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# *Rec*BC, *sbc*B independent, $(AT)_n$-mediated deletion of sequences flanking a *Xenopus laevis* $\beta$ globin gene on propagation in *E. coli*

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ABSTRACT

Plasmids containing sequences 3' of the adult $\beta$1 globin gene of Xenopus laevis are unstable on propagation in a range of E.coli host strains. Up to 300 bp of Xenopus DNA are lost by rec A independent recombination between $(AT)_{37}$ and $(AT)_{17}$ sequences. Additionally, smaller deletions occurring in or around the $(AT)_{37}$ sequence are observed. Deletion of these potential cruciform structures occurs in the absence of exonuclease I, exonuclease V and exonuclease VIII as the same pattern of deletion events is observed in recA recBC sbcB and recBC sbcA recE strains.

## INTRODUCTION

In a recent analysis of simple sequence repeats in the Xenopus laevis globin gene locus we reported the presence of an $(AT)_{34}$ sequence within the first intron of a tadpole $\alpha$ globin gene and an $(AT)_{23}$ sequence ~450 bp 5' of the adult $\beta$1 globin gene (1,2). Hybridisation experiments using a synthetic $(AT)_n$ copolymer probe under stringent conditions detected restriction fragments containing these $(AT)_{34}$ and $(AT)_{23}$ sequences.Hybridisation was also observed to sequences ~4.5 kb 3' of the adult $\beta$1 gene. We report here that the region 3' of the adult $\beta$1 gene which hybridises to the synthetic $(AT)_n$ probe contains two separate $(AT)_n$ tracts, $(AT)_{37}$ and $(AT)_{17}$, ~180 bp apart. High frequency recA independent deletion of these $(AT)_n$ tracts and the intervening 180 bp of DNA is observed in a range of E.coli strains.

Homologous recombination in E.coli is catalysed by recA protein. Analysis of genetic recombination in E.coli strains deficient in recA revealed the presence of two alternative recombination pathways (35). The recBC pathway requires functional exonuclease V, the product of the recB and recC genes, and exonuclease I, the product of the sbcB gene. The second pathway for recA independent recombination requires exonuclease VIII, the product of the recE gene which is regulated by sbcA. The role of exonucleases in catalysing recA independent recombination is little understood (see Discussion in ref.36).

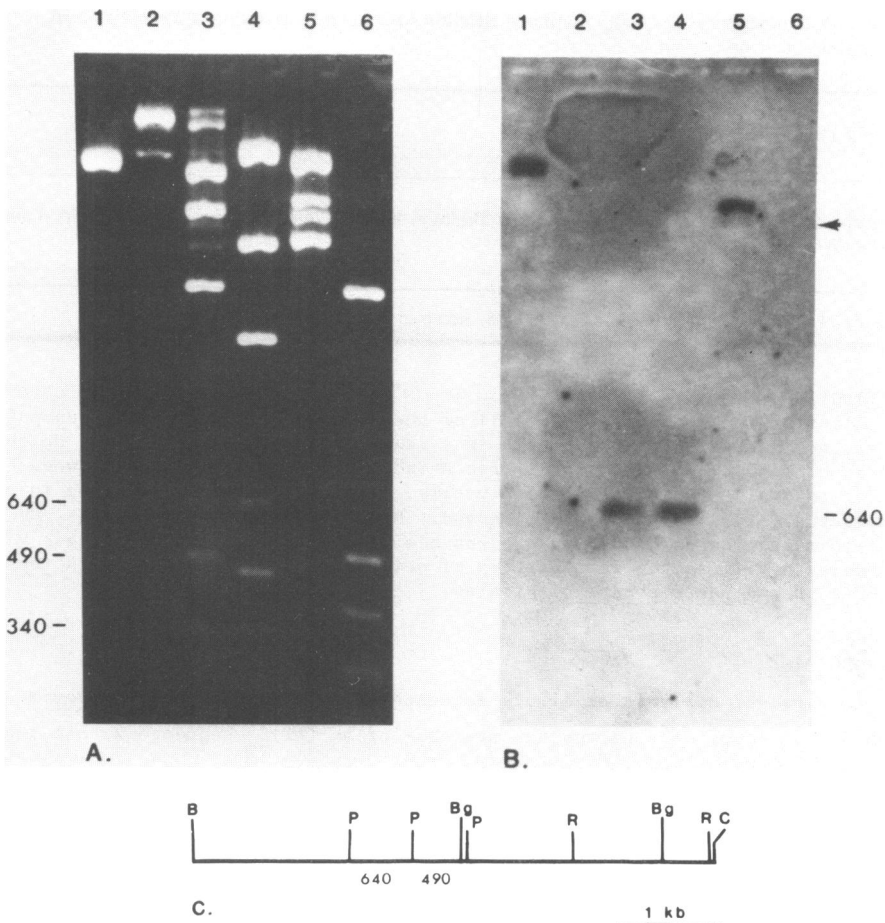


Figure 1. $(AT)_n$ sequences are deleted from plasmid pXGBC5.6 on propagation in E.coli.
PANEL A Ethidium bromide stained 1.2% agarose gel of the following digests: Lane 1 pXG540 (24) Bam HI. Lane 2 Charon 4 Bam HI. Lane 3 pXGBC5.6 Pst I+Bam HI (partial). Lane 4 pXGBC5.6 Pst I + Bam HI + Bgl II. Lane 5 pXGBC5.6 BamHI + Bgl II. Lane 6 pAT153 Hinf I. Sizes of pXGBC5.6 fragments are indicated in base pairs. PANEL B The same digests after transfer to Biodyne A nylon membrane (Pall) and hybridisation to $(AT)_n$ copolymer as described in reference 2 (final washing stringency $1.5^n$X SET at 55°C). PANEL C Restriction map of the subclone pXGBC5.6. The sizes of the small Pst I fragments are indicated in base pairs.

Recent studies have suggested that long inverted repeats in phage or plasmid vectors can be propagated in recBC sbcB E.coli strains(3-6). Deletion of the $(AT)_{37}$ and $(AT)_{17}$ inverted repeats 3' of the Xenopus $\beta$1 globin gene is

observed in CES201, a recA recBC sbcB strain. $(AT)_n$ mediated deletion is also observed in strains deficient in the sbcA recE pathway of recombination. These results are discussed with reference to the instability of long inverted repeats in E.coli and cruciform formation in vivo.

## METHODS AND MATERIALS

E.coli strains used are listed in Table I. All strains used were tested for their sensitivity to u.v. light as described in Maniatis et al. (7). The sbcB genotype of strain CES201 was confirmed by assaying the u.v. sensitivity of CES201 transformed to tetracycline resistance with the plasmid pDR1453 which carries a functional copy of the recA gene ( a kind gift of Dr.Richard Kolodner ). CES201 transformed in this way was less sensitive to u.v. irradiation than a recBC strain.

Transformation and DNA isolation. Bacterial cultures grown to an $OD_{600}$ of 0.3 were rendered competent by treatment with $CaCl_2$ as described by Cohen et al. (8) except for DHI cells which were made competent by the procedure of Hanahan (9). pXGBC5.6 DNA was used at a concentration of 100ng $ml^{-1}$. Plasmid DNA was isolated from small scale cultures (2 ml) after overnight growth at 37°C in L broth supplemented with 100 μg $ml^{-1}$ ampicillin by the method of Birnboim & Doly (10). Large scale plasmid preparations from 1 litre cultures with chloramphenicol amplification were performed by a scaled up version of the NaOH/SDS procedure described by Ish-Horowicz & Burke (11). Plasmid DNAs were purified by two consecutive bandings on CsCl/ethidium bromide gradients.

DNA transfer and $(AT)_n$ copolymer hybridisation were performed as described in reference 2.

DNA end labelling and sequencing. pXGBC5.6 DNA digests were 3' end labelled using T4 DNA polymerase and $^{32}P$ dCTP (3000 Ci $mmol^{-1}$) as described in reference 7. After removal of free label by chromatography over Sephadex G50 (Pharmacia) restriction fragments were recovered from Low Gelling Temperature (LGT) agarose gels after very brief illumination with long wave u.v. light. DNA was recovered from melted gel slices by phenol extraction and ethanol precipitation. Chemical degradation sequencing was by the method of Maxam and Gilbert (12).

## RESULTS

### $(AT)_n$ hybridising sequences are deleted on propagation in E. coli HB101.

Shown in Figure 1C is a restriction map of the insert in clone pXGBC5.6, which contains a 5.6 kb Bam HI-Cla I fragment subcloned from the cosmid cXG$\alpha\beta$ 4.2 into pAT153 (13).Restriction enzyme digestion of pXGBC5.6 plasmid DNA with Pst I (+ BamHI +/- Bgl II) revealed 640 and 340 bp Pst I fragments present in non-stoichiometric amounts compared to the 490 bp Pst I fragment (Figure 1A, lane 3) or the slightly smaller Pst I-Bgl II fragment (Figure 1A, lane 4). Digestion with Bam HI and Bgl II revealed non-stoichiometric 2.9 and 2.6 kb fragments (Figure 1A, lane 5) and linearisation of pXGBC5.6 at its unique Bam HI site yields two fragments differing in size by 300 bp (not shown). These observations are consistent with a deletion of 300 bp within the 640 bp Pst I fragment to give two populations of plasmid molecules, one 'full-length' and

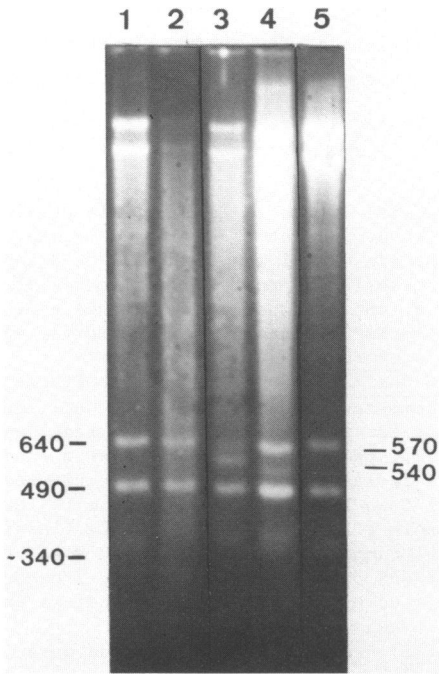


Figure 2. High frequency recA independent deletion of pXGBC5.6 sequences in E.coli HB101.
The pXGBC5.6 glycerol cell stock was streaked to single colonies on L agar + ampicillin and plasmid DNA prepared from four colonies was digested with Pst I (lanes 1-4) and run on a 1.2% agarose gel alongside a large scale plasmid preparation cut with Pst I (lane 5). The cell cultures used to prepare DNA were tested for their sensitivity to u.v. light and shown to be recA.

one 300 bp shorter. The digests of Figure 1A were transferred to Biodyne nylon membrane and hybridised to a nick translated $(AT)_n$ copolymer probe under conditions shown previously to be specific for long stretches of strictly alternating A and T residues (2). The autoradiograph in Figure 1B shows that $(AT)_n$ sequences of sufficient length to hybridise are present in the non-stoichiometric 640 bp Pst I fragment but not in the 340 bp Pst I fragment. Similarly the 2.9 kb Bam HI-Bgl II fragment hybridises with the $(AT)_n$ probe whereas the 2.6 kb Bam HI-Bgl II fragment hybridises only weakly (marked with an arrowhead in Figure 1B,lane 5). Hence the deletion event in pXGBC5.6 has removed almost all the $(AT)_n$ hybridising sequences from the plasmid.

High frequency rec A independent deletion of $(AT)_n$ sequences in HB101.

If there had been a single deletion event during growth giving rise to two populations of plasmids it should be possible to isolate E.coli cells carrying the 'full length' pXGBC5.6 plasmid and other E.coli cells carrying the deleted version of the plasmid. To test this hypothesis the glycerol cell-stock of pXGBC5.6 was streaked to single colonies on L agar + ampicillin and four randomly picked colonies were grown in 2 ml of L broth + ampicillin at 37°C overnight. Plasmid DNA isolated from these cultures by a 'miniprep' procedure is shown in Figure 2 after digestion with Pst I (lanes 1-4) . All four DNA preparations contain varying amounts of the non-stoichiometric 640 bp Pst I fragment and other, smaller non-stoichiometric PstI fragments which we show below are derived from the 640 bp Pst I fragment by deletion . The DNA preparations of lanes 3 and 4 contain 570 and 540 bp Pst I fragments. Careful examination of Pst I digested pXGBC5.6 from a large scale plasmid preparation show these fragments are present in low abundance (Figure 4, lane 10). The non-stoichiometric 340 bp fragment runs as a much more diffuse band than the marker fragments in this size range. Analysis of Pst I digested pXGBC5.6 on sequencing gels after 3' end labelling shows that the smallest pXGBC5.6 Pst I fragment is a heterogenous collection of fragments 340-380 bp in size (data not shown).

The cell cultures maintaining the 640 bp and 570 bp Pst I fragments (Figure 2, lanes 1 & 3) undergo further deletion events on continued propagation to give non-stoichiometric Pst I fragments of ~340bp. Growth temperature does not appear to have any effect on the deletion process as the pattern of deletion observed is not altered by growth at 27°C (data not shown). The 640bp Pst I fragment of pXGBC5.6 was gel purified and ligated to Pst I cut pAT153 DNA and introduced by transformation into E. coli JM101 and DH1. All twelve ampicillin resistant transformants analysed had undergone deletion within the 640bp Pst I fragment to give 570, 540 and ~340 bp Pst I fragments (data not shown). Thus deletion of sequences within the 640 bp Pst I fragment is not dependent upon the presence of flanking X. laevis sequences. All cell cultures used for DNA isolation were tested for their sensitivity to u.v. light and shown to be recA (not shown). The deletion of pXGBC5.6 sequences is therefore recA independent.

Determination of the sequences deleted from pXGBC5.6.

Pst I digested pXGBC5.6 DNA prepared from HB101 was 3' end labelled with T4 polymerase and the 640, 570, 540 and ~340 bp fragments were purified from an LGT agarose gel. The 3' end labelled fragments were restriction mapped

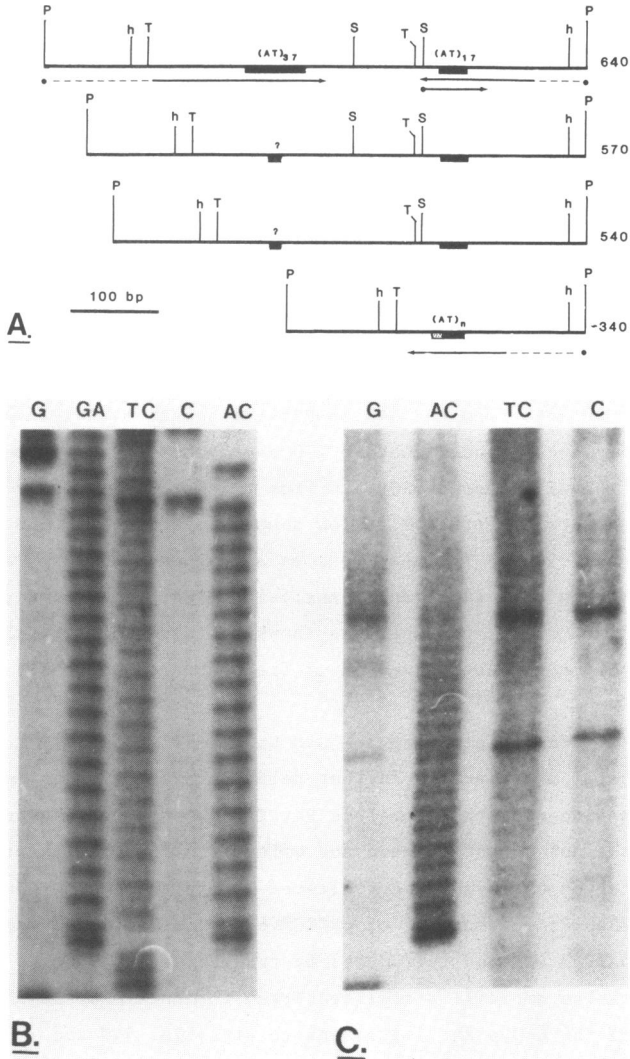


Figure 3. Determination of sequences deleted in pXGBC5.6.
3' end labelled pXGBC5.6 Pst I fragments were isolated as described in Methods and Materials and restriction maps for the enzymes Hae III (h), Taq I (T) and Sau 3A (S) are shown in panel A. The extent of sequencing of 3' end labelled DNA fragments is indicated by solid arrows below the restriction map of 640 and 340 bp fragments. Panel B shows Maxam & Gilbert sequence determinations of a 180 bp Pst I - Sau 3A fragment derived from the 3' end labelled 640 bp Pst I fragment. The 3' end labelled Pst I fragments of 340 - 380 bp were cut to completion with Taq I and subjected to chemical degradation sequencing. The relevant region of the autoradiograph is shown in panel C. The origin of the heterogeneity in the sequence is discussed in the text.

using partial and complete digestion with Hae III, Sau 3A and Taq I. The restriction maps presented in Figure 3A show Hae III and Taq I sites proximal to the Pst I sites that are common to all four fragments. The 70 bp deletion which gave rise to the 570 bp Pst I fragment lies within the 200 bp Taq I - Sau 3A fragment and the 100 bp deletion that gave the 540 bp Pst I fragment removed one of the internal Sau 3A sites. The strategy used to derive sequence from the 640 bp and ~340 bp Pst I fragments is shown in Figure 3A. There are two $(AT)_n$ tracts within the 640 bp Pst I fragment,one consisting of 74bp of strictly alternating A-T,$(AT)_{37}$, and a shorter $(AT)_n$ tract, $(AT)_{17}$, separated by 180 bp of intervening DNA. Analysis of the ~340bp Pst I 3' end labelled fragments on a sequencing gel shows that the deletion events within the 640bp Pst I fragment generate a series of Pst I fragments of 340 - 380 bp, differing in size by 2 nucleotides (not shown). Maxam & Gilbert sequence analysis of this mixed population of Pst I fragments is shown in Figure 3C. The sequence up to the start of the $(AT)_{17}$ run is identical to that of the 640bp Pst I fragment (Figure 3B). After an unambiguous run TTT AA$(TA)_9$ there is a band in all four tracks. We interpret this as meaning that the nucleotides A,T,G and C are present at this position in different subpopulations of ~340bp Pst I fragments. After this nucleotide the run $(AT)_{5-8}$ can be discerned before the sequence becomes unreadable. Thus the recA independent deletions which gave rise to the ~340bp fragments are the result of recombination events between the $(AT)_{37}$ and $(AT)_{17}$ sequences which act to remove most but not all the $(AT)_n$ sequences from pXGBC5.6.

### $(AT)_n$ sequences in pXGBC5.6 are deleted in a wide range of E.coli strains

The stability of the pXGBC5.6 640 bp Pst I fragment was analysed after

Table I

| Strain | Reference | Relevant markers |
|---|---|---|
| HB101 | 30 | recA13 |
| JM101/103 | 31 | sbcA15 |
| N4178-15 | 32 | recB gyrB226 |
| SD104-14 | 32 | gyrB226 |
| AB2480 | 33 | recA uvrA |
| 159 | 33 | uvrA |
| DH1 | 9 | recA1 gyrA96 |
| CES200 | 34 | recB21 recC22 sbcB15 |
| CES201 | 34 | recAΔ recB21 recC22 sbcB15 |
| JC8679 | 36 | recB21 recC22 sbcA23 |
| JC9604 | 36 | recB21 recC22 sbcA23 recA56 |
| JC8691 | 36 | recB21 recC22 sbcA23 recE159 |

Genotypes of E.coli strains used as hosts for pXGBC5.6. Recombination competence was assayed by sensitivity to u.v. light as described in Materials and Methods.

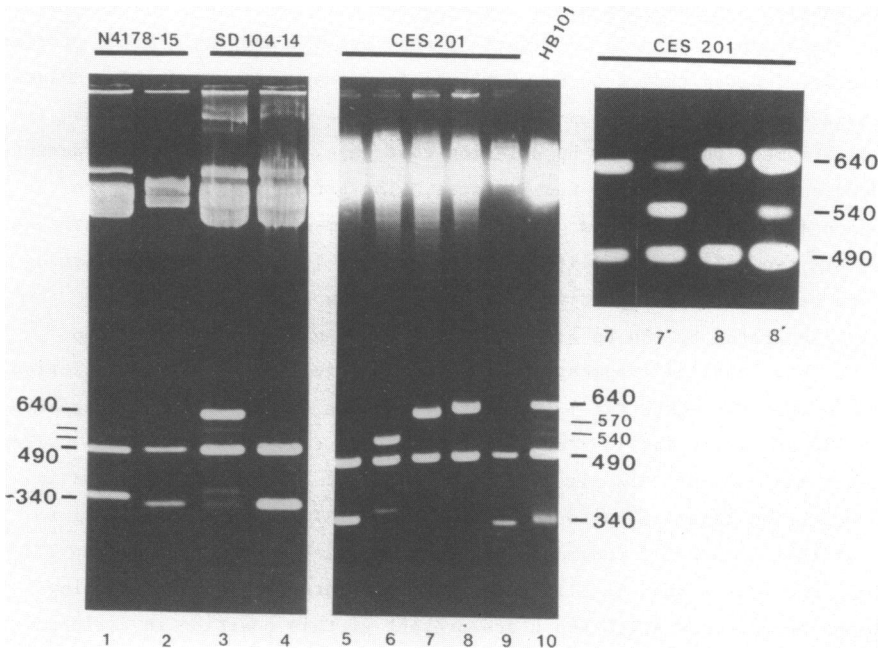


Figure 4. Instability of the 640 bp Pst I fragment in a range of E.coli strains.
pXGBC5.6 plasmid DNA was introduced into the E.coli hosts listed in Table I by transformation as described in Methods and Materials. Ampicillin resistant colonies were picked into 2ml of L broth + ampicillin, grown at 37°C overnight and plasmid DNA prepared by a "miniprep" procedure. After digestion with Pst I, DNAs were analysed on 1.5% agarose gels alongside the pXGBC5.6 DNA from HB101 which was used for transformation. Representative examples of N4178-15, SD104-14 and CES201 transformants are shown. Sizes of Pst I fragments are indicated in base pairs. Cell cultures used to prepare the DNAs of lanes 7 and 8 were subcultured and miniprep DNAs were analysed after digestion with Pst I (7 -> 7' and 8 -> 8').

introduction into a range of E.coli host strains deficient in recombination and repair enzymes other than recA (Table I). The large scale plasmid preparation of pXGBC5.6 grown in HB101 was introduced into bacterial strains rendered competent by treatment with $CaCl_2$ or $RbCl_2$ (DH1). Examples of pXGBC5.6 'miniprep' DNA samples digested with Pst I are shown in Figure 4. Several recent reports have suggested that inverted repeats in plasmid or phage vectors can be stably maintained in recBC sbcB E.coli strains(3-6). pXGBC5.6 was introduced by transformation into the recA recBC sbcB strain, CES201. Of the five CES201 transformants shown in Figure 4 only one maintains the 640bp Pst I fragment without deletion (lane 8). One clone carries a small

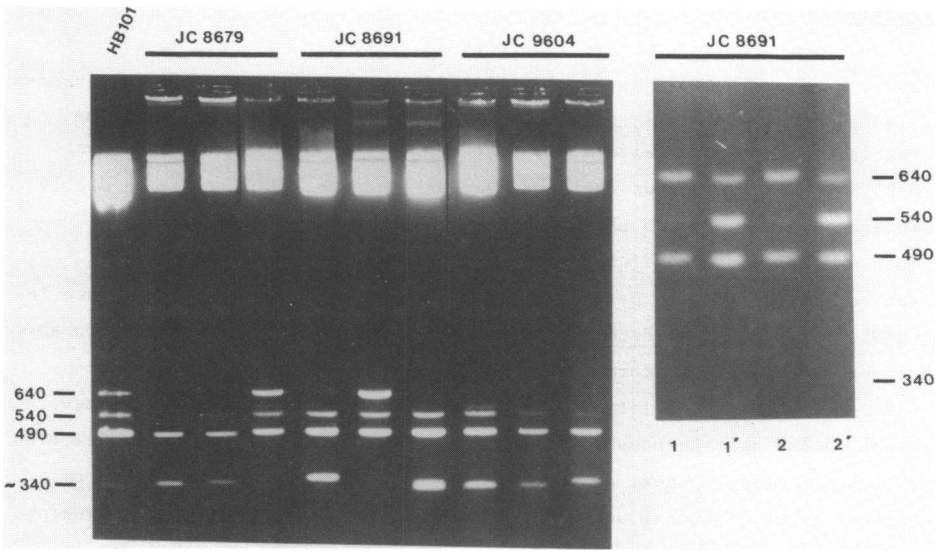


Figure 5. Deletion of $(AT)_n$ sequences does not proceed through the recE pathway.
pXGBC5.6 plasmid DNA was introduced into strains JC8679, JC8691 and JC9604 by $CaCl_2$ transformation. Representative miniprep DNAs digested with Pst I are shown. JC8691 transformants which carried only the 640bp Pst I fragment (lanes 1 and 2) were subcultured in 10ml of L broth + ampicillin and resultant plasmid DNAs were analysed after Pst I digestion (lanes 1' and 2'). Sizes of Pst I fragments are given in base pairs.

deletion of ~20bp (lane 7) while other clones contain 540,380,340 and 320bp Pst I fragments. It could be argued that the deleted fragments observed are not the result of deletion in CES201 but rather preferential uptake of shorter plasmids at transformation or loss of larger plasmids during growth. The clones with 640 and ~620bp Pst I fragments were subcultured and mini-prep DNAs analysed after Pst I digestion. Both 640 and ~620 bp Pst I fragments undergo deletion upon further growth, even in the recA recBC sbcB strain CES201 (Figure 4 inset, 7-> 7', 8-> 8'). Similar results were obtained using the recBC sbcB strain, CES200 (not shown).

To investigate the role of the recE pathway in deletion of $(AT)_n$ sequences, plasmid pXGBC5.6 was transformed into the E.coli strains JC8679, JC8691 and JC9604 (see Table I). Representative DNA preparations from ampicillin resistant transformants are shown in Figure 5. The few transformants which contained only the 640bp Pst I fragment gave 540 and ~360 bp deletion products on continued growth (eg. Figure 5: 1,1'; 2,2').

DISCUSSION

Long palindromic sequences generated *in vitro* can not be propagated in plasmid or phage vectors in *E.coli* (14-19). Shorter inverted repeat sequences (< 70 bp) have been stably maintained in bacterial plasmids and have the potential to extrude cruciform structures *in vitro* under torsional stress (20-23,37). We have shown that the *Xenopus* sequence, $(AT)_{34}$, can be stably maintained in the plasmid pXG540 in *E.coli* HB101 and that this $(AT)_{34}$ sequence extrudes a cruciform structure *in vitro* at moderate levels of negative supercoiling . Determination of the specific linking difference ($\sigma$) of pXG540 prepared from *E.coli* HB101 showed that the $(AT)_{34}$ sequence probably does not extrude a cruciform structure *in vivo* (24).

*Rec* A independent deletion events in *E.coli* have been observed in several systems including transposable element excision (16,18,25-27). Such deletion events occur between short direct repeats often separated by kilobases of intervening DNA. Several authors have emphasised the importance of flanking inverted repeats in *rec* A independent recombinations between short direct repeats (16,25). The $(AT)_{37}$ and $(AT)_{17}$ sequences in pXGBC5.6 are unique in that they constitute both perfect direct repeats and perfect inverted repeat sequences. Although we can not measure the frequency with which $(AT)_n$ sequences are deleted on propagation in *E.coli*, deletion must occur at relatively high levels as nearly all small scale cultures analysed contain deletion products of the 640 bp Pst I fragment (Figure 4).

*In vivo* cruciform formation followed by endonucleolytic cleavage and ligation is one possible explanation of the deletion products observed in Figure 3C. If both the $(AT)_{37}$ and $(AT)_{17}$ sequences extrude cruciform structures in the same plasmid DNA molecule, single strand specific nuclease cleavage followed by exonucleolytic trimming and ligation could produce the deletion products observed in Figure 3C. A single nucleotide introduced at ligation, similar to that observed in *Drosophila* copia circles (28), could explain the sequence heterogeneity in deletion products after the $AA(TA)_9$ sequence. We have observed S1 and Bal 31 nuclease cleavage at the hairpin loop of the cruciform formed by the $(AT)_{34}$ of the *Xenopus* $\alpha$T1 globin gene *in vitro* (24). This $(AT)_{34}$ cruciform is also a substrate for the T4 enzyme endonuclease VII, cleavage occurring at each end of the $(AT)_{34}$ sequence. If a similar resolvase enzyme is found in *E.coli*, cleavage 5' of the $(AT)_{37}$ and $(AT)_{17}$ blocks followed by ligation would leave an intact $(AT)_{17}$ in some recombinant molecules. Such molecules can be discerned in the sequence gel of Figure 3C.

Plasmids isolated from E.coli have a specific linking difference ($\sigma$) of -0.05 to -0.06. Recent experiments have suggested that the $\sigma$ value of plasmids in vivo may be lower than that observed for isolated plasmids (29,38). We calculate the free energy of formation of the $(AT)_{34}$ cruciform in the $\alpha$T1 globin gene to be 13.8 kcal $mol^{-1}$, significantly lower than that observed for other inverted repeats in plasmid vectors, the $(AT)_{34}$ sequence forming a cruciform at moderate levels of supercoiling, $\sigma$ = -0.025. We have never observed deletion of the $(AT)_{34}$sequence in HB101,whereas the $(AT)_{37}$ sequence in pXGBC5.6 undergoes recA independent deletion at high frequency. The $(AT)_{37}$ cruciform probably has an even lower free energy of formation than the $(AT)_{34}$ sequence and is more likely to be extruded in E.coli. Thus, the deletion of the $(AT)_{37}$ sequence may be a consequence of cruciform formation in vivo. The experiments of Figures 4 & 5 show that deletion of the $(AT)_{37}$ sequence occurs in the absence of functional alleles for recA recBC sbcB and recE. Hence, the deletion of $(AT)_n$ sequences in E.coli would appear to proceed through a recA independent recombination pathway which does not require exonucleases I, V or VIII.

The deletion of X.laevis DNA sequences on cloning in plasmid vectors reported here serves to emphasise that torsional stress during cloning may select against certain eukaryotic sequences, even in a recBC sbcB background.

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