**The EMBO Journal** Vol.16 No.9 pp.2528–2534, 1997

# Near-simultaneous DNA cleavage by the subunits of the junction-resolving enzyme T4 endonuclease VII

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**In common with a number of other DNA junction-resolving enzymes, endonuclease VII of bacteriophage T4 binds to a four-way DNA junction as a dimer, and cleaves two strands of the junction. We have used a supercoil-stabilized cruciform substrate to probe the simultaneity of cleavage at the two sites. Active endonuclease VII converts the supercoiled circular DNA directly into linear product, indicating that the two cleavage reactions must occur within the lifetime of the protein–junction complex. By contrast, a heterodimer of active enzyme and an inactive mutant endonuclease VII leads to the formation of nicked circular product, showing that the subunits operate fully independently.**

*Keywords*: cruciform/DNA–protein interaction/DNA supercoiling/Holliday junction/recombination

## Introduction

Four-way helical junctions in DNA are important intermediates in homologous (Holliday, 1964; Broker and Lehman, 1971; Sigal and Alberts, 1972; Sobell, 1972; Potter and Dressler, 1976, 1978; Orr-Weaver *et al.*, 1981) and certain site-specific (Hoess *et al.*, 1987; Kitts and Nash, 1987; Nunes-Düby *et al.*, 1987; Jayaram *et al.*, 1988; McCulloch *et al.*, 1994) recombination events, where they serve as substrates for a number of proteins that are targeted primarily to the branched structure of the DNA. Junction-selective nucleases are required to resolve four-way junctions back to regular duplex species, and such resolving enzymes have been isolated from bacteriophage-infected eubacteria (Kemper and Garabett, 1981; de Massey *et al.*, 1984), *Escherichia coli* (Connolly *et al.*, 1991; Iwasaki *et al.*, 1991; Sharples and Lloyd, 1991; Sharples *et al.*, 1994), yeast (Symington and Kolodner, 1985; West and Korner, 1985) and mammalian cells (Elborough and West, 1990; Hyde *et al.*, 1994). These enzymes are fundamentally structure-selective; complexes formed between four-way DNA junctions and the enzymes T7 endonuclease I (Duckett *et al.*, 1995), T4 endonuclease VII (Pöhler *et al.*, 1996) or CCE1 of yeast (White and Lilley, 1996) are not displaced by 1000-fold excesses of duplex DNA of the same sequence.

We have shown that for T4 endonuclease VII (Pöhler *et al.*, 1996), T7 endonuclease I (M.J.Parkinson and D.M.J.Lilley, unpublished data) and yeast CCE1 (White and Lilley, 1996), the enzymes bind to the four-way junction in the form of dimers. The phage enzymes tend to cleave predominantly at two positions within the junction (Duckett *et al.*, 1988; Mueller *et al.*, 1988), suggesting that each subunit makes a single cleavage. The *E.coli* resolving enzyme RuvC also tends to introduce pairs of cleavages within the core of a junction that can undergo a limited degree of branch migration (Bennett *et al.*, 1993). The crystal structure of this protein has been solved (Ariyoshi *et al.*, 1994). It is a dimer of subunits, wherein clusters of acidic residues shown to be important for catalytic activity (Saito *et al.*, 1995) are separated by ~30 Å between the two subunits. This would indicate that the two active sites are likely to act independently. One could envisage this occurring in two extreme ways. First, the enzyme might bind, and both strands become cleaved within a very short interval, i.e. giving effectively simultaneous cleavage of the two sites. Alternatively, the first site might be cleaved, followed later by cleavage at the second site. The latter model might even allow for dissociation of the enzyme followed some time later by the binding of a new molecule that introduces the second cleavage. Using cloverleaf-type junctions, Mueller *et al.* (1990) showed that two cleavages are introduced within the same junction molecule by T4 endonuclease VII, but Kemper and colleagues (Pottmeyer and Kemper, 1992) have introduced what they term a 'nick and counter-nick' model, implying uncoordinated cleavage at the two sites.

We have used a self-limiting cruciform substrate to demonstrate that the two strands of the junction are cleaved within the lifetime of the junction–enzyme complex.

## Results

### *The use of a supercoil-stabilized cruciform structure as a self-limiting substrate for T4 endonuclease VII*

A cruciform structure is a pair of hairpin loops that may be formed by intrastrand base-pairing in an inverted repeat sequence in DNA. The region at the base of the stem loops is a four-way helical junction that is equivalent to the Holliday junction, and may act as a substrate for enzymes that are selective for DNA junctions (Mizuuchi *et al.*, 1982a; Lilley and Kemper, 1984). Cruciform structures are thermodynamically unstable in relaxed DNA (Mizuuchi *et al.*, 1982b; Courey and Wang, 1983; Lilley and Hallam, 1984), and require the free energy of negative supercoiling to maintain a stable existence (Gellert *et al.*, 1979; Lilley, 1980; Panayotatos and Wells, 1981). Therefore, they are only observed in supercoiled circular DNA. When the cruciform is nicked by an enzyme, the topological constraint of the circular DNA is no longer in existence, and the superhelical stress is released from the molecule. The cruciform is therefore destabilized, and

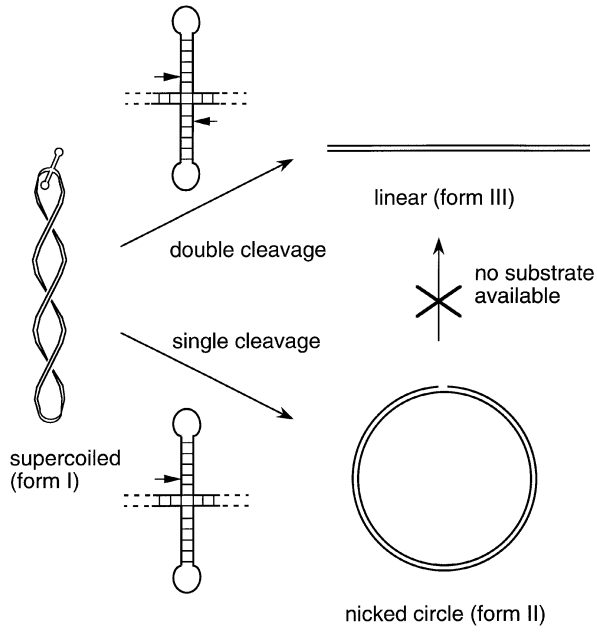


**Fig. 1.** The products in the cleavage of a supercoil-stabilized cruciform structure by a junction-resolving enzyme. This schematic shows the principle of the experiments performed in this paper. The cruciform structure is intrinsically unstable and requires the free energy of negative supercoiling to maintain its existence. Upon cleavage of the DNA, the topological constraint is lost and the cruciform is reabsorbed as regularly base-paired DNA. Thus, cleavage of a single strand by a resolving enzyme leads to formation of a nicked circle, and the reaction cannot proceed further as the substrate no longer exists. Only by effectively simultaneous cleavage of two strands of the cruciform junction can linear DNA result.

consequently reabsorbed as regular duplex DNA. For this reason, the cruciform acts as a self-limiting substrate for the enzyme generating the cleavage, because the action of the enzyme leads to the destabilization and disappearance of the substrate.

Two distinct products of cleavage of the cruciform four-way junction are possible, as illustrated in Figure 1. In general, resolving enzymes introduce two symmetrically related cleavages into four-way DNA junctions (Duckett *et al.*, 1988; Mueller *et al.*, 1988; Parsons *et al.*, 1990; Bennett and West, 1995), consistent with the dimeric character of junction-bound resolving enzymes (Pöhler *et al.*, 1996; White and Lilley, 1996). If the junction is cleaved at both sites simultaneously, the product will be a linear (form III) DNA molecule, whereas if it is cleaved at a single site the product is a nicked circle (form II). However, once a single cleavage has released the superhelical constraint in the molecule, the cruciform substrate disappears, and is no longer available for further reaction. Since linear, nicked circular and supercoiled circular DNA are readily separated by agarose gel electrophoresis, it is relatively straightforward to distinguish the products of the reaction and thus deduce the nature of the cleavage reaction by the dimeric enzyme.

### *Cleavage of a cruciform structure by active T4 endonuclease VII*

For these studies we chose to use a plasmid pAT25*tetA* (Figure 2A), which contains the inverted repeat $(AT)_{25}$ (Bowater *et al.*, 1994). Like other alternating adenine–thymine sequences (Greaves *et al.*, 1985), this 50 bp tract

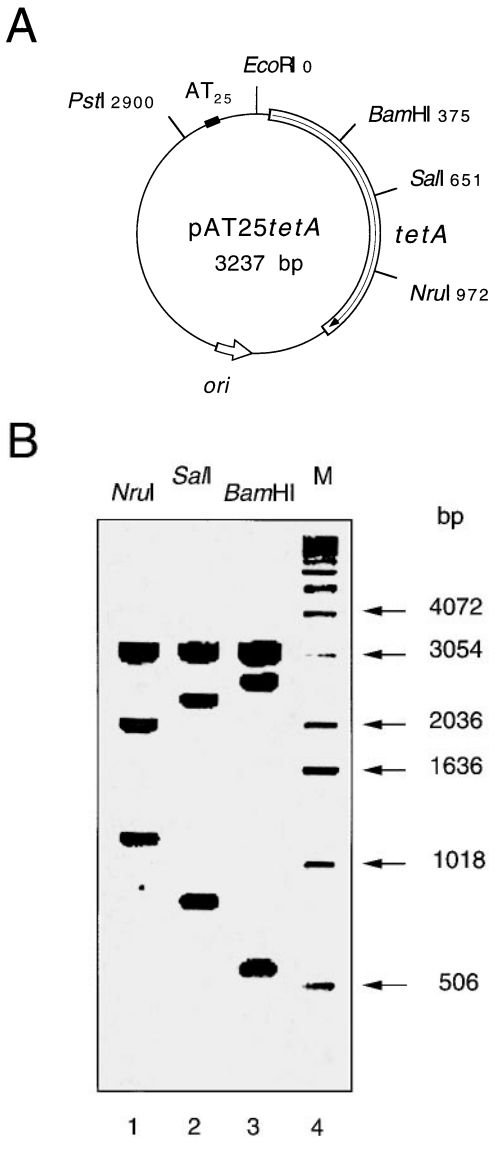


**Fig. 2.** pAT25*tetA*, a plasmid containing an $(AT)_{25}$ cruciform structure. (**A**) Map of pAT25*tetA*. This plasmid contains a 50 bp tract of alternating adenine–thymine sequence, that forms a cruciform structure when the DNA is negatively supercoiled. The $(AT)_{25}$ sequence is located within a 330 bp *Eco*RI–*Pst*I segment, and is indicated by a black box. The location of restriction enzyme sites used in the analysis are indicated. (**B**) Cleavage of the $(AT)_{25}$ cruciform in pAT25*tetA* by T4 endonuclease VII. 14 μg supercoiled pAT25*tetA* was incubated with 67 pmol protein A-endonuclease VII H38T in 60 μl reaction buffer, followed by complete digestion of aliquots with a restriction enzyme for which there is a unique site located in the plasmid. The products of digestion were separated by agarose gel electrophoresis, and the lengths of the DNA fragments deduced by interpolation from a set of marker DNA fragments (sizes shown on right). Note that in each case two DNA fragments shorter than full-length pAT25*tetA* were generated, indicating a single major site of cleavage by endonuclease VII. Calculation of the fragment lengths shows that the target of the resolving enzyme was the $(AT)_{25}$ sequence (see text). A negative image of the ethidium bromide-stained gel is presented. Lane 1, endonuclease VII-cleaved pAT25*tetA* after cleavage with *Nru*I; lane 2, endonuclease VII-cleaved pAT25*tetA* after cleavage with *Sal*I; lane 3, endonuclease VII-cleaved pAT25*tetA* after cleavage with *Bam*HI; lane 4, mixture of DNA fragments to act as size markers.

forms a cruciform structure in plasmid DNA that is negatively supercoiled ($-\sigma > 0.03$), without any discernible kinetic barrier to the extrusion reaction. T4 endonuclease

VII H38T is active in the cleavage of DNA junctions as an N-terminal protein A fusion (Giraud-Panis and Lilley, 1996). Supercoiled pAT25*tetA* was incubated with this enzyme, and the resulting DNA cleaved to completion with the restriction enzymes *Nru*I, *Sal*I or *Bam*HI, and the products analysed by electrophoresis in an agarose gel (Figure 2B). In each case the products are two fragments with a combined length equal to that of the complete plasmid (within the experimental error imposed by the relatively low resolution of agarose gel electrophoresis). Analysis of the fragment sizes (*Bam*HI 0.6, 2.7 kbp; *Sal*I 0.8, 2.5 kbp; *Nru*I 1.1, 2.2 kbp; probable experimental error = 5%) confirms that the target of the endonuclease VII H38T is the $(AT)_{25}$ cruciform structure.

### *T4 endonuclease VII converts the plasmid directly to linear product*

Uncleaved supercoiled pAT25*tetA* and the two potential immediate products of the cleavage reaction, nicked circular and linear plasmid, may be separated and identified by electrophoresis in an agarose gel. pAT25*tetA* was incubated with increasing quantities of protein A-endonuclease VII H38T under standard conditions (20°C, 5 min), and the products examined by electrophoresis (Figure 3). It can be seen that, with increasing extent of digestion, the supercoiled substrate disappears, with the concomitant appearance of linear plasmid. Some nicked circular DNA is present, but the amount of nicked circles as a proportion of total DNA does not change with the extent of digestion; this is quantified in Figure 3B. When the reaction was performed using a fixed quantity of the enzyme for increasing length of time, once again a direct conversion of supercoiled substrate to linear product was observed (Figure 3C).

The results shown are not influenced by either the H38T mutation or the protein A fusion. The cleavage of supercoiled pAT25*tetA* was repeated using wild-type sequence endonuclease VII expressed as an oligohistidine fusion (Figure 4). Once again, as the extent of digestion increases, we see direct conversion of supercoiled substrate to linear product.

These results indicate that the resolution reaction proceeds by near-simultaneous cleavage of the two strands of the cruciform junction.

### *Unilateral cleavage of a cruciform structure by heterodimeric T4 endonuclease VII*

We sought to test our ideas concerning the mechanism of cleavage by dimeric endonuclease VII using an inactive mutant. We have shown previously that endonuclease VII E86A is inactive in the cleavage of DNA junctions, although it retains normal affinity and selectivity of binding to junctions (Pöhler *et al.*, 1996). Our preparations of endonuclease VII E86A are completely devoid of any nuclease activity using supercoiled pAT25*tetA* as substrate; no significant conversion to either nicked circular or linear product was found in the standard incubation (Figure 5A).

We have shown previously that, while endonuclease VII E86A can be quantitatively crosslinked in dimeric form in free solution, it readily undergoes subunit exchange which is complete in less than 5 minutes (Pöhler *et al.*, 1996). A mixture of endonuclease VII H38T and endonuclease VII E86A can also freely exchange subunits.

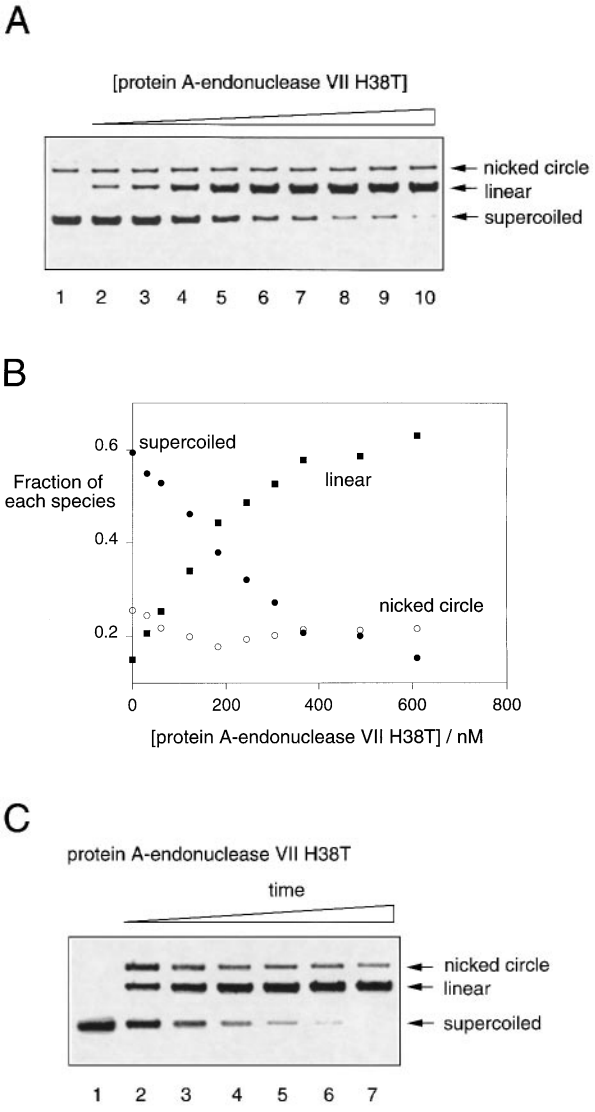


**Fig. 3.** The products of cleavage of a supercoil-stabilized cruciform junction by active endonuclease VII. (**A**) Products of digestion of pAT25*tetA* with increasing quantity of protein A-endonuclease VII H38T. Individual aliquots of 1.4 μg of supercoiled pAT25*tetA* were incubated in 10 μl reaction buffer for 5 min at 20°C with the quantities of protein A-endonuclease VII H38T indicated below. The reactions were terminated and the DNA electrophoresed in a 1% agarose gel. A negative image of the ethidium bromide-stained gel is presented. The following quantities of enzyme were used: lane 1, no enzyme; lanes 2–10: 30.5, 61, 122, 183, 244, 305, 366, 488 and 610 nM protein A-endonuclease VII H38T respectively. (**B**) Quantitation of digestion products. The relative quantities of the different species of pAT25*tetA* were determined by densitometry of the photographic negative from (A), and have been plotted as a function of enzyme concentration. Symbols: ●, supercoiled substrate; ■, linear plasmid; ○, nicked circular plasmid. (**C**) Products of digestion of pAT25*tetA* with protein A-endonuclease VII H38T as a function of time. 8.2 μg of pAT25*tetA* was incubated with 42 pmol of protein A-endonuclease VII H38T in 85 μl reaction buffer at 20°C. 10 μl aliquots were removed at intervals, the reaction terminated and the DNA electrophoresed in a 1% agarose gel. A negative image of the ethidium bromide-stained gel is presented. Lane 1, no protein. Aliquots were taken at the following times: lanes 2–7: 20 s, 40 s, 60 s, 80 s, 100 s and 120 s, respectively.

When we incubated either protein A-endonuclease VII H38T or oligohistidine-endonuclease VII E86A with radioactively labelled four-way DNA junction, we observed discrete retarded complexes on electrophoresis in poly-

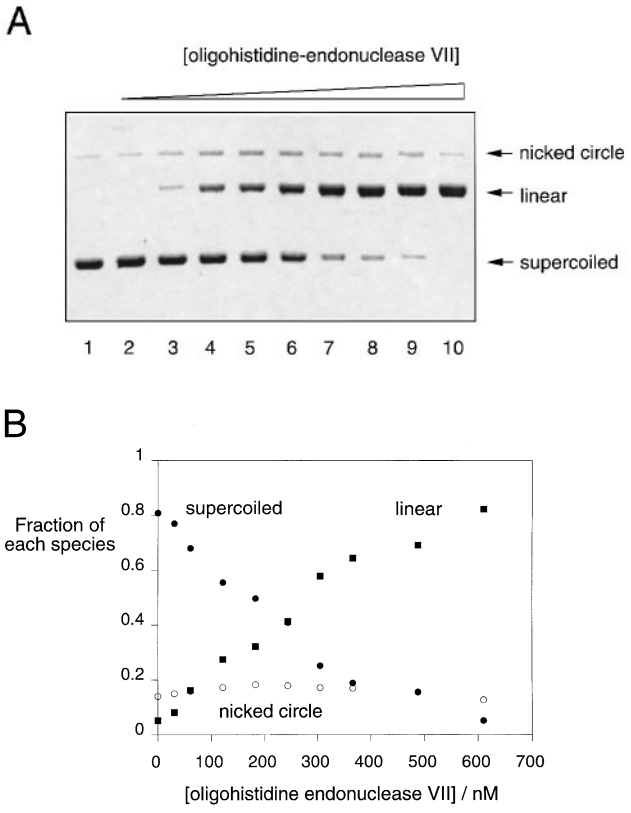


**Fig. 4.** The products of cleavage of a supercoil-stabilized cruciform junction by wild-type sequence endonuclease VII. (**A**) Products of digestion of pAT25*tetA* with increasing quantity of oligohistidine-endonuclease VII. Individual aliquots of 1.4 μg of supercoiled pAT25*tetA* were incubated in 10 μl reaction buffer for 5 min at 20°C with the quantities of oligohistidine-endonuclease VII indicated below. The reactions were terminated and the DNA electrophoresed in a 1% agarose gel. A negative image of the ethidium bromide-stained gel is presented. The following quantities of enzyme were used: lane 1, no enzyme; lanes 2–10: 30.5, 61, 122, 183, 244, 305, 366, 488 and 610 nM oligohistidine-endonuclease VII, respectively. (**B**) Quantitation of digestion products. The relative quantities of the different species of pAT25*tetA* were determined by densitometry of the photographic negative from (A), and have been plotted as a function of enzyme concentration. Symbols: ●, supercoiled substrate; ■, linear plasmid; ○, nicked circular plasmid.

acrylamide (Figure 5B). When we carried out the binding reaction with a mixture of these two proteins, we observed a third complex of intermediate mobility. This species must result from the binding of a protein A-endonuclease VII H38T, oligohistidine-endonuclease VII E86A heterodimer to the DNA junction, thus indicating that the two different mutant proteins have undergone subunit exchange in solution.

The endonuclease VII H38T–endonuclease VII E86A heterodimer contains one active and one inactive subunit. It might therefore be expected that this would generate a single cleavage in a cruciform junction if the active subunit acts autonomously within the heterodimer. To test this we incubated supercoiled pAT25*tetA* with a fixed quantity of protein A-endonuclease VII H38T (active) and an increasing quantity of oligohistidine-endonuclease VII E86A (inactive) under standard conditions (Figure 6). At a high ratio of active to inactive protein, the product of the reaction is linear DNA, as before. However, as the extent of inactive mutant rises, the product increasingly changes

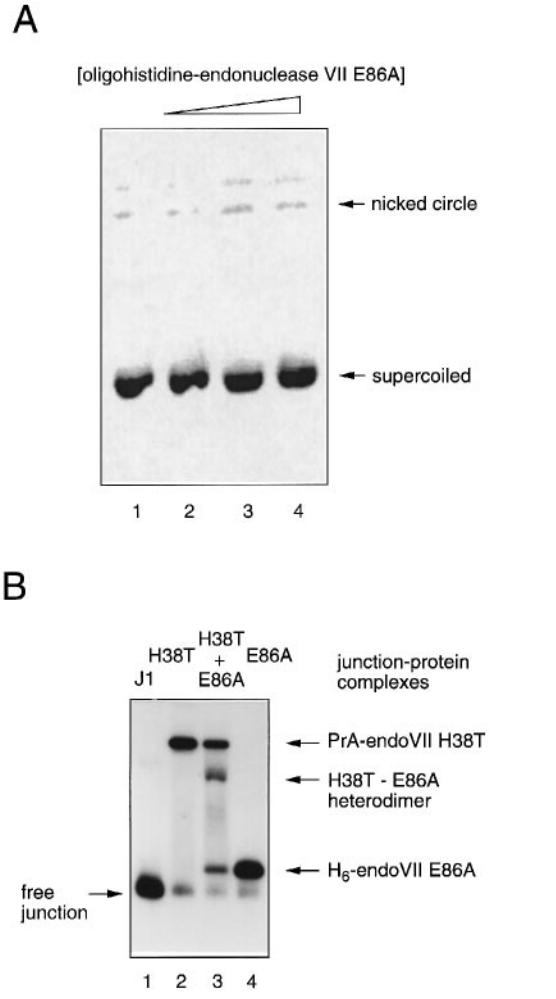


**Fig. 5.** Formation of a heterodimer between active and inactive endonuclease VII. (**A**) Endonuclease VII E86A is inactive in the cleavage of supercoiled pAT25*tetA*. 700 ng of pAT25*tetA* were incubated for 5 min in reaction buffer at 20°C with increasing quantities of oligohistidine-endonuclease VII E86A. The reaction was terminated and the DNA subjected to electrophoresis in an agarose gel. A negative image of the ethidium bromide-stained gel is presented. Note that even with the highest concentration of protein, no conversion of supercoiled plasmid into either nicked circles or linear DNA has occurred. The DNA was incubated with: lanes 1–4: no protein, 62.2 nM, 311 nM, 3.11 μM oligohistidine-endonuclease VII E86A, respectively. (**B**) Examination of heterodimer formation between endonuclease H38T and E86A mutants. The electrophoretic retardation method used previously to demonstrate dimer formation by endonuclease VII E86A was used (Pöhler *et al.*, 1996). In this we use fusion peptides of different sizes, in order to distinguish the different bound complexes. Thus, protein–junction complexes are formed with protein A-endonuclease VII H38T, oligohistidine-endonuclease VII E86A, and a mixture of the two proteins preincubated for 5 min. Pure protein A-endonuclease VII H38T and oligohistidine-endonuclease VII H38T lead to single retarded species, the former being more retarded because of the greater mass of the fused protein A polypeptide compared with the oligohistidine fusion. The same species are found with the mixture of proteins, but in addition a new species of intermediate mobility is observed, arising from the binding of a heterodimer of the two proteins. In the binding experiments, 0.19 nM junction 1 (5′ $^{32}$P-labelled in the h strand) was incubated with protein for 10 min, before loading onto a 6% polyacrylamide gel and electrophoresis in the presence of 5 mM EDTA to prevent cleavage. The autoradiograph is presented. Lane 1, junction without added protein; lane 2, junction incubated with 10 nM protein A-endonuclease VII H38T; lane 3, junction incubated with 10 nM protein A-endonuclease VII H38T + 20 nM oligohistidine-endonuclease VII E86A mixture; lane 4, junction incubated with 20 nM oligohistidine-endonuclease VII E86A.

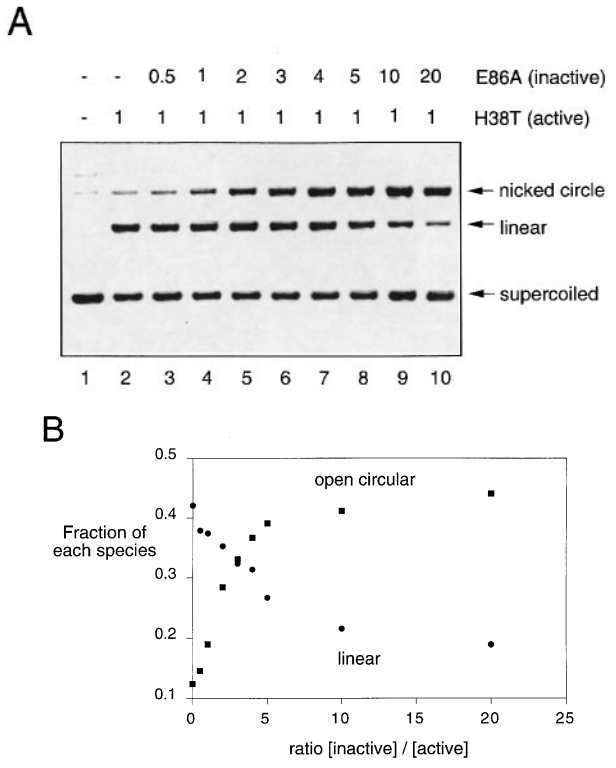


**Fig. 6.** The products of cleavage of a supercoil-stabilized cruciform junction by an active–inactive heterodimer of endonuclease VII. (**A**) Products of digestion of pAT25*tetA* with varying molar ratios of protein A-endonuclease VII H38T (active) and oligohistidine-endonuclease VII E86A (inactive). 31.1 nM of supercoiled pAT25*tetA* was incubated with 62.2 nM protein A-endonuclease VII H38T plus the variable quantities of oligohistidine-endonuclease VII E86A indicated below in 10 μl reaction buffer for 5 min at 20°C. The reactions were terminated and the DNA electrophoresed in a 1% agarose gel. A negative image of the ethidium bromide-stained gel is presented. Note the decrease in linear product as the ratio of inactive mutant protein increases, with concomitant increase in nicked circular product. Lane 1, pAT25*tetA* with no protein; lane 2, pAT25*tetA* incubated with protein A-endonuclease VII H38T alone; lanes 3–10: pAT25*tetA* incubated with protein A-endonuclease VII H38T plus 31.1 nM, 62.2 nM, 124.4 nM, 186.6 nM, 248.8 nM, 311 nM, 622 nM, 1.24 μM, respectively, of oligohistidine-endonuclease VII E86A. (**B**) Quantitation of digestion products. The relative quantities of the linear and nicked circular products of pAT25*tetA* digestion were determined by densitometry of the photographic negative from (A), and have been plotted as a function of ratio of inactive/active proteins. Symbols: ●, linear plasmid; ■, nicked circular plasmid.

from linear to nicked circular pAT25*tetA* DNA. Since we have shown that there is free exchange of subunits, statistically the majority of active endonuclease VII subunits will be part of an active–inactive heterodimer as the proportion of inactive protein increases, and under these conditions the active subunit acts to introduce a single cleavage within the cruciform junction. As predicted previously (Figure 1), this results in the formation of a nicked circular plasmid which can no longer serve as a substrate because the cruciform is reabsorbed on the release of negative supercoiling.

### *Non-complementation of inactive mutants of endonuclease VII*

We have isolated a number of inactive mutants of endonuclease VII; the mutations E86A (Pöhler *et al.*, 1996), H41T and D40N (Giraud-Panis and Lilley, 1996) are all totally inactive in DNA cleavage, although all bind to DNA junctions with unaltered selectivity. Using electrophoretic retardation techniques equivalent to that above we have shown that each mutant can exchange subunits with the other mutants (data not shown), and we have gone on to test the activity of the mixed mutants in the cleavage of four-way DNA junctions. In no case did we observe the recovery of any activity in the mixed mutant species (data not shown).

## Discussion

A heterodimer of active and inactive subunits of endonuclease VII converts the substrate into nicked circular product. This indicates that the active subunit is active in the presence of an inactive partner. We have also performed mixing experiments in which inactive mutants of endonuclease VII were mixed in pairs. None of the pairwise combinations was active in the cleavage of DNA junctions. The lack of any complementation of mutations suggests that the active sites comprise amino acids from a single subunit. Taken together with the new data on the nicking of the cruciform substrate, this indicates that the subunits of the dimeric enzyme act completely independently of one another. In the structure of the *E.coli* resolving enzyme RuvC (Ariyoshi *et al.*, 1994) the putative active sites are separated by ~30 Å, implying independence of action in this enzyme, too.

Active endonuclease VII converts a cruciform-containing supercoiled plasmid directly to linear DNA. As the results with the heterodimeric protein indicate, a single cleavage would lead to loss of the cruciform substrate and thus the end-product of the reaction would be a nicked circular species. To achieve the formation of linear DNA, both strands must be cleaved within the lifetime of the junction—protein complex. Since the subunits act independently of each other, this implies that either the two strands are cleaved so rapidly on binding that they are effectively cut simultaneously, or that the protein holds the nicked cruciform so as to prevent the loss of negative supercoiling until the second cleavage has been made. The results clearly exclude the possibility of unilateral cleavage by a dimeric enzyme, dissociation of the protein followed later by reassociation by a second dimer. If the protein has to hold a nicked cruciform together between cleavages, it must do so against a loss of supercoiling free energy in excess of 50 kcal/mol. A further corollary to these conclusions is that the normal preparations of endonuclease VII, both wild-type sequence and active mutants, must be highly active, as any loss of activity would result in the formation of active–inactive heterodimers that would generate nicked circular product.

In summary, T4 endonuclease VII acts by the independent action of two subunits, whereby both active sites carry out cleavage reactions within the lifetime of the DNA–protein complex. Unilateral cleavage of DNA junctions would have the potential to leave nicks in the DNA which might be mutagenic. Bilateral cleavage within the lifetime of the complex ensures productive resolution into duplex species, and prevents the formation of potentially dangerous lesions in the DNA.

## Materials and methods

### Plasmid DNA

pAT25*tetA* is a deletion derivative of pAT34*tetA* (Bowater *et al.*, 1994), derived by cloning a 330 bp fragment containing an $(AT)_{25}$ sequence between the *Pst*I and *Eco*RI sites of pAT153. pAT25*tetA* was transformed in *E.coli* HB101. The bacteria were grown in 500 ml of M9 medium complemented with 0.2% (w/v) glucose, 0.5% (w/v) casamino acids, 2 μg/ml thiamine, 1 mM $MgSO_4$ and 0.001% (w/v) thymidine, to an absorbance of $A_{660}$ = 0.6, and the plasmid amplified by treatment with 150 μg/ml chloramphenicol overnight. Cells were lysed with NaOH and SDS and, after neutralization, plasmid DNA was precipitated with isopropanol and isolated using the wizard maxiprep resin (Promega) following the manufacturer's protocol. Plasmid DNA was purified using isopycnic CsCl–ethidium bromide centrifugation and supercoiled DNA recovered by side puncture (Murchie and Lilley, 1992). Ethidium bromide was removed by extraction with n-butanol and the DNA subjected to extensive dialysis. This resulted in DNA of which >80% was closed circular supercoiled (form I). DNA concentrations were measured spectrophotometrically at 260 nm, using an extinction coefficient of $\varepsilon = 6.5\times10^3\ M^{-1}\ cm^{-1}\ nt^{-1}$.

### Four-way DNA junction

Oligonucleotides were synthesized using β-cyanoethyl phosphoramidite chemistry (Beaucage and Caruthers, 1981; Sinha *et al.*, 1984) implemented on a 394 DNA/RNA synthesizer (Applied Biosystems). Fully deprotected oligonucleotides were purified by gel electrophoresis in 10% (w/v) polyacrylamide (acrylamide:bisacrylamide, 30:1) containing 7 M urea in 89 mM Tris–borate, 89 mM boric acid, 2 mM EDTA (TBE buffer). DNA-containing bands were excised and the pure oligonucleotides electroeluted and recovered by precipitation in ethanol. In order to construct a four-way junction (J1–30) based on the central sequence of our junction 1 (Duckett *et al.*, 1988), four oligonucleotides were synthesized, of sequence:

b strand: 5′-CCCGTCCTAGCAAGCCGCTGCTACCGGAGG-3′
h strand: 5′-CCTCCGGTAGCAGCGAGAGCGGTGGTTGGG-3′
r strand: 5′-CCCAACCACCGCTCTTCTCAACTGCAGTGG-3′
x strand: 5′-CCACTGCAGTTGAGAGCTTGCTAGGACGGG-3′

Stoichiometric quantities of the four oligonucleotides, including a radioactively 5′ $^{32}$P-labelled h strand, were heated together at 85°C in 50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermine, 0.1 mM EDTA for 3 min and slowly cooled overnight. The junction was purified by gel electrophoresis in 5% polyacrylamide (30:1), and the DNA recovered by excision of the junction-containing band and electroelution.

### Preparation of T4 endonuclease VII and mutants

Endonuclease VII of wild-type sequence was expressed from a plasmid constructed by inserting a synthetic endonuclease VII gene (Giraud-Panis *et al.*, 1995) into pET-19b, cloned in HMS174 (DE3) (Giraud-Panis and Lilley, 1996). Oligohistidine-endonuclease VII E86A was expressed from a pQE30-based vector in M15 (pREP4) (Pöhler *et al.*, 1996). Protein A-endonuclease VII H38T, D40N and H41T were expressed from a pK19-based vector in JM101 (Giraud-Panis and Lilley, 1996). Cells were grown to an absorbance of $A_{660}$ = 0.5 and then induced by addition of 1 mM IPTG for 2 h. Cells were harvested by centrifugation, and lysed by sonication. Protein A fusions were purified essentially as described by Giraud-Panis and Lilley (1996); briefly, the protein was precipitated from the cleared lysate by ammonium sulfate precipitation, and purified by ion exchange chromatography (Poros HS) using a gradient of NaCl. Oligohistidine-endonuclease VII E86A was purified as described in Pöhler *et al.* (1996); briefly, the cleared lysate was applied to a nickel–NTA resin (Quiagen) and eluted using a gradient of imidazole, before further purification by ion exchange chromatography as above. Oligohistidine-endonuclease VII (wild-type) was purified from cleared lysate by application to a Fractogel EMD chelate column charged with nickel ions, and eluted using a gradient of imidazole. All proteins were dialysed for 2 h against 1 l 50 mM Tris–HCl, pH 7.4, 1 mM DTT, 50% glycerol. Protein concentrations were determined by the Bradford method, calibrated against a previous amino acid analysis of endonuclease VII-H41T (Giraud-Panis *et al.*, 1995). Concentrations are reported for monomeric species throughout.

### Other enzymes

Restriction enzymes (Promega) and polynucleotide kinase (Biolabs) were used as recommended by the manufacturer.

### T4 endonuclease VII cleavage reactions

Reactions were performed at 20°C in 50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 100 μg/ml BSA, 1 mM DTT (reaction buffer) in a total volume of 10 μl. The reaction was terminated by addition of 2 μl of a solution of 300 mM EDTA, 1 mg/ml proteinase K followed by incubation for 3 min at 20°C. The samples were loaded on 1% agarose and electrophoresed overnight at 30 V in TBE buffer.

*Time course of cleavage reaction.* pAT25*tetA* (8.2 μg) was incubated with 42 pmol of protein A-endonuclease VII H38T in a total volume of 85 μl in reaction buffer at 20°C. 10 μl aliquots were removed at intervals, the reaction terminated by addition of 2 μl of a solution of 300 mM EDTA, 1 mg/ml proteinase K, and incubated for 3 min at 20°C before electrophoresis.

*Cleavage reactions with heterodimeric endonuclease VII.* A range of mixtures of protein A-endonuclease VII H38T and oligohistidine-endonuclease VII E86A were incubated together for 5 min in reaction buffer + 50 mM NaCl, from 0.62 pmol of protein A-endonuclease VII H38T alone to 0.62 pmol of protein A-endonuclease VII H38T + 12.4 pmol of oligohistidine-endonuclease VII E86A. 700 ng of pAT25*tetA* were added, and incubated for 5 min at 20°C in a final volume of 10 μl. The cleavage reaction was terminated by addition of proteinase K and EDTA as above.

*Analysis of plasmid DNA following reaction with endonuclease VII.* Each digested sample was loaded on a 1% agarose gel in TBE buffer and electrophoresed either for 16 h at 30 V or for 5 h at 70 V. DNA was stained using 1 μg/ml ethidium bromide, destained extensively in water, and photographed under UV illumination using Kodak Tri-X Pan film. Negative photographs were scanned densitometrically, and quantified using ImageQuant (Molecular Dynamics). The data are presented as negative images taken from the densitometry.

### Mapping the endonuclease VII cleavage site in pAT25tetA

pAT25*tetA* (14 μg) were digested in 60 μl of reaction buffer by 67 pmol of protein A-endonuclease VII H38T for 5 min at 20°C. Protein was removed by phenol extraction, and the DNA precipitated by ethanol. The DNA was divided into three portions; 2.5 μg were digested with *Nru*I, 3.6 μg were digested with *Sal*I, and 5.6 μg were digested with *Bam*HI. The restriction-digested DNA was precipitated by ethanol, and loaded onto a 1% agarose gel and electrophoresed for 5 h at 70 V in TBE buffer. The sizes of restriction fragments were calibrated against a set of standard DNA fragments of known length (1 kb ladder, Gibco-BRL) by interpolation from a regression fit to a semilogarithmic plot.

### Electrophoretic retardation analysis

0.19 nmol of radioactively 5′ $^{32}$P-labelled J1–30 in 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM DTT, 100 μg/ml BSA, 5 mM EDTA (binding buffer) were incubated for 10 min at room temperature with either 10 nmol of protein A-endonuclease VII H38T, 20 nmol of oligohistidine-endonuclease VII E86A or a mixture of 10 nmol protein A-endonuclease VII H38T + 20 nmol oligohistidine-endonuclease VII E86A (previously incubated together for 5 min) in a total volume of 10 μl. 2 μl of a 30% (w/v) solution of ficoll and marker dyes was added, and the samples loaded on a 6% (w/v) polyacrylamide (acrylamide:bis, 20:1) gel in TBE buffer. Electrophoresis was performed at 4°C for 5 h at 220 V. The gel was dried and subjected to autoradiography using X-ray film (Konica AX).

## Acknowledgements

We thank our colleagues Drs Richard Pöhler and Malcolm White for discussion and the Cancer Research Campaign for financial support.

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*Received on December 18, 1996; revised on January 15, 1997*