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Polyamines Favor DNA Triplex Formation at Neutral pH†

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ABSTRACT: The stability of triplex DNA was investigated in the presence of the polyamines spermine and spermidine by four different techniques. First, thermal-denaturation analysis of poly[d(TC)]·poly[d(GA)] showed that at low ionic strength and pH 7, 3 μM spermine was sufficient to cause dismutation of all of the duplex to the triplex conformation. A 10-fold higher concentration of spermidine produced a similar effect. Second, the kinetics of the dismutation were measured at pH 5 in 0.2 M NaCl. The addition of 500 μM spermine increased the rate by at least 2-fold. Third, in 0.2 M NaCl, the mid-point of the duplex-to-triplex dismutation occurred at a pH of 5.8, but this was increased by nearly one pH unit in the presence of 500 μM spermine. Fourth, intermolecular triplexes can also form in plasmids that contain purine·pyrimidine inserts by the addition of a single-stranded pyrimidine. This was readily demonstrated at pH 7.2 and 25 mM ionic strength in the presence of 100 μM spermine or spermidine. In 0.2 M NaCl, however, 1 mM polyamine is required. Since, in the eucaryotic nucleus, the polyamine concentration is in the millimolar range, then appropriate purine·pyrimidine DNA sequences may favor the triplex conformation in vivo.

Triplex structures were first described over 20 years ago (Glaser & Gabbay, 1968; Morgan & Wells, 1968). In the more usual form, they consist of T-A-T and C-G-C^+ base triads in which the third pyrimidine strand winds up the major groove of an A-form helix with Hoogsteen pairing. This necessitates protonation of one of the cytosines, and the two pyrimidine strands are antiparallel. Although no X-ray crystallographic structure is yet available, recent NMR studies support this simple model (Rajagopal & Feigon, 1989; de los Santos et al., 1989). These requirements tend to restrict triplex formation to polypurine·polypyrimidine sequences (pur·pyr DNA) although some mismatches can be tolerated (Hanvey et al., 1989; Griffin & Dervan, 1989). In addition, a low pH is generally required because of the protonated cytosine, which has a pK_a of 4.5 in the free state. Thus synthetic DNAs such as poly[d(TC)]·poly[d(GA)] will dismutate to a triplex at pHs below 6 (Lee et al., 1979). Although this result seems to preclude a physiological role for triplexes, several lines of evidence are suggestive of their presence in eucaryotic cells.

First, pur·pyr tracts, some of which are over 100 base pairs in length, represent up to 1% of certain eucaryotic genomes (Hoffman-Lieberman et al., 1986; Manor et al., 1988). Thus the concentration of potential triplex-forming sequences in the nucleus is quite substantial. Second, many pur·pyr tracts are found in the 5′-flanking region of genes, and these regions are sensitive to single-strand specific nucleases both in vitro and in vivo (Schon et al., 1983; Larsen & Weintraub, 1982). By necessity, triplex formation from two duplexes requires the extrusion of a single strand (Htun & Dahlberg, 1988, 1989). Third, a triplex-specific antibody binds to certain regions of mouse, human, and polytene chromosomes (Lee et al., 1987; Burkholder et al., 1988; and unpublished observations). The binding can be abolished by the addition of competing triplex but not with *Escherichia coli* DNA, for example, in which triplexes cannot be detected (Lee et al., 1989). Although this evidence is compelling, it is not clear what factors might be responsible for stabilizing triplexes under physiological conditions.

Potential triplex stabilizers include negative supercoiling, which promotes intramolecular triplex formation (also called H-DNA) in plasmids containing pur·pyr inserts (Lyamichev et al., 1986; Wells et al., 1988; Htun & Dahlberg, 1989). However, in the normal range of superhelix densities (≈ -0.05), a pH less than 7 is still required (Htun & Dahlberg, 1988; Shimizu et al., 1989). Alternatively, triplexes containing 5-methylcytosine or phosphorothioate groups are stable at neutral pH, but there is no evidence for their presence in pur·pyr tracts (Lee et al., 1984; Povsic & Dervan, 1989; Latimer et al., 1989). Cations are also known to alter triplex

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stability (Maher et al., 1990). For example, Na^+ increases the T_m of T-A-T-containing triplexes but decreases the T_m of triplexes containing more than 70% C-G-C^+ (Lee et al., 1984; Latimer et al., 1989). To date there have been few reports dealing with the effect of polycations (Moser & Dervan, 1987; Kohwi & Kohwi-Shigematsu, 1988). Since the negative charge density on a triplex is higher than a duplex, positive ions should bind more tightly to the former. Therefore the duplex-to-triplex equilibrium might be shifted in favor of triplex formation. For this reason, we have studied the effect of the polyamines spermine and spermidine on triplex formation and stability.

Polyamines are ubiquitous in living cells (Tabor & Tabor, 1976, 1984). In eucaryotes, spermine and spermidine are present in millimolar concentrations and may be as high as 5 mM in the nucleus (Sarhan & Seiler, 1989). Their concentration varies during the cell cycle, and they are thought to play a role in cell proliferation (Pegg, 1988). Because spermine and spermidine carry four and three positive charges, respectively, they bind strongly to duplex DNA and may influence the secondary structure (Cohn, 1978; Russell et al., 1983). Binding to triplexes has also been demonstrated (Murray & Morgan, 1973). The results reported here demonstrate that polyamines do indeed have a profound effect on the stability of triplexes. Physiological concentrations of spermine cause triplex formation at pH 7, and thus, in the eucaryotic nucleus, the triplex conformation may actually be preferred.

Materials and Methods

DNA. The synthetic DNAs poly[d(TC)]·poly[d(GA)] and poly[d(TG)]·poly[d(CA)] were prepared as described previously (Lee et al., 1984). The plasmids p913 and pTC45 were gifts were from Dr. D. E. Pulleyblank, Toronto. The plasmid pTC45 contains a $(TC)_{45}$ insert in the polylinker region of p913 (Pulleyblank et al., 1985). Poly[d(TC)] was prepared by depurination of the duplex (Harwood & Wells, 1970).

Thermal Denaturation Profiles. These experiments were performed at 260 nm on a Gilford 260 spectrophotometer equipped with a thermal programmer. Samples of 300 μL containing 15 μg of DNA were heated at a rate of 0.5 °C/min, and the absorbance was monitored automatically every 15 s. The buffer was either 10 mM Tris-HCl, pH 8, or 10 mM HEPES, pH 7, supplemented with 5 mM NaCl and 0.1 mM EDTA.

Kinetics. This assay relies on the fluorescence of intercalated ethidium bromide (Morgan et al., 1979; Lee et al., 1979). Since the drug does not bind to a triplex, the amount of duplex remaining in a mixture of the two forms can be estimated. Briefly, poly[d(TC)]·poly[d(GA)] at 50 μg/mL was incubated at 20 °C in 0.2 M NaCl and 50 mM sodium acetate buffer, pH 5. At various times, 20-μL samples were removed and added to 2 mL of a pH 5 ethidium fluorescence assay solution as described previously (Lee et al., 1979). In this buffer, the rate of dismutation is very slow because of the presence of ethidium and also because of the low DNA concentration. Consequently, the percent of duplex remaining can be estimated by comparison with the initial value.

Effect of pH on the Duplex Dismutation. Duplex poly[d(TC)]·poly[d(GA)] was initially converted to a triplex by incubation at pH 4.5 until the pH 5 ethidium assay showed no further drop in fluorescence. Aliquots were then diluted 2-fold by addition to an equal volume of a buffered solution (with or without added spermine) such that the final concentrations were 0.2 M NaCl and 50 mM of an appropriate buffer in the pH range of 5–10. The final pH of these solutions were measured individually. For *equilibrium conditions*, aliquots were heated at 60 °C for 2 h; control experiments showed that under these conditions equilibrium is reached in less than 1 h. For *metastable conditions*, aliquots were treated for 10 min at 20 °C. After these treatments, the percent of duplex DNA present in each sample was estimated by the pH 5 fluorescence assay as described above. Controls showed that the addition of low concentrations of spermine did not reduce the fluorescence significantly.

Triplex Formation in Plasmids. The plasmid DNA (0.45 μg) was incubated for 2 h with poly[d(TC)] (0.034 μg) in either (a) 75 mM HEPES, pH 7.2, ionic strength 25 mM, (b) 75 mM HEPES, pH 7.2, with 0.2 M NaCl, ionic strength 200 mM, or (c) 70 mM Tris-HCl, pH 8.4, ionic strength 25 mM. Polyamines were added at the concentrations shown. After incubation at 50 °C for 2 h, samples were treated with 250 mM NaCl. Electrophoresis was performed in 1% agarose gels in 40 mM sodium acetate, pH 5. Control experiments showed that there was no significant redistribution of plasmids after addition to the gel.

Results and Discussion

Triplex formation is readily demonstrated in thermal-denaturation profiles since the triplex has a higher T_m than the duplex. In addition, the relative proportions can be estimated from the hyperchromicity of each transition. At pH 8 (Figure 1a), poly[d(TC)]·poly[d(GA)] melts at 64 °C, but as the spermine concentration increases a second transition becomes apparent, and at 3 μM spermine it accounts for about half of the hyperchromicity. This second transition is due to triplex formation (Lee et al., 1984). As expected, the T_m of both the duplex and triplex increases with increasing spermine concentration. At pH 7 (Figure 1b), a second transition, due to the triplex, is observed even in the absence of spermine, but it requires the dissolution of the duplex and is only observed after the duplex has melted. With 3 μM spermine at pH 7, however, the duplex transition virtually disappears, and only the melting of the triplex is observed with a T_m of about 95 °C. Thus, at least at this low ionic strength (about 10 mM), micromolar concentrations of spermine cause dismutation of the duplex to a triplex at pH 7. It is also apparent in Figure 1b that the T_m of the triplex increases by 25 °C with 3 μM spermine but the increase for the duplex is less than 10 °C. Therefore, spermine must bind more tightly to the triplex. Spermidine also favors triplex formation (Figure 1c), but 10-fold higher concentrations are required. The difference in T_m between the duplex and triplex at 30 μM spermidine is only about 10 °C compared to a ΔT_m of 25 °C in the presence of 3 μM spermine. Figure 1d demonstrates that nonpurine·pyrimidine DNAs such as poly[d(TG)]·poly[d(CA)] do not form triplexes even at the highest spermine concentrations that can be used before precipitation occurs.

The effect of polycations on the rate of triplex formation was also investigated. In order to keep the transitions below 100 °C, low ionic strengths must be used in melting experiments, but for kinetic studies 0.2 M NaCl was added to approximate physiological ionic strengths. This also allows the use of much higher spermine concentrations before precipitation occurs. Clearly, the binding of spermine is antagonized by sodium ions (see also below). In Figure 2, after 60 min at pH 5 in 0.2 M NaCl, about half of the duplex has dismutated to the triplex. The addition of 500 μM spermine increases the rate considerably, so that after 60 min only about 30% of the duplex remains. From the initial slopes of these curves, it can be estimated that spermine increases the rate by at least 2-fold. Magnesium at 5 mM also increases the rate,

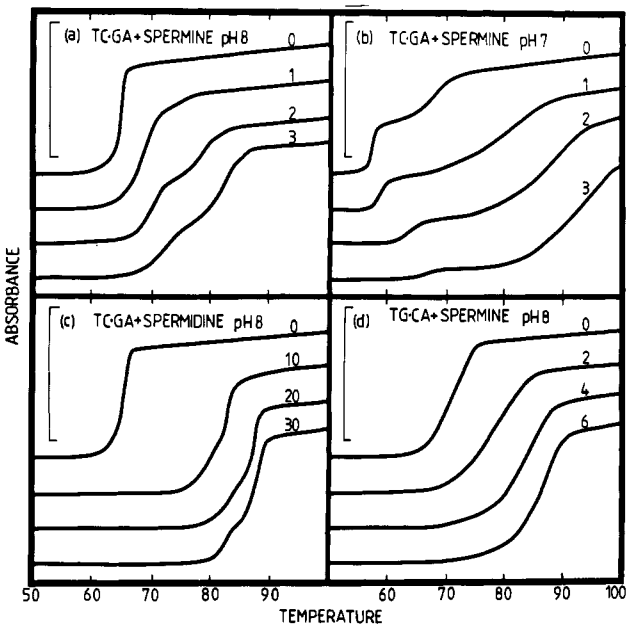

FIGURE 1: Effect of polyamines on the thermal denaturation profiles of synthetic DNAs. The bar represents a hyperchromicity of 30%. (a) Poly[d(TC)]·poly[d(GA)] at pH 8 with 0, 1, 2, and 3 μM spermine; (b) the same at pH 7; (c) Poly[d(TC)]·poly[d(GA)] at pH 8 with 0, 10, 20, and 30 μM spermidine; (d) Poly[d(TG)]·poly[d(CA)] at pH 8 with 0, 2, 4, and 6 μM spermine.

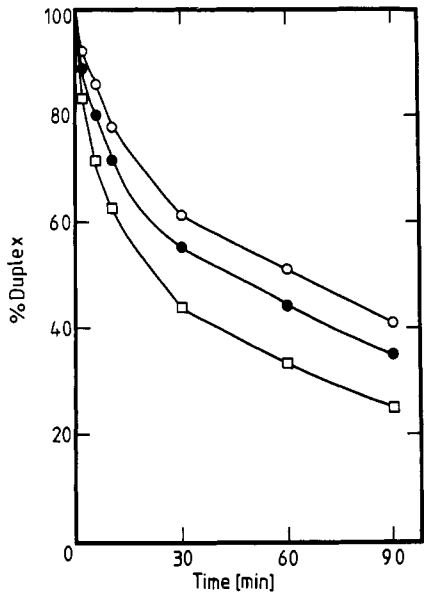

FIGURE 2: Kinetics of dismutation of poly[d(TC)]·poly[d(GA)] at pH 5 with 0.2 M NaCl. The percent of duplex was measured as a function of time by a pH 5 ethidium fluorescence assay. Symbols: O, control; ● +5 mM $MgCl_2$; □, +500 μM spermine.

but the effect is much smaller.

An increase in the rate of dismutation with spermine gives no information about the duplex-to-triplex equilibrium, but a direct method of investigating this aspect is shown in Figure 3. The percent of duplex was measured with 500 μM spermine and 0.2 M NaCl as a function of pH. Under equilibrium conditions (filled circles), the midpoint (pH_m) of the duplex to triplex transition occurs at about pH 6.6. Once formed, however, the triplex remains metastable (open circles) at much higher pHs, and the transition gives a pH_m of 8.2. In the absence of spermine, the equivalent pH_ms for the equilibrum and metastable conditions are 5.8 and 7.2, respectively (Table I). In the presence of 100 μM spermine, intermediate values

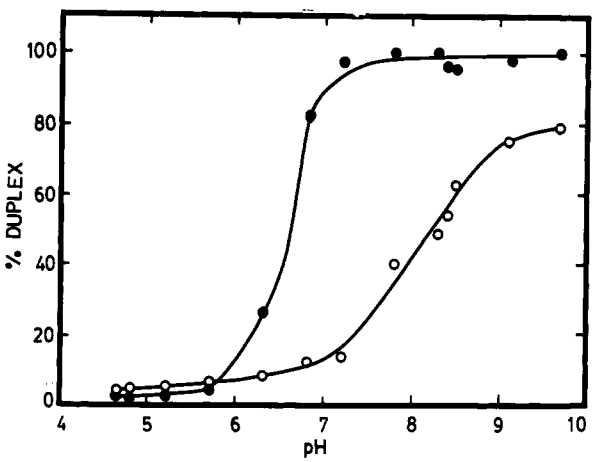

FIGURE 3: Effect of spermine on the pH-dependent dismutation of poly[d(TC)]·poly[d(GA)] in 0.2 M NaCl and 500 μM spermine. The percent of duplex was determined from the pH 5 ethidium flourescence assay. For equilibrium conditions (●) the DNA was heated at the indicated pHs for 2 h at 60 °C before measuring the percent of duplex. For the metastable conditions (O), the triplex was formed at pH 4.5 and then incubated for 10 min at 20 °C. The pH_m is defined as the midpoint of the duplex-to-triplex transition.

Table I: The pH_m of the Duplex to Triplex Dismutation with Spermine[a]

spermine (μM)	equilibrium pH_m	metastable pH_m
0	5.8	7.2
100	6.4	7.9
500	6.6	8.2

[a] The pH_m was determined from midpoint of plots of percent of duplex as a function of pH as described in the legend to Figure 3.

are obtained. Thus, 500 μM spermine raises the pH_m by about one pH unit into the physiological range. In the nucleus of some cells, the spermine concentration may be 10-fold higher, resulting in even more preference for the triplex conformation (Sarhan & Sieler, 1989). It was not possible to assess these high concentrations because of problems with DNA precipitation.

The experiments described above utilized a duplex that dismutated to give a triplex and a free purine strand. Removal of this extra purine strand would be expected (by simple mass action) to push the equilibrium even further in favor of the triplex. Triplexes can also form in plasmids containing pur·pyr inserts (Htun & Dahlberg, 1989), and plasmids can become linked together via triplex formation upon the addition of an homologous polypyrimidine (Lyamichev et al., 1988; Lee et al., 1989). In this system there is no extra purine strand. The effect of spermine and spermidine on the formation of linked plasmids is shown in Figure 4. At pH 7.2 and 25 mM ionic strength in the absence of a polyamine, poly[d(TC)] has no effect on plasmid pTC45, which contains a 45 base-pair insert of (TC)·(GA). Upon addition of 50 μM spermine, some smearing is observed, and, at 100 μM spermine, discrete bands appear that are due to dimers, trimers, tetramers, etc., of the plasmid linked through the single-stranded polypyrimidine. As has been discussed elsewhere (Lyamichev et al., 1988; Lee et al., 1989), this linkage is most likely due to direct triplex formation between poly[d(TC)] and the insert on the plasmid rather than interaction with the fold-back triplex or H-form. Good evidence for an intermolecular triplex is also provided in Figure 4 since the open circular forms of the plasmid participate in multimer formation just as readily as the supercoiled forms. The H-form or intramolecular triplex, on the other hand, is stimulated by supercoiling. At higher spermine

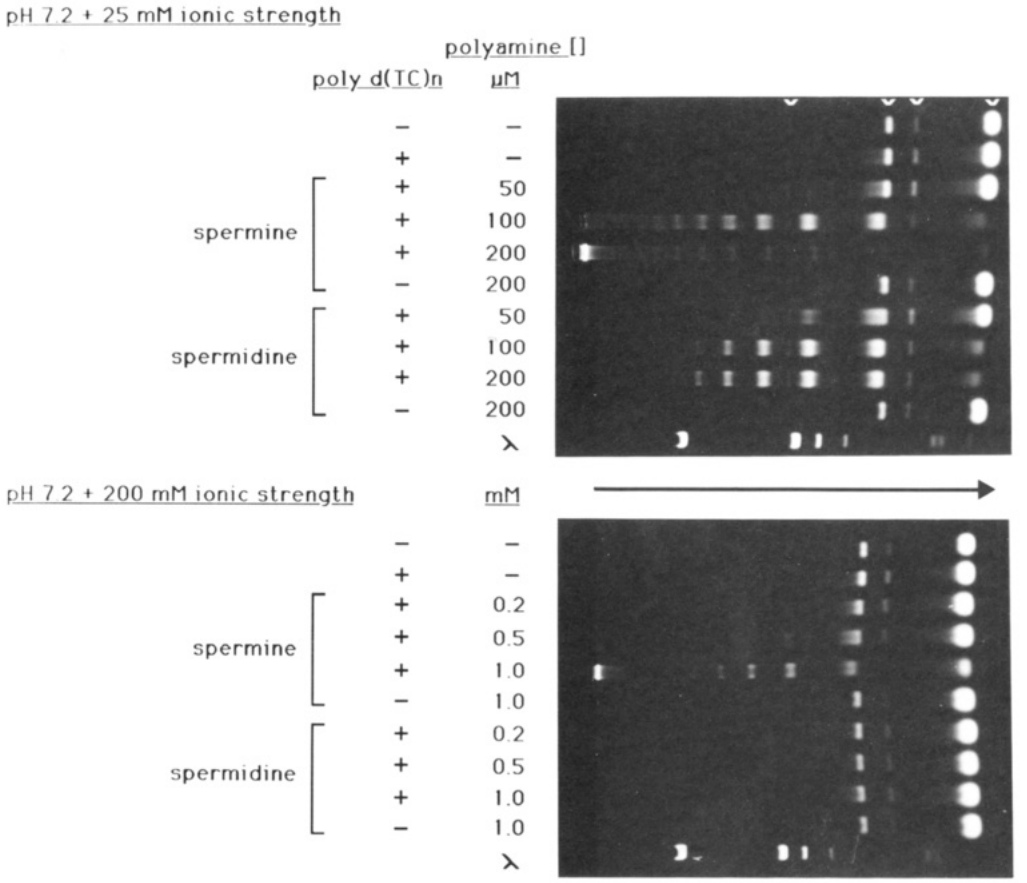

FIGURE 4: Polyamine-induced complex formation between pTC45 and poly[d(TC)]: (upper panel) pH 7.2 and 25 mM ionic strength; (lower panel) pH 7.2 and 175 mM NaCl, 200 mM ionic strength. After incubation for 2 h at 50 °C, the samples were analysed on 1% agarose gels and stained with ethidium. The white arrows in the upper panel show the positions of (from right to left) supercoiled plasmid, open circular plasmid, supercoiled dimer, and open circular dimer. The black arrow shows the direction of electrophoresis from left to right.

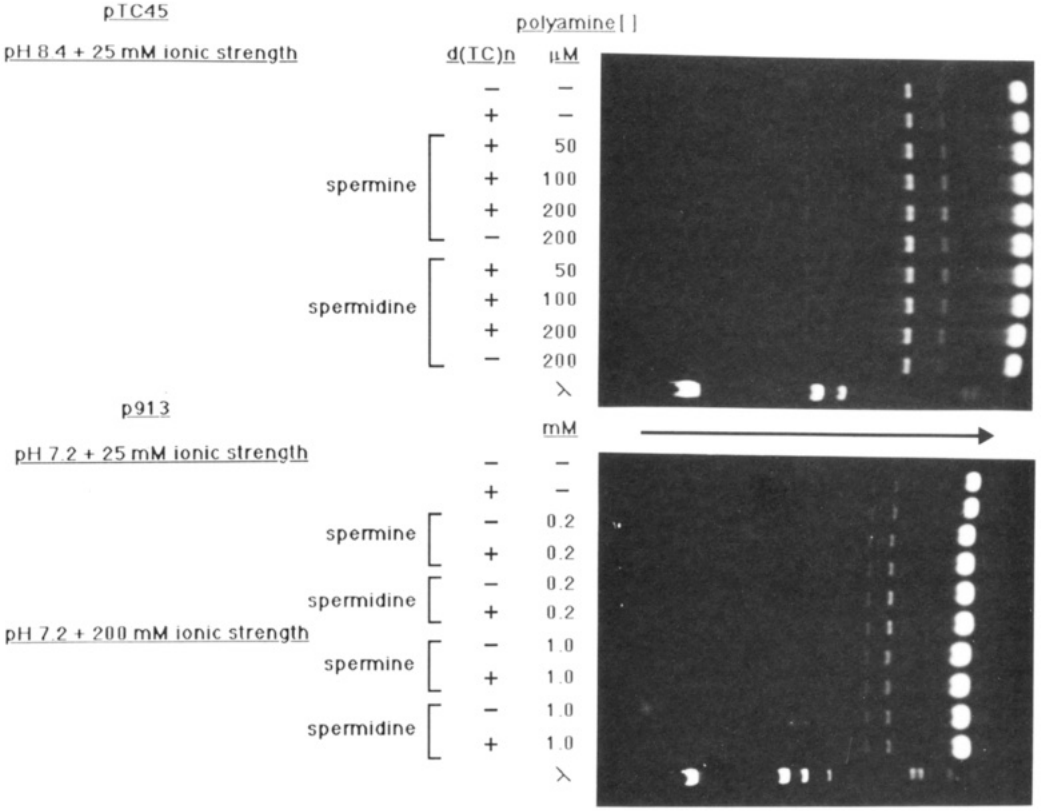

FIGURE 5: Control experiments: (upper panel) pTC45 and poly[d(TC)] at pH 8.4 and 25 mM ionic strength; (lower panel) p913 and poly[d(TC)] at pH 7.2 and 25 mM or 200 mM ionic strength. See legend to Figure 4.

concentrations, the complexes are shifted to higher multimers, some of which do not enter the gel. Multimer formation, as expected for a triplex-mediated event, is pH dependent and occurs much more readily at lower pHs and is not observed at pH 8.4 (Figure 5). The parent plasmid p913, which does not contain an insert, is unaffected by the addition of poly-[d(TC)] even with 200 μM spermine (Figure 5). Spermidine also promotes intermolecular triplex formation but is less effective at producing the higher multimers than spermine (Figure 4). Figure 4 also demonstrates the antagonism of increasing the ionic strength. At 200 mM ionic strength, 0.5 mM spermine is required before plasmid dimers are observed, and with spermidine dimer formation can just be observed at 1 mM. This result is consistent with the melting experiments (Figure 1) that also demonstrated that spermine promotes triplex formation at lower concentrations than spermidine.

Thus by four different techniques polyamines have been shown to stabilize the triplex conformation. It is difficult to mimic in vitro the conditions inside the nucleus because of problems with DNA precipitation in the presence of millimolar concentrations of polyamines. By extrapolation, however, from the results above, it seems reasonable to suggest that triplexes would be stable and may even be the preferred conformation for pur·pyr sequences in the eucaryotic nucleus. A role for polyamines in chromosome condensation seems plausible since upon bindng to DNA they would reduce the repulsion between the phosphate backbones on adjacent duplexes. A similar explanation may also apply to the preferential stabilization of triplexes by polyamines since the positive charge density of a triplex is higher than a duplex, at least for triplexes containing T-A-T base triads.

It is intriguing that pur·pyr sequences represent as much as 1% of some eucaryotic genomes (Hoffman-Lieberman et al., 1986; Manor et al., 1988). The results presented here suggest that these sequences may interact via triplex formation to form loops of DNA. Thus polyamines and triplexes may play a direct role in the structural organization of chromosomes. In addition, pur·pyr motifs that interact with nuclear factors have been located in the promoter regions of several growth related genes, including the c-Ki-*ras* and c-*myc* protoncogenes (Davis et al., 1989; Hoffman et al., 1990). Triplex structures that can be formed in vitro in these regions may be stabilized in vivo by polyamines and regulate the transcription of these genes. In support of this idea, inhibition of polyamine synthesis has been found to decrease the transcription of c-*myc* (Celone et al., 1990). An involvement in chromosome structure and regulation of transcription are both consistent with a role for polyamines in cell proliferation.

Registry No. Poly[d(TC)]·poly[d(GA)], 29627-66-5; spermine, 71-44-3; spermidine, 124-20-9.

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