Plasmid Dimerization Mediated by Triplex Formation between Polypyrimidine–Polypurine Repeats†

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ABSTRACT: The ability of independent pyr·pur tracts to participate in triplex formation has been investigated in linear plasmids. The pyr·pur tract could be positioned at the ends of the plasmids or internally by a suitable choice of restriction enzyme. Dimer formation between plasmids was monitored by mobility shifts on agarose gels as well as by direct visualization in the electron microscope. Linear dimers and X and Y structures were observed. Control experiments showed that a pyr·pur tract was essential and was consistent with triplex formation in which the two pyrimidine strands were antiparallel. These structures were formed at pHs between 4 and 6, but once formed they remained stable up to pH 7. Spermine was required for formation of dimers at low ionic strength, but once formed the dimers remained stable in the absence of spermine. Additional linear plasmids were constructed with pyr·pur tracts at both ends; these formed structures at pH 4 which had mobilities identical to those of open circles. Triplex formation of this type may serve as a good model for loop formation in eukaryotic chromosomes.

Intramolecular triplex formation of the pyr·pur·pyr type has been well documented in plasmids [reviewed in Palecek (1991) and Yagil (1991)]. This structure, termed H DNA, is formed within a pyr·pur tract by folding of the DNA upon itself with triplex formation in one half while the other half becomes single-stranded (Thrier & Leng, 1972; Lyamichev et al., 1985; Lee et al., 1984). The pyr·pur tract must contain a center of symmetry because the two pyrimidine strands in the triplex are antiparallel (Rajagopal & Feigon, 1989; de los Santos et al., 1989). As well, triplex formation is favored by negative supercoiling and a pH below 7 (Palecek, 1991; Yagil, 1991; Htun & Dahlberg, 1988, 1989). There is considerable interest in H DNA because of the possibility that it may be involved in the regulation of transcription at least in some eukaryotic genes (Davis et al., 1989; Hoffman et al., 1990)).

Except with synthetic DNA, intermolecular triplexes have only been demonstrated by the addition of a single-stranded polypyrimidine to plasmids containing pyr·pur tracts (Lee et al., 1989; Lyamichev et al., 1988; Moser & Dervan, 1987). In this case, the plasmids can become linked together if the polypyrimidine is sufficiently long (Lee et al., 1989). Again, there is considerable interest in these ideas because of the possibility of controlling gene expression by the addition of a suitable "third strand" or for DNA isolation by triplex-affinity-capture (Cooney et al., 1990; Takashi et al., 1992).

Another possibility, which is the subject of this paper, is intermolecular triplex formation between two isolated pyr·pur tracts or between two tracts on different plasmid molecules. The sequences of the plasmids used in this work are shown in Figure 1. By cutting with either *Bam*HI or *Bgl*I, linear plasmids were generated with pyr·pur tracts either at the end or in the middle of the DNA. Two plasmids, pKHa1 and pKHa2, contain two tracts, and cutting with *Xba*I places them at opposite ends of a linear DNA. It should be noted that p3PU and p3PY contain asymmetrical inserts which cannot form triplexes with themselves but should be able to form an intermolecular triplex with each other. The structures which are expected to be formed by these plasmids are also shown in Figure 1. These include linear dimers, X and Y dimers, and circles.

MATERIALS AND METHODS

Plasmids. The plasmids p913, pTC45, p3PU, and p3PY were gifts from Dr. D. E. Pulleyblank, Toronto. pTC45 contains a $(TC)_{22}T$ insert in the polylinker region of p913 (Pulleyblank et al., 1985). The sequences of the pyr·pur tracts in p3PU and p3PY are shown in Figure 1A. pKHa1 and pKHa2 were prepared by cloning pyr·pur fragments from p3PU and p3PY into pUC19 (Yannisch-Perron et al., 1985) by standard cloning techniques (Sambrook et al., 1989). The p3PY pyr·pur sequence was cloned into the *Xba*I–*Kpn*I site of M13mp19 (Yannisch-Perron et al., 1985). This chimera (M13/PY) served as a common intermediate for both pKHa1 and pKHa2. For the M13 clone of pKHa1, the p3PU pur·pyr tract was then cloned into the *Xba*I–*Pst*I site of this intermediate. We obtained a synthetic opposite orientation p3PU pur·pyr tract complete with *Xba*I and *Pst*I ends from the Regional DNA Synthesis Laboratory at the University of Calgary. This DNA was cloned into M13/PY to produce the M13 clone of pKHa2. After confirmation of the correct sequence of these two clones from the M13 single-stranded DNA, the *Pvu*II fragments were subcloned into pUC19.

Dimer Assays. Linearized plasmids (0.5 μg in 10 μL equivalent to 75 μM in base pairs) were incubated for 12 h with 50 μM spermine–4HCl and 1 mM EDTA in buffers at pHs 4–7 (25 mM sodium acetate, pH 4.0 or 5.0, 56 mM MES–NaOH, pH 6.0, and 56 mM BES–NaOH, pH 7.0) prior to electrophoresis. In experiments with $MgCl_2$ and $ZnCl_2$, EDTA was omitted from the incubation buffers. The final ionic strength of these reactions, unless supplemented with NaCl, was 25 mM (Na^+). Electrophoresis was performed in 1% agarose gels (unless otherwise stated) at a voltage of 3.3 V/cm in running buffers at pHs 4–7 (40 mM sodium acetate, pH 4 or 5, 90 mM MES–NaOH, pH 6.0, and 90 mM BES–NaOH, pH 7.0) plus 1 mM EDTA. After electrophoresis, gels were stained with ethidium bromide and photographed under UV light.

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A

p3PY (y) 5'-TCCC (CTTCCT)$_6$ GGGA-3'

p3PU (u) 5'-TCCC (GAAGGA)$_6$ GGGA-3'

pTC45 (t) 5'-TCCC T(CT)$_{22}$ GGGA-3'

pKHa1 5'-TCCC (TCCTTC)$_6$ GGGATCTCTAGAGATCCC (CTTCCT)$_6$ GGGA-3'
Xba I

pKHa2 5'-TCCC (GAAGGA)$_6$ GGGATCTCTAGAGATCCC (CTTCCT)$_6$ GGGA-3'
Xba I

B

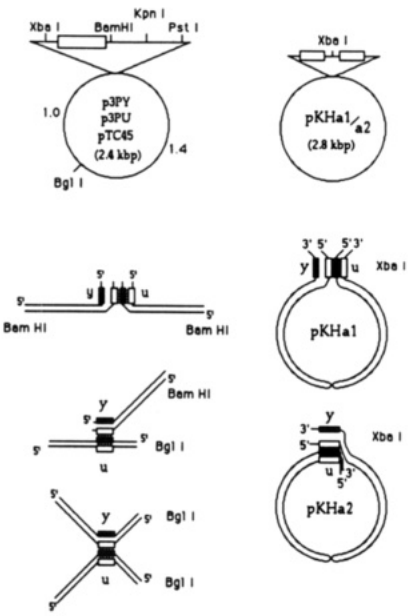

FIGURE 1: Plasmids and potential structures at low pH. (A) Sequences of the pyrimidine·purine tracts in the five plasmids. (B) Diagrams of plasmids with unique restriction sites and potential structures formed from them after linearization with *Bam*HI or *Bgl*I (p3PU, p3PY, and pTC45) and *Xba*I (pKHa1 and pKHa2). Open and filled boxes represent the pyrimidine and purine strands, respectively.

Electron Microscopy. Plasmid samples treated as above at pH 4.0 were diluted to 5 μg/mL. A mixture of 25 μL of DNA and 10 μL of cytochrome *c* (1 mg/mL aqueous solution) was spread on the surface of 0.5 M ammonium acetate, 10 mM acetate buffer, pH 4, and 1 mM EDTA in a 35-mm plastic Petri dish. Formvar–carbon-coated grids were touched to the surface of the spread DNA, rinsed in 95% ethanol, and air-dried. The grids were carbon–platinum shadowed for visualization of the DNA in a Philips 410 electron microscope operating at 40 kV. Length measurements of the observed DNA structures were made on electron micrographs, printed at a uniform magnification, using a map measuring wheel.

RESULTS

Preliminary experiments were performed on *Bam*HI-cut plasmids which had been incubated at pH 4 with 50 μM spermine. These were then electrophoresed on 1% agarose gels at pH 4 in the absence of spermine. As shown in Figure 2, pTC45 and the mixture of p3PY and p3PU give an extra band at 4.9-kb whose mobility is consistent with it being a linear dimer. There are also faint bands at slower mobilities which are presumably some form of trimer or multimer. Neither p913 nor p3PY and p3PU alone show any evidence of dimerization. Thus, dimer formation is not caused by the reannealing of sticky ends but is dependent on pyr·pur tracts in which the two pyrimidine strands are antiparallel. Under these conditions, dimerization is rapid, and after 15 min no

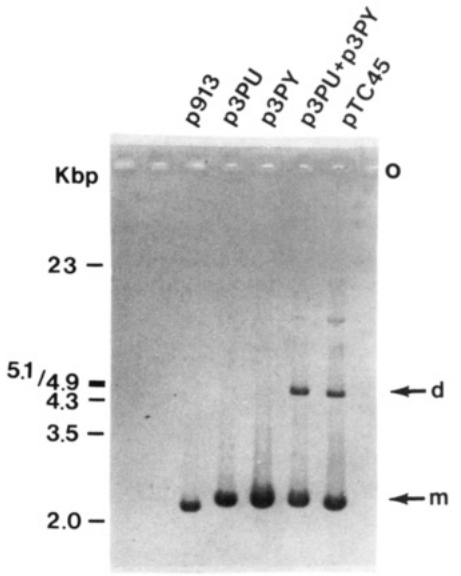

FIGURE 2: Plasmid dimer formation at pH 4.0. Plasmids were incubated as described under Materials and Methods, run on a 1.0% agarose gel at pH 4.0, and then stained with ethidium bromide. Dimer and monomer forms of the plasmids are indicated by (d) and (m), respectively. The origin of the gel is indicated by (o).

further changes in the distribution of species were observed. Control experiments showed that dimer formation was not occurring under the conditions of electrophoresis (i.e., in the absence of spermine). However, once formed, the dimers were extremely stable under these conditions. Indeed, dimer complexes eluted from these gels at low pH can be kept at pH 5.0 for several days at 4 °C without significant conversion to plasmid monomers (data not shown). As well, attempts to convert all the DNA to dimers failed even after prolonged incubation at elevated temperatures. Thus, there is a rapid equilibrium between duplex and triplex in the presence of spermine but in its absence the triplex is locked in place (see also Figure 7). In order to investigate the type of equilibrium with spermine, the absolute concentration of plasmids and the ratio of the two plasmids were varied over a 10-fold range and the amount of dimer formation was estimated on gels as described above (data not shown). The results were consistent with simple mass action in which two plasmids are in equilibrium with the dimer: plasmid 1 + plasmid 2 $\Leftrightarrow$ dimer.

The effect of pH on dimerization is investigated in Figure 3. A mixture of p3PU cut with *Bgl*I and p3PY cut with *Bam*HI, which is expected to form a Y structure, was incubated at various pHs before being electrophoresed at pH 4. Panel A shows that dimerization occurs most readily at pH 4, but even at pH 6 about 10% of the DNA has an altered mobility. In panel B, the plasmid was incubated at pH 4 but then electrophoresed at increasing pHs. In this case, dimers are still present at pH 7. This is a clear demonstration of hysteresis which can be observed in many nucleic acid systems (Lee et al., 1984; Hampel et al., 1991; Rich et al., 1984).

The various structures, notably linear dimers and X and Y, can be distinguished on agarose gels. Lane 1 of Figure 4 shows electrophoresis of a mixture of four linear plasmids (p3PU cut with *Bam*HI or *Bgl*I, and p3PY cut with *Bam*HI or *Bgl*I) after incubation at pH 4 with 50 μM spermine. Two bands can now be resolved from the monomer. Lane 2 (a mixture of *Bgl*I cut p3PU and p3PY) only reveals the slower moving band of the doublet; this structure must be an X because both plasmids have the pyr·pur tract in the middle. The linear dimer is shown in lane 3 for comparison. A mixture of pTC45

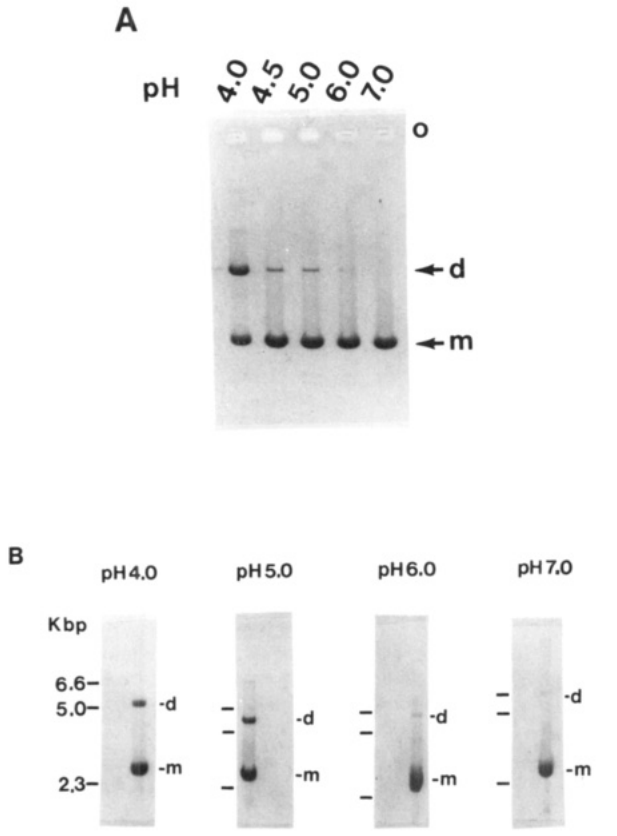

FIGURE 3: Dimer formation is pH dependent and exhibits hysteresis. (A) A mixture of p3PY cut with *Bam*HI and p3PU cut with *Bgl*I was incubated at the indicated pH with 50 μM spermine and run at pH 4.0. (B) For hysteresis experiments, plasmids were incubated at pH 4.0 with 50 μM spermine and run at the indicated pH.

cut with *Bam*HI or *Bgl*I also shows a doublet (lane 4) but the X structure does not form readily (lane 5). The linear dimer of pTC45 is in lane 6 for comparison. The slower moving band of the doublet in lane 4 is therefore most likely to be a Y structure. Mixtures of p3PU and p3PY in which one plasmid has the tract in the middle and the other at the end can only form Y structures. This is confirmed in lanes 7, 8, and 9. It is interesting to note that the two Y structures (lanes 8 and 9) have different intensities. Figure 5 shows that dimer formation is less favored as the tract is moved away from the end of the linear DNA. Therefore, in general, linear dimers and Y structures will be favored over X structures.

Verification of the existence of these structures was obtained by direct visualization, using electron microscopy. The DNA, in 10 mM acetate buffer at pH 4, was spread on a hypophase of 0.5 M ammonium acetate, and although these are not ideal conditions for preservation, clear identification of the structures was possible. Typical electron micrographs of a linear plasmid, linear dimer, Y structure, and X structure are shown in Figure 6, panels a–d, respectively. In general, the frequency of occurrence of each form within a sample correlated with that expected from the relative intensities observed on the agarose gels (Figure 4). Thus, linear dimers accounted for about 20% of the molecules in the electron micrographs, and X structures were about 5%. Y structures were particularly prominent on gels after incubation of *Bam*HI-cut p3PY with *Bgl*I-cut p3PU (lane 8, Figure 4) and also accounted for about 50% of the structures seen by electron microscopy of this sample. Three-membered junctions were observed with high frequency on the grids of this sample even under conditions where a complete Y structure could not be defined (i.e., in regions containing high concentrations of DNA). Normally, three-membered junctions are much less frequent than cross-overs. Length measurements of the various structures were performed on electron micrographs (Figure 6). As expected, the dimer is twice the length of the control and the arms of the X are in the ratio of 1.4 to 1. Similarly, the three arms of the Y are in the ratio of 2.4:1.4:1, consistent with the position of the *Bgl*I restriction site in relation to the pyr·pur tract (Figure 1). This is compelling evidence for the joining of these molecules at the pyr·pur tract.

As shown in Figure 7 at low ionic strength, spermine is required for Y dimer formation (panel A). Above 50 μM

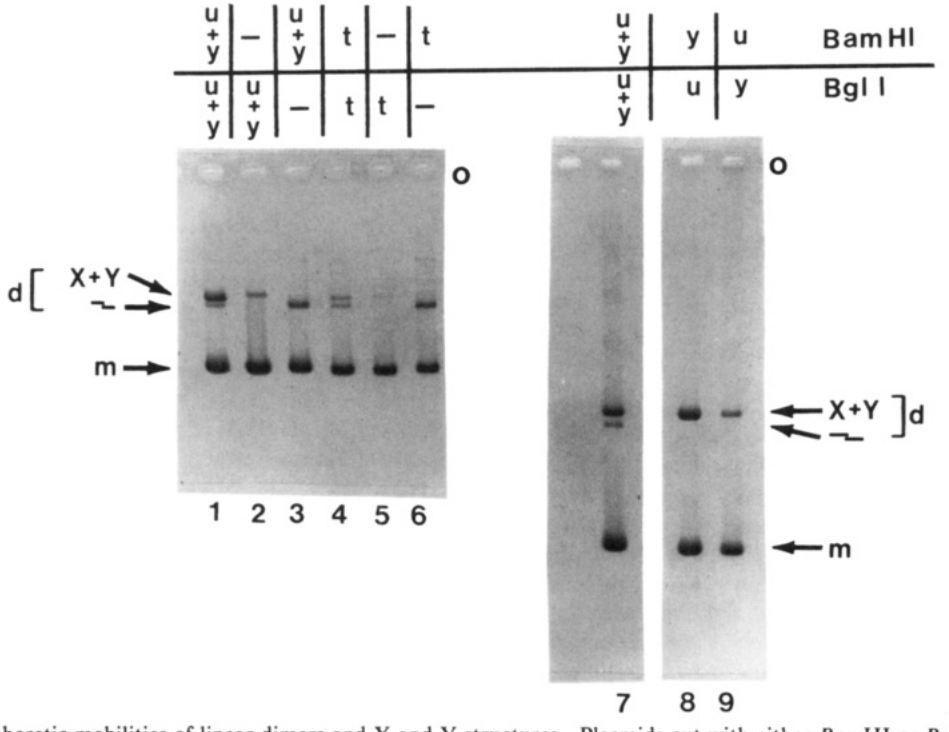

FIGURE 4: Electrophoretic mobilities of linear dimers and X and Y structures. Plasmids cut with either *Bam*HI or *Bgl*I were incubated at pH 4.0 with 50 μM spermine in the combinations indicated where (u) is p3PU, (y) is p3PY, and (t) is pTC45.

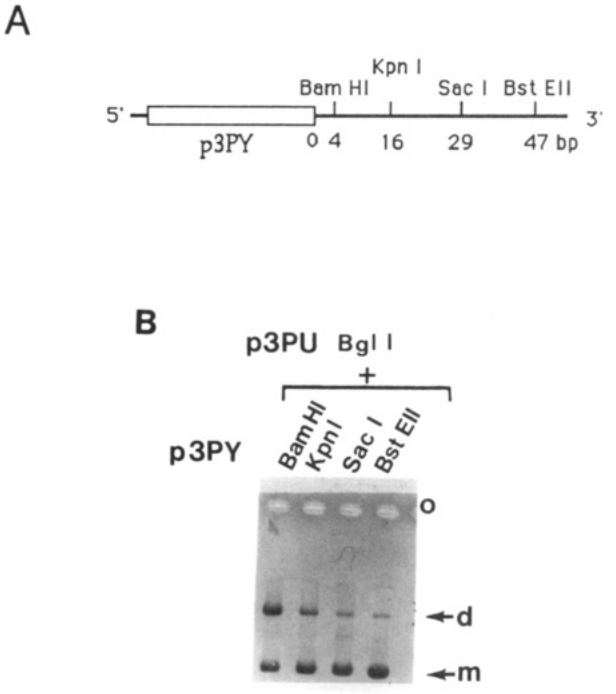

FIGURE 5: Y-dimer formation dependent on the proximity of the donated strand to the end of the plasmid. (A) Map of the unique restriction sites close to the pyr·pur tract (open box) in p3PY. (B) Formation of Y-dimers between p3PU cut with *Bgl*I and p3PY cut by *Bam*HI, *Kpn*I, *Sac*I, or *Bst*EII. Incubations of cut plasmids were carried out at pH 4.0 with 50 μM spermine.

spermine at low ionic strength, precipitation occurs. With a fixed concentration of spermine, increasing amounts of NaCl lead to a marked decrease in dimer formation (panel B). The antagonistic effect of high ionic strength and spermine on triplex formation has been shown in previous reports (Hampel et al., 1991). However, it is clear that at 150 mM NaCl some dimers form even in the absence of spermine and they are quite prominent at even higher NaCl concentrations (panel C). As a control, lane 9 shows the linear dimer and lane 10 is p913, again demonstrating that a pyr·pur tract is required. At intermediate ionic strengths, the formation of dimers could be restored by increasing the spermine concentration (panel D). The condition of 200 mM NaCl and 500 μM spermine is of some interest because higher order structures (trimers and tetramers) are clearly visible. The divalent cations Mg^{2+} and Zn^{2+} were also used to promote triplex-mediated dimerization; however, both were less effective than spermine in this capacity (data not shown). Furthermore, neither was capable of promoting triplex formation above pH 4.0.

We have also designed a model for intramolecular triplex interactions between two separated pyr·pur sequences on the same DNA. The pyr·pur tracts from p3PU and p3PY were cloned into the same vector as shown in Figure 1. If these plasmids (pKHa1 and pKHa2) are linearized between the two tracts with *Xba*I, a linear DNA with pyr·pur sequences at each end is produced. Figure 8 demonstrates that these linear plasmids form dimers upon incubation at pH 4 as well as another form which has a mobility identical to that of open circular plasmids. This structure whose formation is pH dependent must be a circle caused by triplex formation between the two ends. The low intensity of this band is not unexpected since dimer formation is favored for plasmids of this length (Cantor & Schimmel, 1980).

DISCUSSION

The ability of independent pyr·pur tracts to participate in triplex formation has been investigated in linear plasmids. Dimer formation could be monitored by mobility shifts on agarose gels and the predicted structures could be observed directly in the electron microscope. Triplexes are implicated in the formation of these structures for several reasons. First, the structures will only form in plasmids which contain pyr·pur tracts and the requirement for antiparallel pyrimidine strands must also be satisfied. Second, the pH and polyamine dependency of these interactions as well as the hysteresis is typical of the behavior of triplexes in other systems. Third, the lengths of the arms of the X and Y structures show that the join is occurring at the pyr·pur tract.

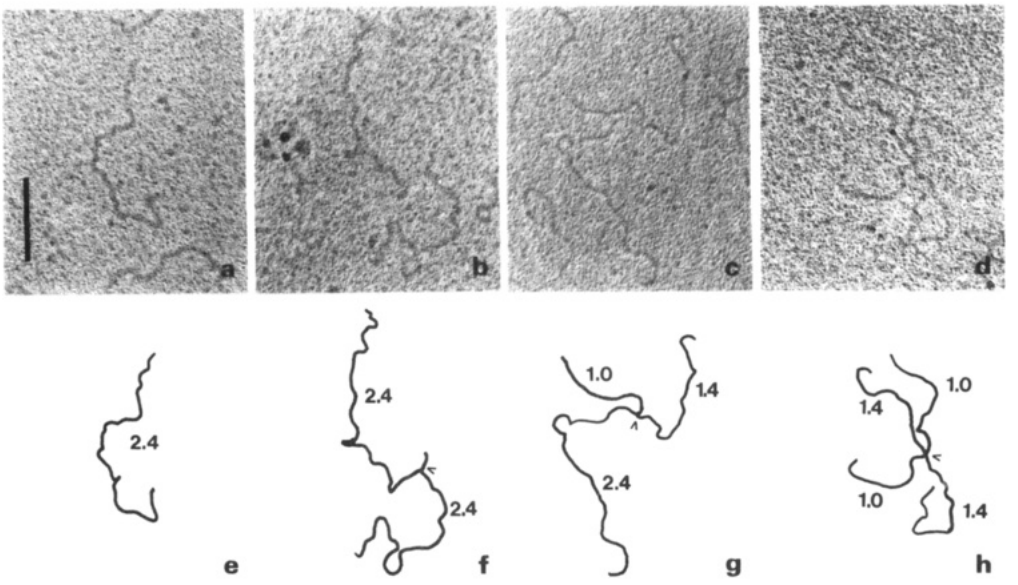

FIGURE 6: Electron micrographs of plasmid dimers produced by triplex formation. Aqueous DNA samples treated at pH 4.0 with 50 μM spermine were diluted and then adsorbed to formvar–carbon-coated grids as described under Materials and Methods. Original photographs of complexes (a–d) or drawings of photographs (e–h) are presented. Experiments consisted of the following plasmids: p3PY alone (a) and (e) (control for monomer length), p3PY cut with *Bam*HI plus p3PU cut with *Bam*HI (b) and (f) (linear dimer), p3PY cut with *Bam*HI plus p3PU cut with *Bgl*I (c) and (g) (Y-dimer), and p3PY cut with *Bgl*I plus p3PU cut with *Bgl*I (d) and (h) (X-dimer). Arrowheads point to positions of putative intermolecular triplexes and numbers refer to the lengths (in kilobase pairs) of the arms of the dimers. The bar in (a) represents 2 μm.

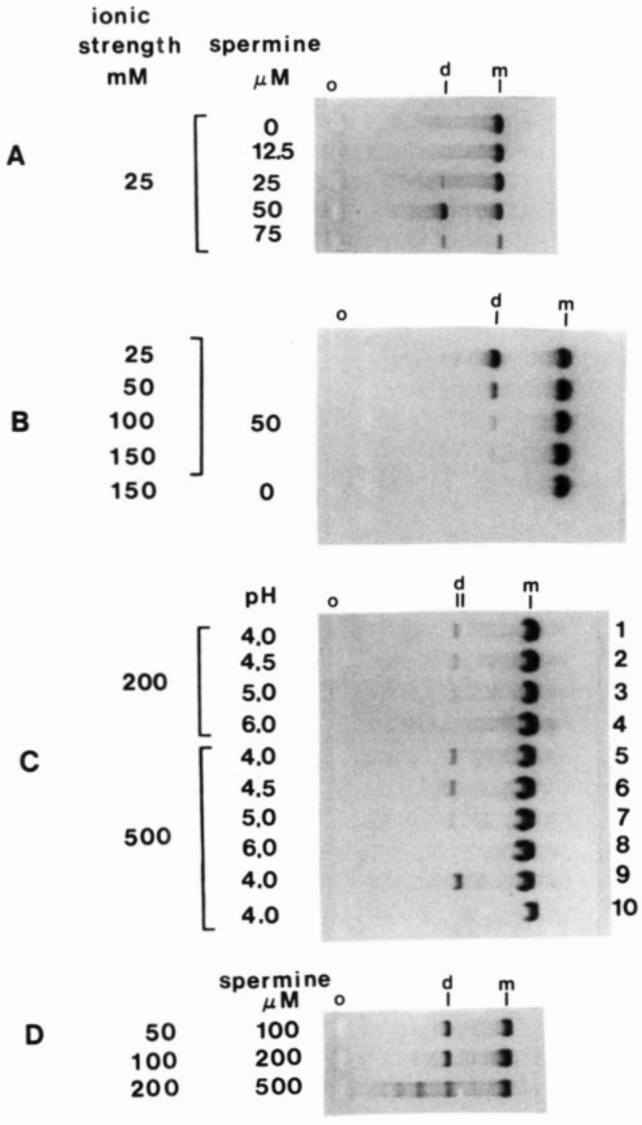

FIGURE 7: Antagonism between ionic strength and polyamine on formation of plasmid dimers. A mixture of p3PY cut with *Bam*HI and p3PU cut with *Bgl*I was incubated at the ionic strengths and spermine concentrations shown and at pH 4.0 (A, B, and D) or at a range of pHs (C). All buffers were 25 mM ionic strength and were made up to higher ionic strengths with NaCl if required.

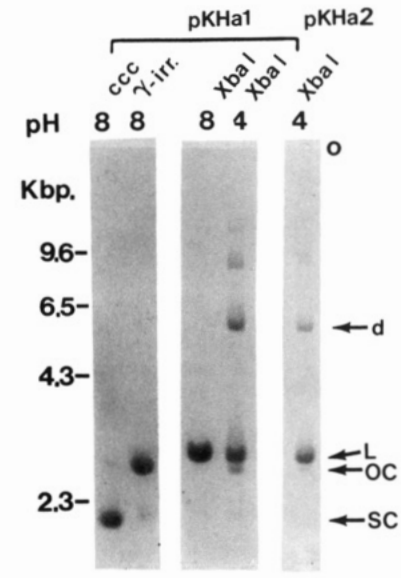

FIGURE 8: Intramolecular circle formation in linear plasmids bearing two pyr·pur inserts. Samples were incubated at pH 4.0 or 8.0 with 50 μM spermine and run on 0.7% agarose gels at pH 4.0. Arrows at the right point to supercoiled plasmid monomers (SC), nicked circular monomers (OC), linear plasmid monomers (L), and triplex-mediated dimers (d).

In both the linear dimers and the Y structure, a free end is available to initiate triplex formation. However, the X structure formed between *Bgl*I-cut p3PU and p3PY does not have a free end. This may be the reason for the lower prevalence of the X compared to Y structures. For *Bgl*I-cut pTC45, very little of the X form could be detected. This may be due to competition with the H form which will also be present in this symmetrical pyr·pur tract at pH 4.0 (Htun & Dahlberg, 1988). With p3PU and p3PY, an H form is not possible because the sequences are not symmetrical. Another interesting feature of the results in Figure 5 is the relative ability of the two different Y-dimers to form. Y-shapes can be formed by mixing p3PU (*Bam*HI cut) with p3PY (*Bgl*I cut) or vice versa as shown in Figure 1. In these structures, we expect that the third strand for the triplex will be donated by the *Bam*HI-cut plasmid since the duplex at the end can be melted more easily. The results in Figure 5, lanes 8 and 9, show that p3PY (*Bam*HI) + p3PU (*Bgl*I) dimer forms more readily than the p3PY (*Bgl*I) + p3PU (*Bam*HI) dimer. We have also observed that this preference is reversed if a restriction site on the opposite side of the plasmid is used to generate linear plasmids (not shown). Thus, there is a difference in the relative formation of Y-dimers which depends on the orientation of the pyr·pur tract at the end of the plasmid. Taken together, this suggests that triplex formation occurs preferentially in the 3′ to 5′ direction with respect to the third strand.

There are several other features of these intermolecular triplexes which might not have been predicted from previous results with other triplexes. For example, poly[d(TC)]·poly-[d(GA)] dismutates to a triplex in the absence of spermine at any ionic strength as long as the pH is lowered to 5 (Lee et al., 1984). However, at low ionic strength, poly-[d(TC)]·poly[d(GA)] may dismutate by an intermolecular mechanism which is not available to our plasmids (Thrier & Leng, 1972). Another difference is that the tracts in the plasmids are much shorter than are found in synthetic DNAs. Second, the equilibrium is established very rapidly in the presence of spermine but the dissociation of the dimer is very slow in its absence as judged from the tight bands on the agarose gels. At low ionic strength, spermine may be required to overcome the charge repulsion between different DNA molecules. Third, the mobilities of the X and Y structures were identical under all conditions and only slightly slower than the linear dimers. There are two factors to be considered; an increased mobility caused by a decrease in the end to end length for X and Y structures and a decreased mobility because they form bent DNA (Crothers et al., 1990). Fourth, circles do not form as readily as linear dimers in pKHa1 and pKHa2. This may be due to several factors such as the short distance between the tracts and the increased persistence length and stiffness of the DNA at low ionic strength (Cantor & Schimmel, 1980). Thus, circle formation is expected to be more favorable in larger plasmids. Experiments to test this idea are now in progress (unpublished results).

Since triplex formation can link two DNAs together or circularize a linear plasmid, it seems reasonable to suggest that this process could occur in chromosomes. Eukaryotic chromosomes contain long pyr·pur tracts which may represent 1% of the genome in some species (Hoffman-Liebermann et al., 1986; Manor et al., 1988; Birnboim et al., 1979). A loop

of DNA held together by triplex formation is simply an X structure, of the type described above, in which two of the arms are infinitely long. It is known that chromosomes contain loops of DNA (Paulson & Laemmli, 1977) and the high polyamine concentration in the nucleus will favor triplex formation (Hampel et al., 1991). Thus, chromosome condensation or at least one level of folding might be mediated by triplex formation between homologous pyr·pur tracts.

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