing peptides are more effective than similar Lys-containing peptides. In the presence of triarginine but not trilysine, the local DNA unwinding consistent with the nucleation of H-DNA is observed in samples with very low superhelical tension. (iii) According to the patterns of CAA reactivity, in the presence of polycations an H-y5 isomer forms at low σ, whereas an H-y3 isomer forms at higher σ. This indicates that polycations do not induce the formation of a particular H-DNA isomer, but rather, they stabilize the isomer that may form at given superhelical density (H-y5 at low σ or H-y3 at higher σ). (iv) The H-DNA-stabilizing effect of cationic peptides diminishes upon increasing their concentrations up to, or above, 1 mM. Increasing salt concentration also has a limited inhibitory effect on H-DNA formation. (v) A slight increase in DNA torsional stress upon polycation binding can only minimally account for the facilitated H-DNA formation in the presence of spermine and peptides. (vi) The H-DNA-stabilizing effects of spermine and lysine-containing peptides generally correlate with their stabilization of intermolecular triplex DNA. However, triarginine has a more pronounced effect on H-DNA than on intermolecular triplex formation.

The available data allow a conclusion that the H-DNA-stabilizing effect of polycations is mostly electrostatic. Their high total charge and/or charge densities may result in a significant neutralization of the repulsion between anionic phosphate groups in the strands of a local triple helix so that H-DNA forms at lower superhelical tension than in the absence of polycations. This effect is significant for $(Arg)_3$, $(Lys)_5$, $(Lys)_3$-Trp-$(Lys)_3$, and spermine, presumably due to their high affinities for triplex DNA and their high charge densities. H-DNA stabilization is virtually nonexistent for $(Lys)_3$ and Lys-Gly-Lys-Gly-Lys, with fewer charges and lower charge densities. Relatively high polycation concentrations ($\geq$1 mM) or increasing salt concentration may result in a partial inhibition of H-DNA formation. A similar destabilizing effect of increasing ionic strength was reported for other H- and H′-DNA forming sequences (*6*, *13*, *46*). Note that the triplex-stabilizing divalent metal cations at excessive (millimolar) concentrations may also inhibit the formation of H-DNA-like structures in supercoiled plasmids (*47*, *48*). Increasing concentrations of monovalent salt, divalent metal cations, or polycations, may stabilize the duplex (*49*, *50*), requiring higher superhelical tension for the local unwinding in order to form the triplex. Thus, peptides may have optimum concentrations at which they stabilize H-DNA. These optimum values depend on the solution ionic strength because, similar to the duplex and intermolecular triplex stabilization (*44*, *51*), there is a competition of stronger (multivalent cations) and weaker but more abundant stabilizers (monovalent cations) for the binding to the triple-stranded part of H-DNA.

The likely scheme of electrostatic H-DNA stabilization by polycations is that a transiently formed triple helix, which

appears due to the supercoil-induced fluctuations in local denaturation, preferentially binds polycations. For example, the affinity of spermine to the triple helix is about 1.5 times greater than to the duplex (*51*). Bound polycations stabilize this transient structure so that the H-DNA-containing molecules are selected and structurally fixed. Thus, the preferential reaction of a polycation with one DNA conformation efficiently shifts the equilibrium to that conformation ("driven equilibrium").

Other mechanisms of H-DNA stabilization may include induction of negative supercoiling in closed circular DNA, similar to the effects of oligocationic minor-groove-binding drugs of the netropsin series (*42, 43*). However, in our experiments the ability of pentalysine, triarginine, and spermine, which may bind in the minor groove of B-DNA (*52, 53*), to increase the torsional stress of supercoiled molecules may have only a minor contribution to H-DNA stabilization. At DNA and polycation concentrations used in chemical probing experiments, spermine and peptides increased the numbers of supercoils by less than one, which was not sufficient to account for observed shifts in H-DNA transition curves.

At least for the DNA sequence studied, the Arg-rich peptides were more effective in H-DNA stabilization than the Lys-rich ones, which might be due to the differences in the interaction of arginine and lysine with DNA. Complexes between phosphates and the terminal ammonium group of lysine and the guanidinium group of arginine have different geometries (*55, 57*). Polylysines interact with the double and triple helices in a similar manner, with the minor groove as a binding site (*54*), so that the terminal ammonium group of lysine can bind to the phosphate group as well as N3 of adenine and O2 of thymine (*50, 55*). Although Arg-rich peptides may also bind at the minor-groove side of the double helix (*52*), they have some preference for G·C base pairs (*50*). [An alternative, major-groove-binding mode of Arg-rich peptides, as proposed for protamine−DNA interaction (*56*), seems less likely. In this case, the terminal guanidinium group of arginine could hydrogen bond to the O6 and N7 of guanine (*50, 55*). But if the Arg-rich peptides were to interact with guanines in the major groove, this would prevent efficient hybridization of third polynucleotide strand at the same bases.] In addition, since spermine and pentalysine stabilize the intermolecular triplex better than triarginine, whereas the trend in H-DNA stabilization is opposite, other modes of peptide−DNA interactions might be involved.

There are at least two more possibilities for the differential interaction with DNA of arginines compared with lysines: (i) Unlike the ammonium group of lysine, upon a supercoil-induced fluctuation in local denaturation of the Py·Pu sequence, the guanidinium group of arginine might form hydrogen bonds at O2 and N3 of cytosine, thereby preventing reassociation of separated strands. Such an unwound DNA conformation might then more easily rearrange into an H-DNA structure. This suggestion is not supported by the results of melting experiments—the duplex is not noticeably destabilized in the presence of triarginine. (ii) Oligoarginines bind more strongly to single-stranded polynucleotides than oligolysines (*58*). Thus, oligoarginine binding to an unwound Pu strand of H-DNA would prevent its reassociation into a double helix. The remaining three strands would stay in the triple-helical structure. The sequence and conformation of a short single-stranded loop at the tip of the triple helix and its interaction with ligands, such as metal cations, significantly affect stability and the particular isomer of H-DNA formed (*59−61*). It is possible that interaction of this loop with oligoarginines is also important for H-DNA stability.

H- (H′-) DNA structures may play a number of roles in cells. These may include local DNA unwinding to facilitate RNA polymerase binding and transcriptional initiation (*16, 19*), DNA bending as a potential means to bring regulatory sequence elements into close proximity to interact with one another (*63*), or chromosome condensation mediated by triplex formation between distant duplex DNA sequences (*64*). H-DNA, which may mediate cellular events, should transiently form and disappear after completing its functions. Therefore, the H-DNA-stabilizing factors might not be available all the time and under all physiological conditions because the formation of a truly stable structure is incompatible with regulation of a changing physiological state (*62*). Among the suitable stabilizing factors whose availability may change during the cell cycle are an increased negative superhelical density and varying concentrations of polyamines and certain proteins. Although up to 1 mM concentrations of polyamines can be detected in the cell (*34−36*), it is not clear what fraction of cellular polyamines is available for the triplex-stabilizing effect, as most of them might be involved in polyamine−RNA complexes (*65*). Our data support earlier suggestions that proteins may induce or stabilize triplex DNA (*37, 62, 66−68*). Moreover, our results may offer an explanation for the unexpected high level of intramolecular triplex detected in *Escherichia coli* given the intracellular pH and superhelical density (*26*). Among the proteins that specifically interact with the triplex-forming Py·Pu sequences are those which bind to the double-stranded Py·Pu tracts (*38, 69−75*), as well as proteins specific to homopurine (*68, 76−79*) or homopyrimidine single strands (*38, 67, 80−83*).

Various modes of protein−DNA interactions that may facilitate or stabilize H-DNA formation are conceivable. Single-strand-binding (SSB) proteins that bind to homopurine (*68, 76−79*) or homopyrimidine single strands (*38, 67, 80−83*) can bind to and "capture" an unpaired DNA strand resulting from Py·Pu tract breathing or denaturation under torsional stress (*38, 79*). Fixing a single strand of H-DNA would force the remaining strands to stay in a triple-stranded complex. In an experiment that may mimic such protein−DNA interaction, hybridization of an unpaired strand with an oligonucleotide stabilized the entire (triple helix plus double helix) structure (*84*). However, neither of the SSB proteins specific to Py or Pu sequences have been tested for H-DNA stabilization. It should be also kept in mind that such a mode of protein−H-DNA interaction may also lead to H-DNA destabilization. For example, SSB protein from *E. coli* required a longer single strand for binding (about 70 nt) than that provided by an unpaired strand in H-DNA. Upon binding to a single-stranded part of H-DNA, this protein extended unpairing in the H-DNA-forming region that resulted in virtual disappearance of H-DNA (*85*).

H-DNA stability may also be affected by interaction with clustered basic amino acid residues in proteins. A few examples of the proteins with clustered basic amino acid residues are histones and prokaryotic histone-like proteins

enriched in lysines (*86*), DNA (cytosine-5-)-methyltransferase of mouse cells with a run of 13 alternating lysine and glycine residues (*87*), arginine-rich protamines, nonhistone chromosomal protein HMG-I/Y with fragments enriched in lysines and arginines, and a human DNA-binding factor that represses transcription (*39*), containing a stretch of arginines and lysines. It is not known which of such proteins (if any) may interact with H-DNA. To mimic interactions of H-DNA and cationic protein domains, we have used Lys- and Arg-rich oligopeptides and demonstrated their ability to shift the equilibrium from B-DNA to H-DNA. At least for the DNA sequence studied, the Arg-rich peptides were more effective in H-DNA stabilization than the Lys-rich ones, which might be due to the differences in interaction of arginine and lysine with DNA. Further work will be required to understand the roles of DNA and protein sequence and conformation of peptide backbone in basic regions in interactions with triple-stranded DNA.

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