Articles

Kilobase-Range Communication between Polypurine·Polypyrimidine Tracts in Linear Plasmids Mediated by Triplex Formation: A Braided Knot between Two Linear Duplexes†

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ABSTRACT: Linear plasmids were constructed containing two pyrimidine tracts that were 0.34 and 0.94 kilobases (kb) from either end and were separated by 2.8 kb. The tracts [$d(TCCTTC)_n$ and $d(CTTCCT)_n$ where $n = 6$ or 12] were designed so as to be able to form triplexes with each other but not with themselves. Upon lowering of the pH to 4 in the presence of spermine, these plasmids form intermolecular dimers and intramolecular loops of 2.8 kb, as judged from mobility changes on agarose gels. A tethered loop could also be formed in a linear plasmid containing two identical tracts by adding an homologous single-stranded oligopyrimidine, but not an oligopurine. In plasmids containing different tracts, the formation of both dimers and loops could be blocked by adding either homologous single-stranded oligopyrimidine but not an oligopurine. Together with the requirement of low pH, these results demonstrate that triplex formation is of the pyr·pur·pyr type. The extent of dimer and loop formation was dependent on the length of the pyrimidine tract; dimers could be detected in plasmids containing the 72 base pair (bp) inserts after incubation at pH 6, but in plasmids containing the 36 bp inserts, a pH of 5 was required. Hysteresis was also evident to a remarkable extent. Once formed at pH 4, loops and dimers remained stable indefinitely at pH 8, suggesting that the structures become topologically trapped. However, the structures were resolved into the component linear plasmids by incubation with nuclease P1. This is the first demonstration of a braided or hydrogen-bonded knot between two linear duplexes and may have implications for chromosomal loop formation.

The biological importance of DNA looping in chromosome condensation and gene regulation is well-documented (Gasser & Laemmli, 1987; Schleif, 1992). The formation of large looped domains in condensed chromosomes has been characterized in prokaryotes and eukaryotes and represents at least one level of DNA condensation (Sinden & Pettijohn, 1981; Paulson & Laemmli, 1977). For gene expression, DNA looping is a potential means to bring regulatory sequence elements into close enough proximity to interact with one another. Proteins have been found to mediate the formation of double-stranded DNA loops in both cases (Schleif, 1992; Hofmann et al., 1989). Another potential means to link specific sequences over long distances is by a direct interaction between two duplexes. Several types of multistranded structures have been described in the past 35 years, but the idea that these structures could anchor a double-stranded DNA loop has received little attention.

Simple repetitive DNA sequences and mirror repeats are capable of dismutation to several types of multistranded structures and altered duplexes [reviewed in Palecek (1991); Yagil, 1991]. In particular, polypurine·polypyrimidine (pur·pyr) sequences are capable of forming three- and four-stranded complexes, depending on the conditions. Among the most studied of these structures in recent years is a triplex composed of a polypyrimidine strand bound in the major groove of a Watson–Crick double helix, so that the two pyr strands are antiparallel. In general, the sequence is limited to $C{\cdot}G{\cdot}C^+$ and T·A·T base triads; however, several studies have indicated that some mismatches are permitted (Griffin & Dervan, 1990; Macaya et al., 1991). The requirement for protonation of cytosine residues accounts for the acidic pH dependence of this structure (Lee et al., 1979), and it has since been verified experimentally by NMR (Rajagopal & Feigon, 1989). In the presence of micromolar concentrations of polyamines (minor groove binders) or substitution with m^5C, triplex formation in synthetic pur·pyr DNAs can be observed at neutral pH (Hampel et al., 1991; Lee et al., 1984). The ability of polyamines to promote triplex formation is particularly interesting since the concentration of spermine in the nucleus is in the millimolar range and is known to be involved in DNA condensation (Sarhan & Seiler, 1989; Hougaard et al., 1987). Furthermore, pur·pyr tracts are abundant in eukaryotes, making up as much as 1% of eukaryotic genomes (Manor et al., 1988).

pur·pyr mirror-sequence repeats in plasmids can also form protonated triplex structures with the aid of negative supercoiling, where the sequence folds back on itself to form a hinge (Lyamichev et al., 1986; Htun & Dahlberg, 1988, 1989). This type of triple helix, known as H-DNA, has been suggested to be involved in gene regulation, since many genes have potential H-DNA-forming sequences in their regulatory regions (Larson & Weintraub, 1982). Indeed, a role for such a triplex in the regulation of hemoglobin gene utilization has been reported (Ulrich et al., 1992). Short stretches of non-pur·pyr DNA (< 20 base pairs (bp)) between pur·pyr tracts

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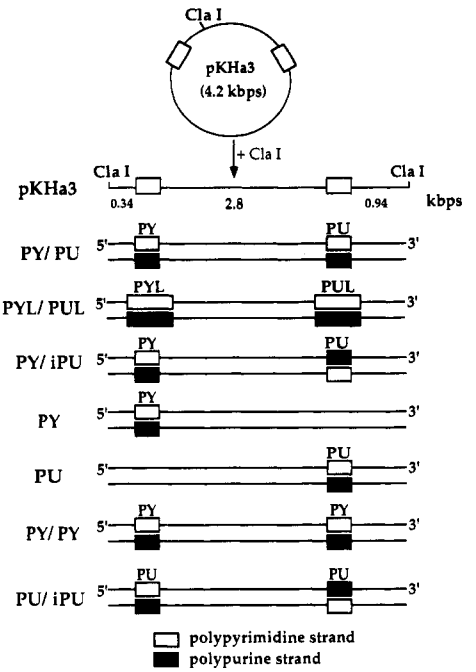
have been shown to become single-stranded upon the formation of an H-DNA (Shimizu et al., 1989). Longer stretches between the tracts can be accommodated if the loop of non-pur·pyr DNA assumes a cruciform structure, which presumably brings the pur·pyr tracts into close proximity without requiring a sharp bend in the DNA (Klysik, 1992). Loops of this type may be important in local gene regulation, but they are unlikely to be involved in long-range chromosome structure.

Another possibility has been suggested where a triplex is formed between two distant pur·pyr sequences to form a large double-stranded loop (Lee et al., 1984, 1987; Burkholder et al., 1988). Such a loop, in principle, could be many kilobases (kb) in length and represent a distinct level of chromosome folding. Evidence for triplex formation in intact chromosomes has come from immunofluorescent studies with triplex-specific monoclonal antibodies (Burkholder et al., 1988, 1991; Agazie et al., 1994). As well, folding events in yeast *Saccharomyces cerevisiae* chromosomes have been observed at low pH using pulsed-field gel electrophoresis (Hampel & Lee, 1993). We have recently demonstrated that two independent pur·pyr tracts positioned at either end of linear plasmids can form a circle mediated by triplex formation (Hampel et al., 1993). In this case, plectonemic problems were minimized because both tracts were at the ends of the linear duplex and, therefore, unwinding could occur during triplex formation. A much better model for loop formation in chromosomes would require that both tracts be embedded in a sea of duplex; then triplex formation between the tracts would result in an Ω circle or loop (see Figure 2). In the present work, linear plasmids were constructed containing two pyrimidine tracts that were 0.34 and 0.94 kb from either end and were separated by 2.8 kb. These plasmids do indeed form Ω loops as well as dimers and, surprisingly, both structures can form a braided or hydrogen-bonded knot.



Plasmid Inserts

PY 5'-CCC(CTTCCT)$_6$ GGG-3'
3'-GGG(GAAGGA)$_6$ CCC-5'

PYL 5'-CCC(CTTCCT)$_{11}$CTTCCGGGG-3'
3'-GGG(GAAGGA)$_{11}$GAAGGCCCC-5'

PU 5'-CCC(GAAGGA)$_6$ GGG-3'
3'-GGG(CTTCCT)$_6$ CCC-5'

PUL 5'-CCC(GAAGGA)$_{12}$ GGG-3'
3'-GGG(CTTCCT)$_{12}$CCC-5'

Oligomers

PYa (L) 5'-(CTTCCT)$_{5/12}$-3'
PUa (L) 5'-(AGGAAG)$_{5/12}$-3'
PYb (L) 5'-(TCCTTC)$_{5/12}$-3'
PUb (L) 5'-(GAAGGA)$_{5/12}$-3'
(L) = /12

FIGURE 1: pKHa3 series of plasmids containing a unique *Cla*I site and sequences of the polypurine·polypyrimidine tracts. It should be noted that the PY tract can only form a triplex (having antiparallel pyrimidine strands) with the PU tract. For the single-stranded oligomers, PYa and PUa are found in the PY duplex and PYb and PUb are found in the PU duplex.

MATERIALS AND METHODS

Plasmids. The sequences and plasmids used in this study are shown in Figure 1. Plasmids were grown in JM109 *Escherichia coli*, and all cloning manipulations followed standard techniques (Sambrook et al., 1989). The plasmids were derived from pKHa1 and pKHa2, which were constructed as previously described (Hampel et al., 1993). pKHa3PY/PU and pKHa3PY/iPU were made by cloning the 1.3-kbp *Rsa*II fragment from M13mp19 (Yanisch-Perron et al., 1985) into the blunt-ended *Xba*I sites of pKHa1 and -a2, respectively. This produced plasmids with a unique *Cla*I site within a 1.3-kbp spacer between the two pur·pyr tracts. pKHa3PYL/PUL was produced by cloning the 3PYL d(CCC(AGGAAG)$_{12}$GGGATCT)·d(CTAGAGATCCC(CTTCCT)$_{12}$GGGGTAC) and 3PUL d(CTAGAGATCCC(GAAGGA)$_{12}$GGGCTGCA)·d(GCCC(TCCTTC)$_{12}$GGGATCT) tracts into the *Kpn*I–*Xba*I and *Xba*I–*Pst*I sites of pUC19, respectively. 3PUL and 3PYL were synthesized by the Regional DNA Synthesis Laboratory at the University of Calgary. The 1.3-kbp spacer was then cloned between the pur·pyr tracts, as described above. pKHa3PU/iPU and pKHa3PY/PY were made by replacing the appropriate sequences from pKHa3PY/PU with tracts removed from p3PU and p3PY (Hampel et al., 1993). In all plasmids the 1.3-kbp spacer was in the same orientation, and all constructs were confirmed by dideoxy sequencing (Sanger et al., 1977). A single base change was observed at the end of the tract in 3PYL. pKHa3PU and pKHa3PY were constructed by removing the 3PY and 3PU sequences, respectively, from pKHa3PY/PU by Bal 31 nuclease digestion. Complete removal of the sequence was determined by restriction digest analysis.

DNA Dimer and Loop Formation. Linearized plasmids (1.6 μg in 10 μL, equivalent to 242 μM in base pairs) were incubated for 12 h at room temperature with 50 μM spermine tetrahydrochloride and 1 mM EDTA in buffers at pH 4–8 [25 mM sodium acetate (pH 4–5), 56 mM MES–NaOH (pH 6), 90 mM BES–NaOH (pH 7), and 33 mM HEPES–NaOH (pH 8)]. In experiments with $MgCl_2$ and $ZnCl_2$, EDTA was omitted from the incubation and gel-running buffers. The final ionic strength of these reactions, unless supplemented with NaCl, was 25 mM (Na^+). Oligonucleotides were made up in distilled water and added to the indicated experiments at an oligonucleotide to plasmid ratio of 1:1 or 1:10. Unless stated otherwise, electrophoresis was performed in 1.8% agarose gels at pH 4 [40 mM sodium acetate (pH 4.0) plus 0.1 mM EDTA]. For some gels, a pH of 8 or 9 was also used [40 mM Tris–acetate (pH 8) plus 20 mM sodium acetate, or 30 mM Tris–acetate (pH 9) plus 20 mM sodium acetate]. Gels were run for 7 h at 10 °C (unless stated otherwise) and a voltage of 3.3 V/cm. Buffers were recirculated every hour to prevent excessive acidification of the positive chamber. After electrophoresis, gels were stained with ethidium bromide and photographed under UV light.

P1 Nuclease and Topoisomerase Assays. Plasmids (10 μg) were incubated in a buffer containing 25 mM sodium acetate, (pH 4), 1 mM EDTA, and 50 μM spermine tetrahydrochloride in 100 μL for 12 h. The buffer was exchanged for STE buffer [100 mM NaCl, 10 mM Tris-HCl

(pH 8), and 1 mM EDTA] for P1 nuclease and topoisomerase I experiments and TE buffer for topoisomerase II experiments on a CHROM SPIN-1000 column (Clontech). Individual samples contained 1 μg of plasmid in 12 μL reaction buffer for all assays.

STE buffer was supplemented with 10 mM $MgCl_2$ for P1 nuclease assays. P1 nuclease was added at the indicated amounts (0, 5, 50, and 500 milliunits) in a volume of 1 μL (1 unit hydrolyzes 1 A_{260} unit of yeast tRNA to acid-soluble products in 15 min at 37 °C). The reactions were incubated for 5 min at room temperature and then stopped by the addition of 50 mM EDTA and placed on ice. Samples were loaded on 1.8% (w/v) agarose gels at pH 8 and run as above.

Topoisomerase I reactions were carried out at concentrations of up to 8 units/μg of plasmid in STE buffer and were incubated for 5 h at 37 °C (1 unit relaxes 0.5 μg of supercoiled ΦX174 RF in 30 min at 37 °C). Topoisomerase II reactions consisted of 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL BSA, and 1 mM ATP (1 unit relaxes 0.3 μg of supercoiled pBR322 in 15 min at 30 °C). Up to 40 units of topoisomerase II was added to each reaction; the reactions were incubated for 12 h at 30 °C and stopped with the addition of 50 mM EDTA. In both topoisomerase I and II experiments, the highest concentration of enzyme used resulted in the complete relaxation of supercoiled pKHa3PY/PU. Samples were loaded on 1.8% (w/v) agarose gels at pH 8 and run as above.

Equilibrium Assays. Linearized pKHa3PY/PU (5 μg) was incubated in buffers at pH 4 or 8 [25 mM sodium acetate (pH 4) and 33 mM HEPES (pH 8)] plus 50 μM spermine and 1 mM EDTA for 12 h in a total volume of 50 μL. These samples were run on a gel as above. The gels were stained, and the bands containing the linearized plasmids, not loops or dimers, were excised with a scalpel. The plasmid was electroeluted from the agarose at 7 V/cm for 3 h in 100 μL TE buffer diluted 1:2. The samples of linearized plasmid were removed from the tubing, and the volume was reduced to 50 μL by evaporation at 37 °C under a stream of air. Plasmids were then incubated with 25 mM sodium acetate (pH 4), 1 mM EDTA, and 50 μM spermine tetrahydrochloride for 12 h at room temperature. Incubated samples at pH 4 or 8 or untreated samples were then loaded onto a 1.8% agarose gel and run as above.

RESULTS

Due to the sequence and strand symmetry requirements for pyr·pur·pyr triplexes, plasmid dimer and loop formation can be carefully controlled. For example, our previous experiments showed that dimer formation could occur between two plasmids only if one carried the sequence 3PU and the other carried 3PY (Hampel et al., 1993). Dimer formation was not observed between plasmids that carried identical asymmetric sequences because the two pyrimidine strands must be antiparallel (Rajagopal & Feigon, 1989). Given these rules, the dimer and looped structures that might be formed are shown in Figure 2. Triplex-mediated loop formation should be observed only in plasmids where 3PU and 3PY are positioned 2.8 kbp apart (pKHa3PY/PU, pKHa3PYL/PUL, and pKHa3PY/iPU). The plasmids pKHa3PY/PY and pKHa3PU/iPU would not be capable of forming such loops, but they should form dimers with one another. Agarose gel electrophoresis was used to separate these structures. It was found by trial and error that, at a gel concentration of 1.8%, all acidic pH-dependent forms of the plasmids had mobilities distinct from those of linear plasmids.

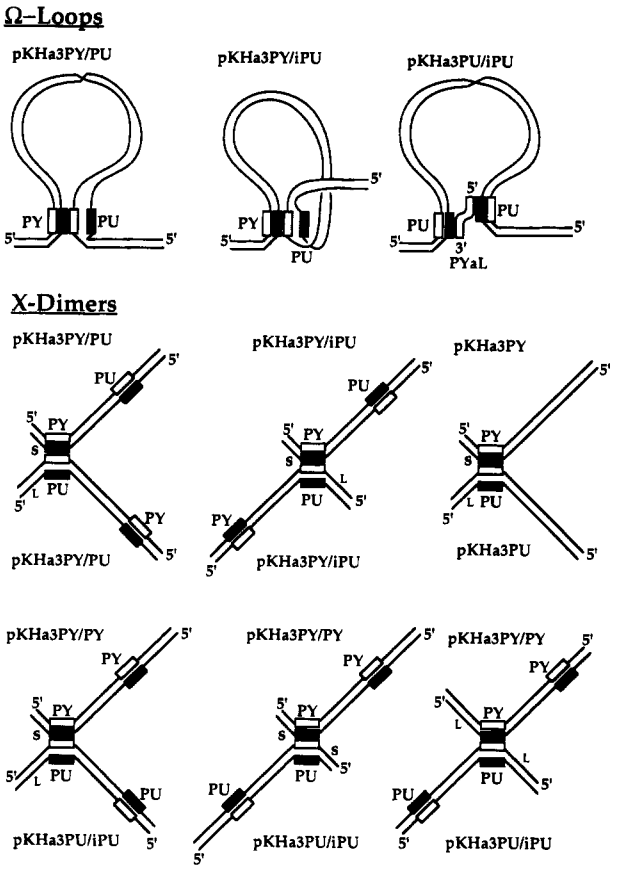


FIGURE 2: Diagrams of potential structures formed from the linear plasmids by interactions between the pyr·pur tracts. Open and filled boxes represent the pyrimidine and purine strands, respectively. Note that three different X dimers are possible, with pKHa3PY/PY plus pKHa3PU/iPU reflecting the different lengths of the arms. A tethered loop is also possible by adding a homologous pyrimidine to pKHa3PU/iPU.

Figure 3A illustrates that pKHa3PY/PU and pKHa3PYL/PUL are converted to a series of structures at pH 4 that any of the other four plasmids alone do not form (Figure 3B). Thus, both 3PU and 3PY sequences are required to be present in the same plasmid for these structures to form. As shown previously (Hampel et al., 1993), the slower moving bands are dimers and multimers. However, the fastest band from pKHa3PY/PU (lane 3) and pKHa3PYL/PUL (lane 5) cannot be a simple dimer since its mobility is faster than that of the open circle. This observation is only consistent with the formation of an intramolecular structure, which requires both the 3PU and 3PY sequences, and is, therefore, designated as an Ω loop. It should also be noted that the mobility of this loop does not correspond to those of any of the dimer bands produced by combining pKHa3PU and pKHa3PY (lane 7) or pKHa3PU/iPU and pKHa3PY/PY (lane 9). The multitude of bands in the latter case (lane 9) is consistent with the fact that the combination of pKHa3PU/iPU and pKHa3PY/PY can produce three X-shaped dimers with arms of different lengths (see Figure 2). Some of the slowest bands, which are found in lanes 3, 5, and 9 but not in lane 7, may also correspond to Ω dimers that require two tracts on each plasmid. It is also clear that having two tracts capable of triplex formation in one plasmid favors dimers (compare lane 9 with lane 7). The amount of dimer in lane 7 is comparable to that in our earlier work with related plasmids (Hampel et al., 1993).

It is known that two open circles containing a pur·pyr tract can be linked or tethered together via triplex formation by the

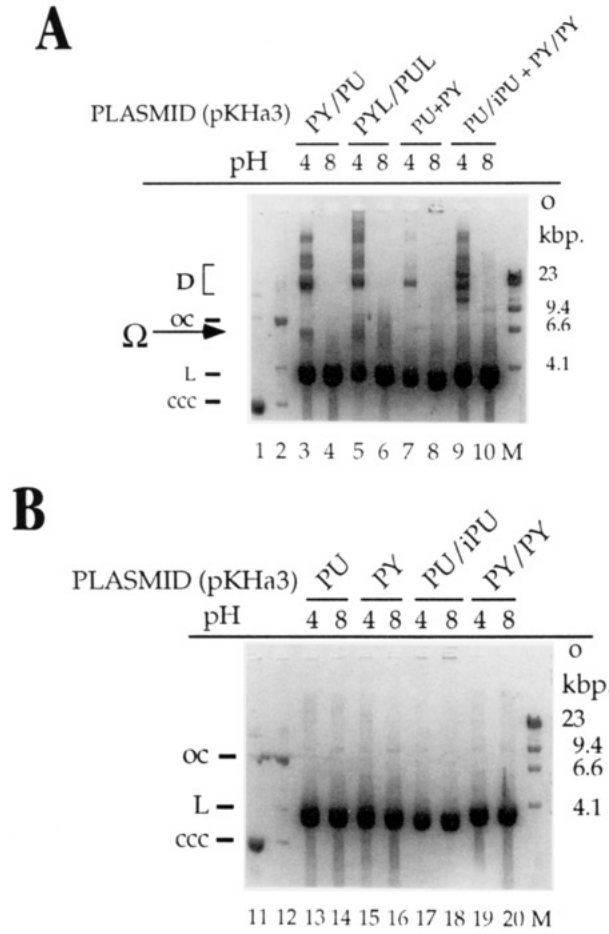


FIGURE 3: Plasmid dimer and loop formation at pH 4. *Cla*I-linearized plasmids were incubated as described under Materials and Methods, run on a 1.8% agarose gel at pH 4, and then stained with ethidium bromide. Covalently closed circular (ccc), open circular (oc), and linear (L) forms of the plasmids are indicated. As well, pH-induced dimer and loop forms are indicated by D and Ω, respectively. The origin of the gel is indicated by O. The marker lane (M) contains phage λ DNA cut by *Hin*dIII. (A) Plasmids or mixtures that do form dimers and/or loops at pH 4; (B) controls that do not form dimers and/or loops at pH 4.

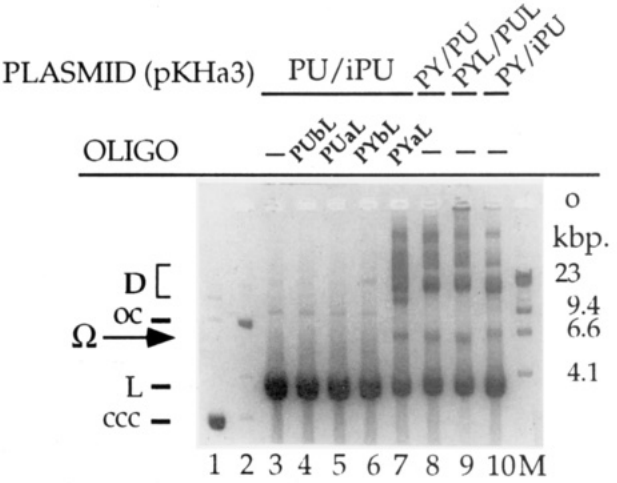


FIGURE 4: Positive control for plasmid looping. *Cla*I-digested pKHa3PU/iPU was incubated at pH 5 in the presence (lanes 4–7) or absence (lane 3) of purine (pur) or pyrimidine (pyr) rich oligonucleotides of either the a or b type sequence. *Cla*I-digested pKHa3PY/PU, pKHa3PYL/PUL, and pKHa3PY/iPU (lanes 8–10) were incubated at pH 4, as in Figure 3.

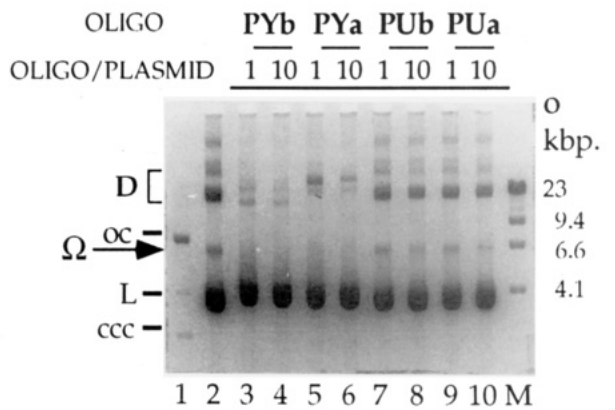


FIGURE 5: Inhibition of plasmid loop formation by blocking pur·pyr tracts. *Cla*I-linearized pKHa3PY/PU was incubated at pH 4 in the presence (lanes 3–10) or absence (lane 2) of added oligonucleotides at the ratios of oligo to plasmid shown.

addition of a homologous single-stranded pyrimidine (Lee et al., 1989; Lyamichev et al., 1988). Thus, it was expected that an Ω loop could also be formed by tethering two tracts in the same plasmid. Figure 4 shows that, in the presence of the PYaL pyrimidine strand at pH 5, pKHa3PU/iPU (lane 7) dismutates to several different structures, including a band that comigrates with the Ω loop formed by pKHa3PY/PU, pKHa3PYL/PUL, and pKHa3PY/iPU (lanes 8–10). This band does not appear if pKHa3PU/iPU is incubated under the same conditions with the other three oligomers. Thus, Ω loops can be formed in two different ways, but the mobilities are the same. As well, inversion of one of the tracts in pKHa3PY/PU to give pKHa3PY/iPU gives an Ω loop with a similar mobility, although the geometry at the site of triplex formation must be different (see Figure 2). Intramolecular triplexes that contain two antiparallel purine strands have also been observed *in vitro* and *in situ* (Kohwi & Kohwi-Shigematsu, 1988; Kohwi, 1992). This class of triplex is not pH-dependent, but requires high concentrations of divalent cations and negative supercoiling. We have not observed dimer or loop formation at neutral pH, even after prolonged exposure of pKHa3PYL/PUL in the presence of 10 mM $MgCl_2$ (data not shown).

Further evidence that both the 3PU and 3PY tracts were directly involved in the Ω loop was acquired by blocking these sequences and examining the distribution of plasmids on agarose gels (Figure 5). Oligonucleotides capable of triplex formation with the pur·pyr tracts in the plasmid were added to the incubation at pH 4. Both pyrimidine oligonucleotides (either the 3PU or 3PY tract) were able to block loop formation in pKHa3PY/PU (lanes 3–6), but the purine oligomers had little effect (lanes 7–10). This result demonstrates that both pur·pyr tracts are required for the Ω loop structure to form. As well, since purine oligomers are ineffective, the involvement of pyr·pur·pur triplexes can be eliminated. This result is interesting in one other respect as well. In contrast to Ω loops, dimer formation in pKHa3PY/PU is not completely eliminated in the presence of the pyr oligomers. Where the ratio of oligomer to plasmid is 1:1 or 10:1 three bands appear, one of which is identical to the dimer formed by pKHa3PY/PU alone. This is attributed to the potential for the oligomers to tether more than one plasmid together via a triplex. It can be concluded that a triplex formed from three strands is energetically more favorable than a triplex formed from the dismutation of two duplexes.

The effect of the length of the tract on loop and dimer formation is investigated in Figure 6. pKHa3PY/PU and pKHa3PYL/PUL were incubated at pH 4–6 for 12 h in the presence of spermine and run on agarose gels at pH 4. The Ω loop forms in both plasmids at pH 4, but only pKHa3PYL/

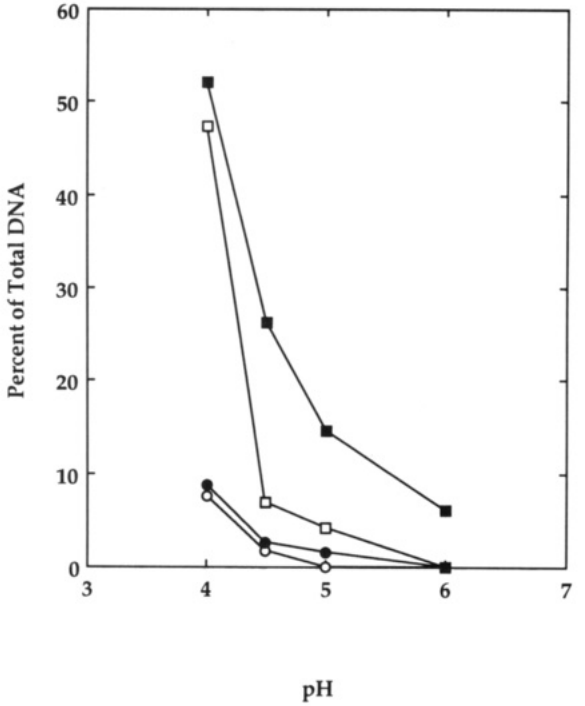


FIGURE 6: Formation of X dimers and loops as a function of pH and the length of the insert. *Cla*I-digested pKHa3PY/PU and pKHa3PYL/PUL were incubated at pH 4–6 and run on a 1.8% agarose gel at pH 4. The percent of each structure was estimated by laser densitometry of photographs of ethidium-stained gels. ■, X dimers from pKHa3PYL/PUL; □, X dimers from pKHa3PY/PU; ●, loops from pKHa3PYL/PUL; ○, loops from pKHa3PY/PU.

PUL forms loops at pH 5. The effect of the length of the pur·pyr insert is also apparent with dimer formation. At pH 6, dimers (but not loops) are able to form from pKHa3PYL/PUL but not pKHa3PY/PU. Therefore, doubling the length of the pur·pyr tract favors triplex-mediated interactions.

One curious feature of dimer and loop formation is that even after 24 h of incubation some linear plasmid remains. Our previous work (Hampel et al., 1993), which was mainly concerned with Y rather than X dimers, suggested that an equilibrium was established between the duplexes and triplex formation. This equilibrium was rapid in the presence of spermine but very slow in its absence (thus allowing the species to be observed on agarose gels) (Hampel et al., 1993). We were concerned that the linear plasmid that remained after extensive incubation at pH 4 might be altered in some way (e.g., a deletion or modification of the tract), so that the conversion to dimers and loops would apparently not go to completion. To test this possibility (Figure 7A), the linear plasmid remaining after extensive incubation at pH 4 can be purified on a gel and reelectrophoresed at pH 8. No dimers or loops are seen in these preparations. However, as shown in Figure 7B, if this same DNA is now reincubated at pH 4, loops and dimers are formed once again (lane 2) to a similar extent as in the control (lane 1). The reverse experiment has also been performed in which excised loops and dimers have been shown to partially reconvert to the linear form at pH 4 (data not shown). This is excellent evidence for an equilibrium process. Another feature of Figure 7B is that the DNA concentration is approximately one-half of that of the other gels, and in this case the loop form is as prominent as the dimers (cf. Figures 3–5). Thus, as expected, intramolecular loop formation is favored at lower plasmid concentrations.

Attempts were made to push the equilibrium to completion by adjusting the incubation conditions. Figure 8 shows the

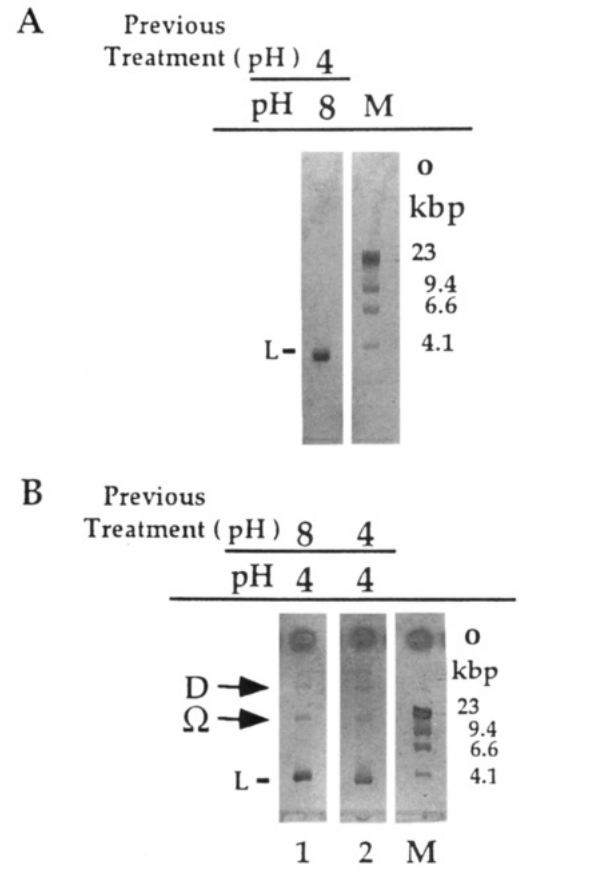


FIGURE 7: Loop and dimer formation is an equilibrium process: (A) linear pKHa3PY/PU DNA that had been cut out of an agarose gel to remove loops and dimers and reelectrophoresed; (B) lane 1, control treated at pH 4, lane 2, linear DNA from A that was treated at pH 4 before being reelectrophoresed.

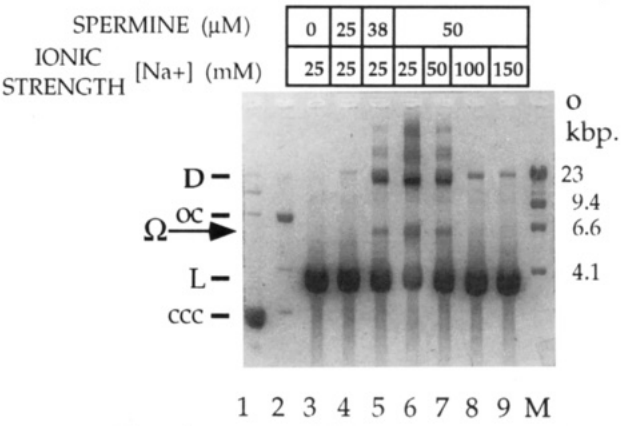


FIGURE 8: Effect of spermine and ionic strength on plasmid loop formation. *Cla*I-digested pKHa3PY/PU was incubated at pH 4 with spermine and NaCl at the indicated concentrations.

formation of dimers and loops from pKHa3PY/PU as a function of spermine concentration and ionic strength. At 50 μM spermine, approximately 70% of the linear plasmids are converted to loops and dimers (see also Figure 6). At higher concentrations of spermine the DNA precipitates, and it becomes impossible to determine the relative amounts of the plasmid forms (data not shown). Divalent cations Mg^{2+} and Zn^{2+} are also able to promote loop and dimer formation at concentrations greater than 5 mM, but the amounts of these forms never exceed 25% of the total plasmid DNA (data not shown). If the ionic strength of the incubation is increased by adding NaCl at the highest concentration of spermine, the amount of dimers and loops decreases dramatically. Thus,

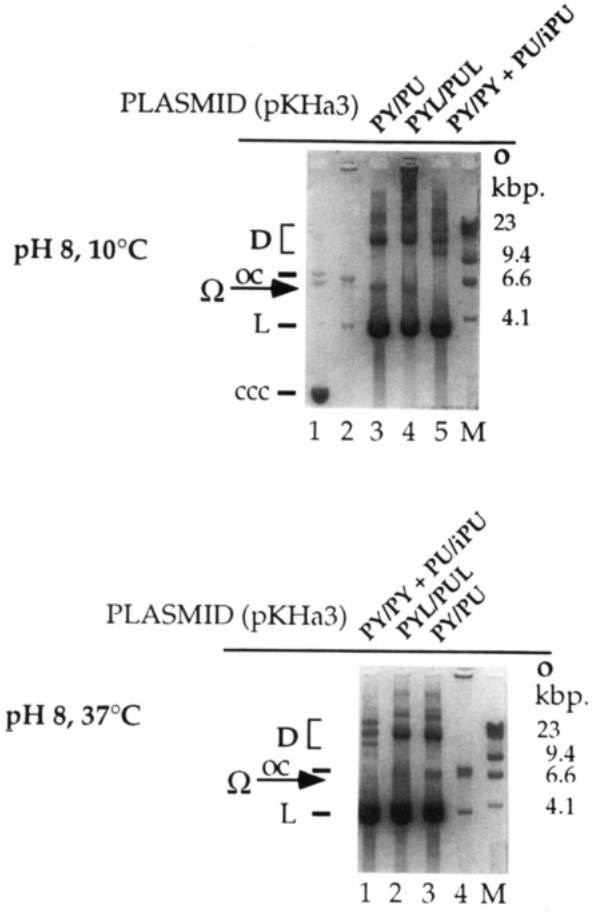


FIGURE 9: *Cla*I-digested pKHa3PY/PU incubated at pH 4 and then run on an agarose gel at pH 8 at either 10 or 37 °C.

X dimers and loops behave in a manner similar to the Y dimers studied previously (Hampel et al., 1993).

pyr·pur·pyr triplexes have also been shown to remain stable at pHs higher than can be used to form them (Lee et al., 1984; Hampel et al., 1991). This manifestation of hysteresis was also tested in the Ω loop model (Figure 9). After incubation at pH 4 in the usual way, the DNA was run on an agarose gel at pH 8. It can be seen that there is no apparent reversion of Ω loops or dimers to the linear form. An identical pattern was observed even after electrophoresis at pH 9 (data not shown). By the use of "spin columns", the original pH 4 buffer could be exchanged rapidly, allowing a variety of other conditions to be investigated. For example, incubation of the Ω loops and dimers at pH 8 at low ionic strength (5 mM) for 24 h at 50 °C had no effect. These results were most unexpected since our previous studies with Y-shaped dimers with the same sequences showed that only a very small fraction remained after electrophoresis at pH 7 and no Y dimers were observed at pH 8 (Hampel et al., 1993). An obvious difference between a Y dimer and an Ω loop or X dimer is that the Y dimer has an end from which plectonemic problems can be disbursed. This suggested that the Ω loop and X dimers might be sensitive to single-strand-specific nucleases. As shown in Figure 10, treatment at pH 8 with increasing concentrations of PI nuclease (lanes 1–4) converts the structures to linear duplexes under conditions where the supercoiled plasmid itself is unaffected (lanes 5–8). On the other hand, incubation with both type I and II topoisomerases under conditions that relaxed supercoiled plasmids led to no increase in the linear form (data not shown).

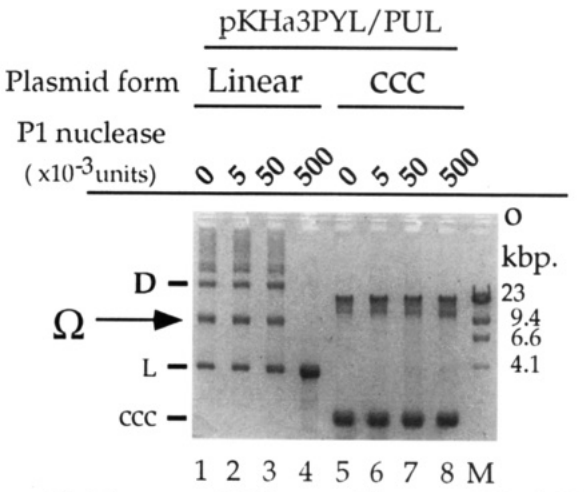


FIGURE 10: Treatment of X dimers and loops at pH 8 with P1 nuclease: lanes 1–4, digestion of pH 4 treated linear plasmid with increasing concentrations of P1 nuclease (0, 5, 50, and 500 milliunits); lanes 5–8, control digestion of supercoiled plasmid with increasing concentrations of P1 nuclease (0, 5, 50, and 500 milliunits). The gel was run at pH 8.

DISCUSSION

We have examined the ability of linear plasmids to form Ω loops, where the base of the loop is held by direct interactions between the separated pyr·pur tracts. In our previous work on this topic, we demonstrated the formation of open circular structures from linear plasmids via an intramolecular triplex if the plasmid carried pur·pyr tracts at either end (Hampel et al., 1993). Ω loop formation does not require free ends and therefore is a more realistic model for how triplexes may be involved in cellular processes such as chromosome condensation or transcriptional control.

The plasmids pKHa3PY/PU, pKHa3PYL/PUL, and pKHa3PY/iPU all formed Ω loops which, as expected, had mobilities slightly faster than those of the open circular forms of the plasmids. If either sequence was not present or both tracts were the same, loops were not observed. X dimers were also formed under these conditions, and at present it is not clear how to favor the formation of Ω loops. The addition of a homologous pyrimidine to a linear plasmid containing two identical tracts also gave rise to a structure with an identical mobility (Figure 4). This tethered loop serves as a good control for the Ω loop.

Blocking of the tracts with a pyrimidine oligomer capable of triplex formation with either tract eliminated loop formation (Figure 5). On the other hand, purine oligomers did not block triplex formation nor did they give rise to tethering. Mixed sequences of the type $d(TTGTGG)_n$ were also tested and were equally ineffective (data not shown). From experiments with short oligonucleotides, it has been suggested that triplexes containing two purine strands or those derived from $d(TG)_n$ sequences can be formed just as readily as the pyr·pur·pyr triplex described here (Pilch et al., 1991). In fact, since these other triplexes do not require a low pH they may be of more relevance physiologically. However, a more recent study by Fossella et al. (1993) demonstrated that not only are T·A·T and C·G·C^+ base triads more stable but they also are more specific than the other 14 combinations. Our results are in agreement, since with the pKHa3 series of plasmids the pyr·pur·pyr triplex predominates and, even under conditions where this triplex will not form (i.e., at neutral pH), the other types are not observed. One explanation is that our pur·pyr tracts are embedded in long duplexes, and triplex formation

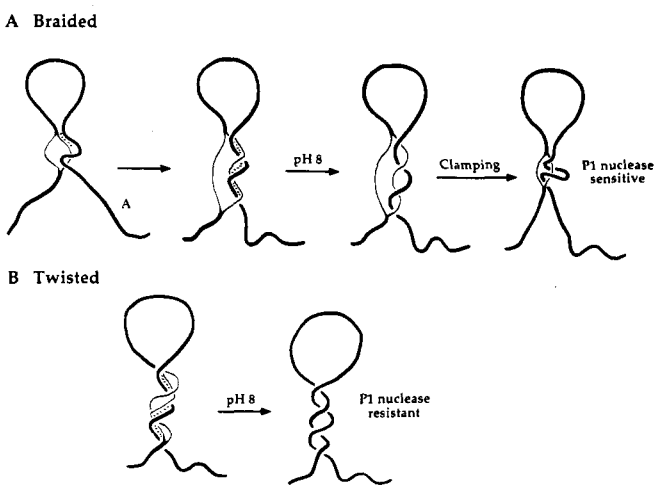


FIGURE 11: (A) Braided and (B) twisted models for X junctions mediated by triplex formation. Thick lines are duplex DNA, and thin lines are single-stranded regions. Triplexes are indicated by dashed lines between duplexes and single-stranded DNA. The end marked A must pass through the bubble once for each turn of the triplex in order to form the braided structure.

might be dependent on the ability of the tracts to find each other. Since the pyrimidine strand of a pyr·pur·pyr triplex is partially protonated, the initial recognition step may be more rapid. Another factor is that pyr·pur·pur triplexes require high Mg^{2+} concentrations, and under these conditions inhibition of breathing of the duplexes may inhibit triplex formation. Formation of the pyr·pur·pyr triplex, on the other hand, occurs under conditions of low pH and low ionic strength, both of which may encourage extensive breathing.

At first sight, it is perhaps suprising that Ω loops can form at all since there are no free ends to allow unwinding. In our experiments, loop formation posed no obvious topological problems since looping was not enhanced by introducing nicks into the linear plasmids (data not shown). However, it is possible to model loop formation of this type without introducing supercoils into the circle. As shown in Figure 11A, formation of the triplex-mediated structures can proceed via a small bubble or a single-stranded region at the top of the tract on the donor duplex. The other pur·pyr tract (the acceptor duplex) can then initiate triplex formation. However, zippering can only occur if one end of the acceptor duplex passes through the bubble once for each turn of triplex (i.e., the duplex tail labeled A in Figure 11A). Thus, although the loop is topologically closed, it does not become supercoiled. A similar mechanism also applies to the formation of X dimers, but in this case supercoiling need not be considered even though the two tracts become interwound. We propose to call this structure a braided knot. In the mathematical sense, it is not a true knot but rather a hydrogen-bonded knot, since theoretically it can be untied without breaking a covalent bond. It is also possible to envisage loop formation in which the two tracts are not interwound (Figure 11B), but here the extruded fourth (purine) strand must also wrap around the triplex without interacting with it.

The production of an interwound knotted triplex gives a simple explanation for the remarkable stability at pH 8. It is not credible to suggest that the triplex remains stable under the harsh conditions that were tested, because even a long synthetic triplex derived from poly[d(TC)]·poly[d(GA)] would revert back to a duplex (i.e., pH 8 at 50 °C for 2 h) (Lee et al., 1984). However, even if the triplex falls apart, the acceptor duplex is still wrapped around the donor pyrimidine strand (Figure 11A). In the absence of triplex formation, repassage of one end of the duplex back through the bubble will be difficult because the donor duplex will reform and clamp down on the acceptor (Figure 11A). Thus, an equilibrium between the Ω loop and the linear form is established at pH 4, but not at pH 8 where the loop is locked in place. This structure can only be resolved by breaking one strand of the donor duplex or completely melting the duplex to the end. This explains the P1 sensitivity at pH 8 since the donor duplex cannot reform an intact helix; part of it must remain single-stranded. A single nick in this region will allow passage of the acceptor duplex, and the structure will resolve into linear duplexes as shown in Figure 10. S1 nuclease at pH 4.5 will also cut the loops and dimers (data not shown), but this result is expected since triplexes are known to be sensitive. The observation of a single-strand-sensitive structure at pH 8, on the other hand, is novel. In contrast, the topology of the twisted form (Figure 11B) would not be altered by nicking. This is the first demonstration of a braided or hydrogen-bonded knot being formed within a single linear duplex or between two linear duplexes. In this sense, the pyr·pur tract in the DNA is acting as an autocatalytic topoisomerase.

The requirement for low pH in the formation of this complex need not be viewed as excluding its relevance *in vivo*. Proteins that bind to a triplex conformation or single-stranded polypurines have been found and may be capable of promoting triplex formation *in vivo* (Collick et al., 1991; Kiyama & Camerini-Otero, 1991). As well, the formation of protonated DNA structures may benefit from documented fluctuations in intracellular pH (Reich et al., 1991). It is noteworthy, therefore, that chromatin aggregation can be promoted by a decrease in pH (Guo & Cole, 1989).

Under standard conditions, topoisomerases apparently have no effect on this structure. In order to relax a supercoiled plasmid, a topoisomerase can bind to any region. To resolve the knot described here, the topoisomerase would have to bind within the structure itself and, thus, much higher concentrations might be needed. *In vivo*, the formation of Ω loops could be regulated by the presence of a topoisomerase or by other nicking–closing activity that was directed to this site. Nicking of the pur·pyr tracts would allow the structure to form more readily in a very long linear chromosome, and sealing that nick once the structure had formed would trap the loop. Removal of the loop could be accomplished by simply nicking the extruded strand from the two duplexes. The matrix to which chromatin loops are anchored contains topoisomerase II, which could provide such nicking and resealing activity (Adachi et al., 1991). Indeed, the condensation and decondensation of eukaryotic chromosomes requires this type II topoisomerase activity, which may in part reflect the involvement of triplex DNA structures in some level of chromosome condensation (Uemura et al., 1987).

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