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Demonstration of remarkable sequence divergence in variants of a complex satellite DNA by molecular cloning

(Recombinant DNA; Bermuda land crab; cloned variants; micro- and macroheterogeneity in closely related DNA sequences)

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SUMMARY

Repeat units of a complex G + C-rich satellite of the Bermuda land crab have been cloned by insertion into either the *Pst*I or *Eco*RI site of pBR322 or the *Eco*RI site of pUC9. While most of the recombinants contained inserts of approx. 2.1 kb, the average size of repeat units seen in cellular satellite digests, several inserts were markedly different in size. Two domains that account for major sequence differences among the satellite variants and that may be 'hotspots' for sequence modification have been subcloned to permit characterization of their secondary and tertiary structures independent of the influence of the other unusual sequences present. One of these domains is striking in its content of simple repeats; one strand is highly biased in pyrimidines which may permit the formation of unusual secondary and/or tertiary conformations. The other subcloned domain is rich in Pu/Py; preliminary data indicate a transition from B→Z DNA in this region.

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Abbreviations: Ap, ampicillin; ARU, cloned satellite of average repeat unit length (2.07 ± 0.1 kb); BAP, bacterial alkaline phosphatase; Bluo-Gal, formula unavailable from manufacturer, related to XGal; bp, base pair(s); BSA, bovine serum albumin; CIAP, calf intestinal alkaline phosphatase; Cm, chloramphenicol; DIM, insert of cloned satellite dimer (4.14 kb); DTT, dithiothreitol; EtBr, ethidium bromide; EXT, insert of cloned extended satellite variant (2639 bp); IPTG, isopropyl-β-D-thiogalactopyranoside; kb, 1000 bp; LB, Luria–Bertani medium; media and buffers, ACH, LM, TAE, TBE, 2 × YT, see MATERIALS AND METHODS, sections **b** and **c**; $-\sigma$, negative superhelical density; nomenclature of clones: p, plasmid, pGl, primary clones of *Gecarcinus lateralis* satellite repeat units inserted into the *Eco*RI (pGlE$_n$) or *Pst*I (pGlP$_n$) site of pBR322; pGlR$_n$, reclones; pGlUC$_n$, pUC9 recombinants; n, clone number; pGlE15 plasmid containing satellite insert RU (an ARU that has been sequenced, 2089 bp); pT463-I, -II, subclones of satellite Pu:Py segments; pZE15, subclone of Pu/Py segment; PA, polyacrylamide; Pu:Py, tracts consisting of purines on one strand, pyrimidines on the other; Pu/Py, alternating purines and pyrimidines; ^{R}, resistance; ^{S}, sensitivity; SC, supercoiled; Tc, tetracycline; TRU, insert of truncated cloned satellite variant (1674 bp).

INTRODUCTION

A G + C-rich (63% G + C) satellite that accounts for 3% of the DNA of the Bermuda land crab *Gecarcinus lateralis* is complex; the average repeat unit of the 16000 copies/genome is 2.07 ± 0.1 kb (LaMarca et al., 1981; Fowler et al., 1985). Restric-

tion mapping of a highly conserved 380-bp *Mbo*I fragment from cellular satellite revealed that approx. 70% of the population of fragments contained sites for eight restriction enzymes in apparently identical positions, while the other 30% had several distinctly different arrangements (Skinner et al., 1982).

Some domains of the satellite are highly conserved while others are not. The latter have unusual sequences; two have been subcloned. One of them is very biased in pyrimidines; in one of the sequenced variants, there is a stretch of 194 bp of which 86% are pyrimidines on one strand. The other domain is very rich in Pu/Py. These two subcloned domains provide model systems for the study of the influence of primary structure on secondary and tertiary structure as well as the influence of specific sequences on the mutability of primary structure (Bonnewell et al., 1983; R.F. Fowler and D.M. Skinner, unpublished).

To obtain pure samples of individual repeat units for sequencing, we cloned the satellite; the presence of unique sites for *Pst*I and *Eco*RI in most of the repeat units facilitated these experiments.

MATERIALS AND METHODS

(a) Cloning strategies and procedures

DNA was extracted from crab tissues and satellite was purified by centrifugation through three sequential CsCl gradients. A shotgun approach was used for the insertion of satellite into the *Eco*RI site of pBR322 and pUC9. Both vector and insert DNAs were digested with *Eco*RI, the vector was subsequently treated with BAP (BRL) or CIAP (Boehringer) and satellite and vector were ligated (ligase, New England BioLabs) in the buffer specified by the manufacturer except that ATP was 4 ×; in some ligations, DDT and ATP were 0.5 × and BSA was omitted. Alternatively, vector and cellular satellite DNA were *Pst*I digested, phosphatase-treated, and gel-purified.

BAP-treated pBR322 was ligated to satellite insert at a molar ratio (insert: vector) of 0.42:1. After incubation for 16 h at 12°C, the ligation products were used to transform $CaCl_2$-treated *Escherichia coli* HB101 (Maniatis et al., 1982). Transformants were selected on LB agar, pH 7.5, plus 10 μg Tc/ml (pGlP clones; for nomenclature, see Abbreviations) or Tc and 20 μg Ap/ml (pGlE clones). Two fragments containing domains of unusual sequences were subcloned by insertion into the *Cla*I site of pBR322. The molar ratio of fragment:vector was 1:1. Transformants selected on LB agar plus Ap were screened for Tc sensitivity.

Satellite insert was ligated to CIAP-treated pUC9 at a molar ratio of 4:1. After incubation for 18 h at 12°C, 1.5 ng of the DNA was added to 0.2 ml of competent JM109 cells (Yanisch-Perron et al., 1985). Competent cells were prepared (Hanahan, 1983) starting with an isolate picked from a minimal medium plate. Transformants (pGlUC clones) were selected on LM agar (1% tryptone, 0.5% yeast extract, 10 mM NaCl, 10 mM $MgSO_4$) plus 25 μg Ap/ml and screened on LM agar plus Ap, 0.4% Bluo-Gal and 40 μM IPTG (Messing, 1983); approx. 800 transformants/ng of DNA were obtained.

(b) Recombinant plasmid preparations

All pGlUC recombinants were grown in 2 × YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl, pH 7.5); other mini-preparations were grown in LB. Plasmid DNA was prepared according to Birnboim and Doly (1979) or Holmes and Quigley (1981). Cultures of 1–300 liters were grown in ACH medium (0.2% glucose, 0.5% Casamino acids, 3% cysteine, 1 × Vogel-Bonner salts, 1 μg thiamine/ml, 10 μg tryptophan/ml), supplemented with the appropriate antibiotic(s); plasmid DNA was amplified for 18 h in the presence of 200 μg Cm/ml. Amplification was enhanced by the presence of 1 mg uridine/ml. Plasmid DNAs were prepared by the method of Clewell and Helinski (1969) followed by two centrifugations in CsCl/EtBr.

(c) Restriction analyses and Southern blots

Restriction enzymes (Boehringer, BRL, Miles, New England BioLabs) were used according to the vendor's specifications. Since *Hpa*II and its isoschizomer, *Msp*I, gave similar results, the two were used interchangeably; both enzymes are referred to in the text as *Hpa*II. *Dpn*I or *Sau*3A, isoschizomers of *Mbo*I, were used instead of *Mbo*I for digestions of satellite DNA cloned in HB101 in which *Mbo*I sites are methylated.

Intact or restricted DNAs were electrophoresed on agarose gels in TAE (40 mM Tris · HCl, pH 7.8, 5 mM Na · acetate, 1 mM EDTA) or PA gels in TBE (90 mM Tris · borate, pH 8.2, 2.5 mM EDTA). Some gels were transferred to nitrocellulose filters (Southern, 1975) and probed with nick-translated cellular or cloned satellite DNAs; DNA was nick-translated with [α-^{32}P]dATP and [α-^{32}P]dCTP using DNA polymerase I. Radioactive gels and Southern blots were exposed to Kodak XAR-5 film.

RESULTS AND DISCUSSION

(a) Satellite repeat units inserted into the *Eco*RI or *Pst*I site of pBR322 or the *Eco*RI site of pUC9

Tc^RAp^S (pGIP clones) and Tc^RAp^R (pGIE clones) were screened (Grunstein and Hogness, 1975) with nick-translated cellular satellite as probe; those that hybridized were further screened by agarose gel electrophoresis. Ap^R pGIUC clones were screened directly (see MATERIALS AND METHODS, section **a**); Lac^- transformants were characterized further. 126 pGIP clones, 1050 pGIUC clones, and 16 pGIE clones were identified. Although most of the clones were ARUs, each of those analysed to date differs from all others in at least 1 bp. Representative pGIE clones are shown in Fig. 1.

Among the pGIE clones (Fig. 1A), three inserts, DIM, which lacks an *Eco*RI site, EXT, and TRU, are distinctly different in size from RU. Nick-translated cellular satellite hybridized to all the cloned pGIE inserts (not shown). Similarly, probe made using another ARU insert (lane 8) hybridized to itself and to all other satellite inserts as well as to undigested pGIE DNA (Fig. 1B), indicative of homology among copies of the satellite. Specificity of hybridization is shown by the absence of radioactive bands in lane 11, which contains a *Hpa*II digest of unlabeled ϕX174 marker DNA.

Although the satellite has been evolutionarily conserved among the Crustaceae, with homology inversely related to taxonomic distance (Graham and Skinner, 1973), evidence for microheterogeneity is accumulating. For example, although most of the pGIE inserts have a single *Pst*I site near their 3′ ends, TRU does not; it is also missing the segment of

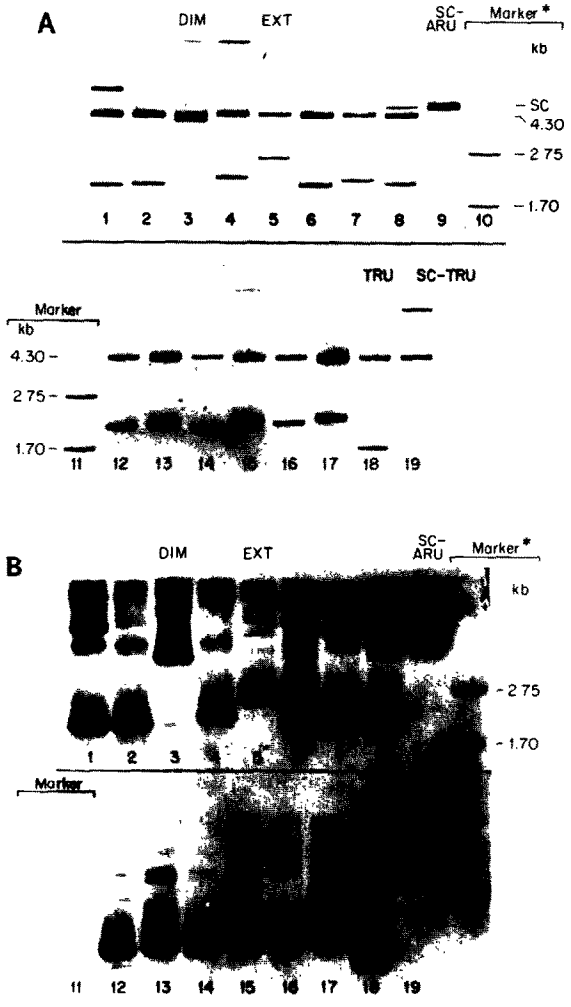

Fig. 1. Recombinant plasmids constructed from *Eco*RI-digested cellular satellite and pBR322. (A) Plasmid preparations of 15 of 16 pGIE clones digested with *Eco*RI, electrophoresed through 1.5% agarose gels in TAE, and stained with EtBr. ARUs are seen in lanes 1, 2, 4, 6–8, 12–17; lane 3, DIM; lane 5, EXT; lane 9, recombinant plasmid in lane 8, undigested; lanes 18 (digested) and 19 (undigested) TRU. Marker DNAs: lane 10, mixture of labeled (*) and unlabeled ϕX174 digested with *Hpa*II; lane 11, as in lane 10 but unlabeled DNA only; 4.3 kb, linear pBR322. (B) Autoradiograms of Southern (1975) blots of gels in (A) hybridized to the nick-translated satellite insert shown in lane 8.

DNA that contains one of two *Mbo*I sites present in most inserts (Fig. 2). In ARUs, one *Mbo*I site is 140 bp from the 5′ end, the other is 240 bp from the 3′ end; *Mbo*I digests of cellular satellite contain 380 bp (140 + 240 bp) fragments which are highly homologous (Skinner et al., 1982). Approx. 50% of the pGIE clones, including EXT, contain a third *Mbo*I site 0.47 kb from the 3′ end. The additional ca. 700 bp in the EXT variant is accounted for by a fivefold

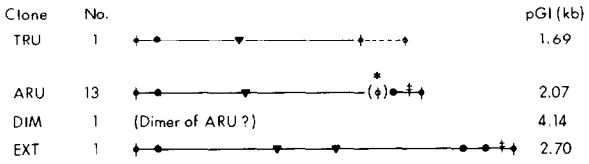

Fig. 2. Diagram of diagnostic restriction enzyme sites in the satellite inserts of 16 pGlE clones. ●, *Eco*RI site; (Φ), position in ARU of 5/6 of an extra *Eco*RI site that truncates TRU, homologous position in EXT not shown; ●, *Mbo*I site; ▼, *Hind*III site; ‡, *Pst*I site; ---Φ, truncated 3′ end of TRU terminated by a postulated *Eco*RI site.

amplification of a 142-bp segment that is 83% homologous to a segment in RU (127 bp) and TRU (130 bp). The two *Hin*dIII sites in EXT (Fig. 2) bracket three tandem copies of the amplified DNA segment (Bonnewell et al., 1983). All other pGlE clones examined have a single *Hin*dIII site approx. 0.8 kb from the 5′ end.

Inserts from the thirteen ARU recovered from the pGlE clones were purified, digested, and electrophoresed on gels; scans of five typical digests are shown (Fig. 3B). Fragments 5 (not seen in these gels), 1, and 7 are all highly conserved among all of the satellite inserts. However, all inserts differ from each other as indicated by the distinct differences in sizes of the other fragments.

Restriction enzyme digests of crude preparations of 126 pGlP clones and 34 pGlUC clones give further evidence of sequence heterogeneity among copies of the satellite. Within each insert there is at least one differently sized fragment from those found in other repeat units (Fig. 3A). It seems unlikely that salts and proteins in the crude plasmid preparations alter the mobility of the restriction fragments since the migration of the pUC9 fragments is constant in all preparations.

Evidence that recombination in the bacterial host or cloning accidents were not the cause of the diversity of the cloned satellite inserts was obtained by recloning a satellite repeat unit. RU excised from pGlE15 was isolated from agarose gels, religated to pBR322 and used to transform *E. coli* HB101. Two recombinants as well as a sample of the original pGlE15 were digested with several restriction enzymes; digests of the three were indistinguishable (Fig. 4). In addition, a number of preparations of RU, TRU, and EXT, as well as other cloned satellite repeat units that have been sequenced or restriction

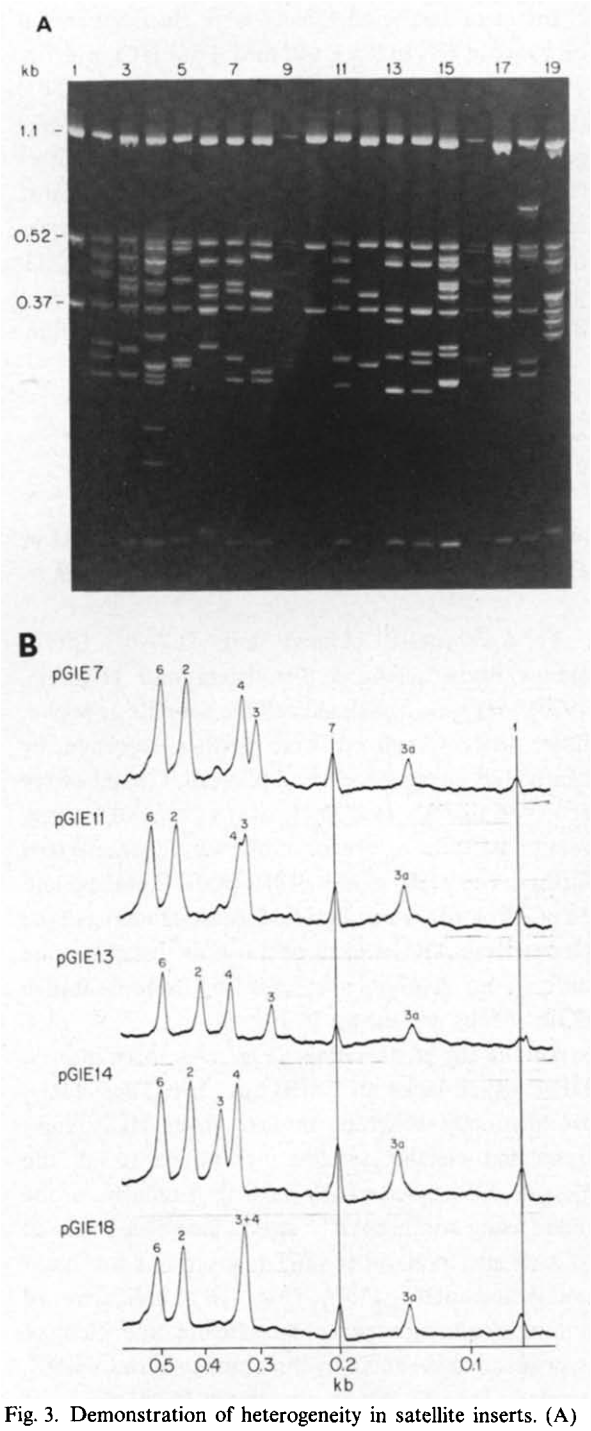

Fig. 3. Demonstration of heterogeneity in satellite inserts. (A) Plasmid DNAs (Holmes and Quigley, 1981) of 17 pGlUC clones were digested with *Hin*fI, electrophoresed on a 7% PA gel and stained with EtBr. Marker DNA, lanes 1 and 10, pUC9 digested with *Hin*fI. (B) Densitometer scans of a gel of purified inserts from five pGlE clones treated as in (A). Very highly conserved *Hin*fI fragments are indicated by vertical lines. Numbers were assigned on the basis of restriction mapping by partial digestion (Smith and Birnstiel, 1976); *Hin*fI digested RU was used as a reference for numbering the fragments (Fowler et al., 1985).

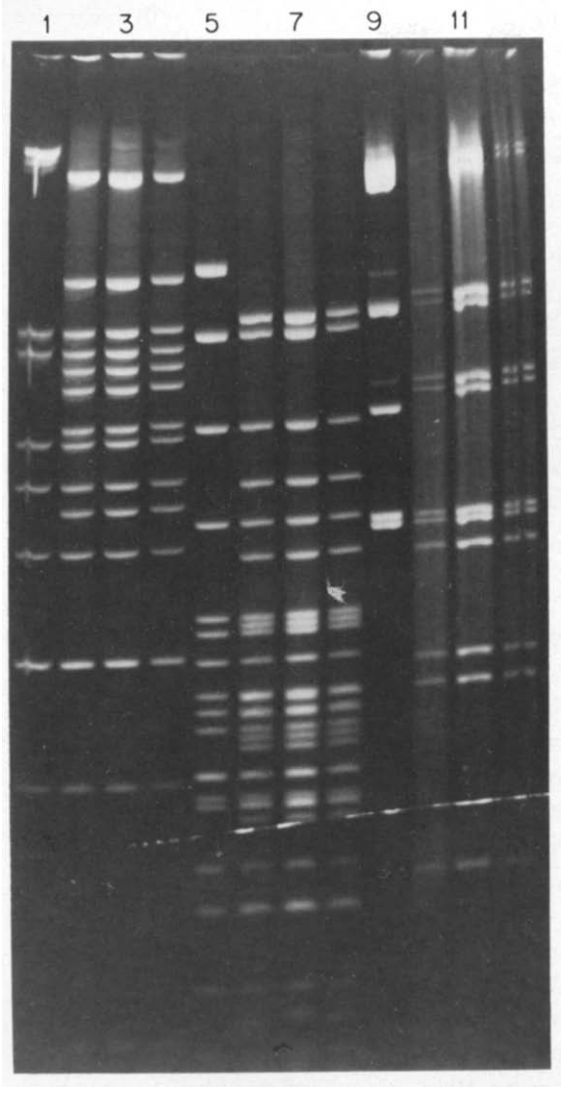

Fig. 4. Identity of restriction fragments in recombinant plasmids containing RU (pGlE15) and recloned RU (pGlR19 and pGlR32). Plasmid DNA was purified, digested, and treated as in Fig. 3A. The restriction enzymes used were: lanes 1–4, *Hin*fI; lanes 5–8, *Hpa*II; lanes 9–12, *Taq*I. Lanes 1, 5, 9, pBR322; lanes 4, 8, 12, pGlE15; lanes 2, 6, 10, pGlR19; lanes 3, 7, 11, pGlR32.

mapped have maintained their constancy following repeated culturing. Finally, the sequences of subclones of two divergent regions containing segments of either Pu:Py (pT463) or Pu/Py (pZE15) were identical to those of the original fragments isolated from the parent satellite insert.

(b) Truncation of satellite repeat units

TRU is truncated by approx. 360 bp at an EcoRI site (Fig. 2); five out of six bases of an *Eco*RI site (CAATTC and AAATTC) are present approx. 360 bp from the 3′ end in RU and EXT, respectively. This region in EXT and RU was sequenced by several strategies to verify that the odd residue was different in both variants. In *Eco*RI-digested, end-labeled preparations of cellular satellite, two discrete populations of fragments (approx. 330 and 360 bp) were seen on autoradiograms (Fowler et al., 1985). The sizes and restriction maps of these fragments are similar to those expected for ends of TRU and other truncated satellite variants that might have originated from an ARU suffering a point mutation that resulted in an additional *Eco*RI site.

(c) Subclone of a repeating Pu:Py domain

Tracts of dG:dC adopt unusual secondary or tertiary conformations under torsional stress (Cantor and Efstratiadis, 1984; Evans et al., 1984; Fowler et al., 1985); they have also been found upstream from active genes (Larsen and Weintraub, 1982; Mace et al., 1983).

A region of RU that contains $(dG:dC)_{22}$ embedded in base-compositionally biased repeating sequences has undergone a 108-bp deletion from EXT and major modifications in TRU. The complete sequence of the region which contains the tract is $[(\text{“CCT”})_{27}CC(CCT)_{15}CTTAAC_3TC_{22}(CGCAC)_5$ $(\text{“CGCAC”})_2]$; sequences related to the sequences listed in parentheses are indicated by quotation marks (Fowler et al., 1985). To study the secondary and tertiary structures associated with Pu:Py independent of the influence of other unusual sequences present in the satellite, the region was purified as a 463 bp fragment from a *Taq*I digest of RU, inserted into the *Cla*I site of pBR322, and cloned. Six recombinants were recovered, pT463-I through VI. pT463-I had the satellite fragment inserted as written above; the other five, of which pT463-II has been studied as a representative, had the fragment inserted in the opposite orientation (Fig. 5).

In native negatively supercoiled pT463-I and pT463-II as well as pGlE15, the repetitive region is sensitive to S1 nuclease. The distribution of S1 nicks suggests that the segment adopts an altered conformation spanning about 100 bp. S1 sensitivity is absent from linearized plasmids, indicating that unusual structures are induced under torsional stress (Fowler et al., 1985). Experiments in progress ex-

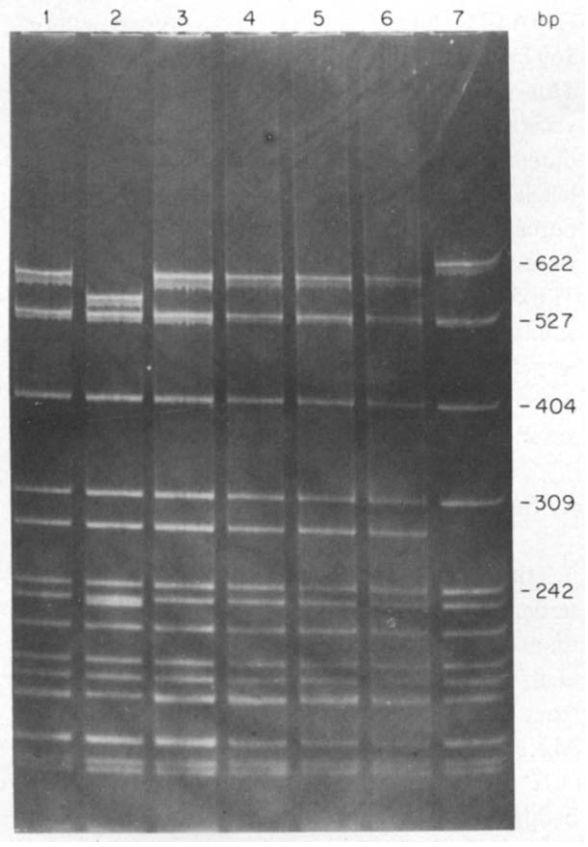

Fig. 5. Recombinant plasmids of an unusual segment of the satellite that contains [("CCT")$_2$7CC(CCT)$_{15}$CTTAAC$_3$TC$_{22}$-(CGCAC)$_5$("CGCAC")$_2$]. The six subclones were digested with *Hpa*II and electrophoresed as in Fig. 3A. Lane 2, one of the six recombinant plasmids containing the fragment of interest inserted as written above. Lanes 1, 3–6, plasmids with the satellite fragment inserted in the reverse direction. Marker DNA, lane 7, pBR322 digested with *Hpa*II.

plore the effects of pH, $-\sigma$, and ionic strength on S1 nicking patterns. The marked sequence divergence among RU, TRU, and EXT in this and other domains sharing similar structural features suggests that such altered conformations may provide specific sites for recombination between repeat units (Fowler et al., 1985).

(d) Subclone of a Pu/Py-rich, potential Z-form region

A tract of $(CA)_{31}$ interrupted by one TA doublet 300 bp 3′ to the K2 V-region of the mouse κ immunoglobulin gene (Nishioka and Leder, 1980) forms Z-DNA when inserted into pBR322 and maintained under negative superhelical stress (Singleton et al., 1984). Long tracts of $(CA)_n$; (n = 30) have also been found near the mouse κ immunoglobulin gene (Gebhard and Zachau, 1983). Interrupted stretches of Pu/Py can still adopt the Z-conformation, although somewhat higher $-\sigma$ are required to drive the transition (Azorin et al., 1983; Singleton et al., 1983). RU, TRU, and EXT contain localized domains that are unusually rich in Pu/Py (Fowler et al., 1985) including Pu/Py of heterogeneous base composition as well as homocopolymer segments such as $(CA)_n$.

Among the three variants, RU contains the longest stretch of Pu/Py (53 bp) of which 48 bp are in alternation with an occasional interruption; several of the Pu/Py blocks are out of phase. This Pu/Py region is absent from TRU since it is located downstream from the extra *Eco*RI site but it is present in the 330 and 360 bp *Eco*RI fragments that appear to be the 3′ ends of truncated satellite repeat units. Since approx. 10 bp of Pu/Py is sufficient to permit Z formation (Singleton et al., 1983), there are at least two potential Z-form sites within this region in RU (9 and 15 bp) but only one in EXT because of a 12-bp deletion (the length of a single helical turn of Z-DNA; Dickerson et al., 1982). It is noteworthy that, except for two nucleotide substitutions that border the deletion in EXT, this segment along with 20 bp upstream and 30 bp downstream has been 100% conserved in RU and EXT (Fowler et al., 1985). The segment also contains several inverted repeats that would permit the formation of cruciform structures (Fowler and Skinner, 1985) in equilibrium with Z-DNA.

The Pu/Py-rich region of RU was recovered in a 171-bp *Msp*I fragment, ligated to pBR322 at the *Cla*I site, and cloned in HB101. Since the Pu/Py-rich domain contains a conserved *Bss*HII site, Ap^RTc^S recombinants were screened by *Bss*HII digestion. 27 pZE recombinants were identified; typical plasmids were 4533 bp (Fig. 6). Two smaller plasmids [Fig. 6, lane 13 (2.9 kb) and one not shown (2.6 kb)] that lack pBR322 sequences from the *Cla*I site (position 23) to the vicinity of the *Ava*I site (position 1424) were also obtained. The deleted plasmids were not linearized by *Bss*HII indicating that the Pu/Py-rich segment of the satellite is absent or modified. These deletions are close to the major Z-DNA segment of pBR322 (positions 1447–1460; Azorin et al., 1983); they could be the consequence of the involvement of Z-DNA in recombination as might have occurred in

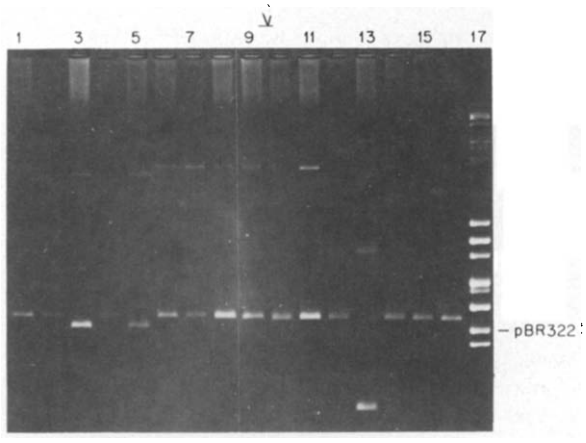

Fig. 6. Agarose gel of SC plasmids from transformants containing a Pu/Py-rich fragment of RU. Lanes 1–16 plasmid preparations (Birnboim and Doly, 1979) from Ap^RTc^S colonies. Marker DNA, lane 17 contains a mixture of SC and linear DNAs of known sizes; SC pBR322 is indicated.

vivo in some of the variants of the satellite. Z-DNA has a tendency to form aggregates (Castleman and Erlanger, 1982); self-association in a plasmid might facilitate strand exchange (Haniford and Pulleyblank, 1983). The deleted plasmids are of interest because some commonly used vectors do not accept long tracts of repeated sequences, either simple satellites (Brutlag et al., 1977) or $(CG)_n$ (n = 25) (Klysik et al., 1982). The vector might even delete segments of its own DNA with the extruded foreign DNA (W. Zacharias, personal communication). We note what appears to be an extreme effect (i.e., large deletions) on the primary sequence of DNA associated with the unusual domains of this crab satellite.

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