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Tn*552* transposase purification and *in vitro* activities

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The *Staphylococcus aureus* transposon Tn*552* encodes a protein (p480) containing the 'D,D(35)E' motif common to retroviral integrases and the transposases of a number of bacterial elements, including phage Mu, the integron-containing element Tn*5090*, Tn*7* and IS*3*. p480 and a histidine-tagged derivative were overexpressed in *Escherichia coli* and purified by methods involving denaturation and renaturation. DNase I footprinting and gel binding assays demonstrated that p480 binds to two adjacent, directly repeated 23 bp motifs at each end of Tn*552*. Although donor strand cleavage by p480 was not detected, *in vitro* conditions were defined for strand transfer activity with transposon end fragments having pre-cleaved 3′ termini. Strand transfer was Mn^{2+}-dependent and appeared to join a single left or right end fragment to target DNA. The importance of the terminal dinucleotide CA-3′ was demonstrated by mutation. The *in vitro* activities of p480 are consistent with its proposed function as the Tn*552* transposase.

Key words: DNA binding/*Staphylococcus aureus*/strand transfer/Tn*552*/transposase

Introduction

Tn*552* is a 6545 bp element that confers resistance to β-lactam antibiotics in the Gram-positive pathogen *Staphylococcus aureus* (Murphy and Novick, 1979; Rowland, 1989; Rowland and Dyke, 1989, 1990). Thus it was intriguing to find homology between the presumptive transposase of Tn*552* and the integrase (IN) proteins of eukaryotic retroviruses and LTR-type retrotransposons (Rowland, 1989; Rowland and Dyke, 1990). The emerging similarities between Tn*552* and certain transposable elements from Gram-negative bacteria (integron-containing elements, Tn*7* and bacteriophage Mu; Figure 1) have provided a further incentive for functional and mechanistic studies on Tn*552* transposition.

Tn*552* and a number of other staphylococcal β-lactamase-encoding elements are believed to have evolved from a common ancestor; they are distributed among members of at least three major classes of large staphylococcal plasmids, and occupy a unique site on the *S.aureus* chromosome (Lyon and Skurray, 1987; Gillespie *et al.*, 1988; Weber and Goering, 1988; Paulsen *et al.*, 1994). Interestingly, there is at least one Tn*552*-like element that carries different antibiotic resistance genes (KG.H.Dyke, personal communication). Two independent insertions of Tn*552* in the *S.aureus* plasmid pI9789 have been characterized; it is striking that each insertion is upstream of a gene encoding a recombinase of the resolvase/invertase family (*binR* and *bin3/sin*). The large palindromic sequence upstream of *bin3/sin* appears to be a preferred target site for Tn*552* and a recombination hotspot (Rowland and Dyke, 1989; Paulsen *et al.*, 1994).

Three of the six proteins encoded by Tn*552* (p480, p271 and BinL; Figure 1) are likely to have a role in transposition (Rowland and Dyke, 1990). p480 was proposed to be the transposase, in the light of its homology with retroelement IN proteins, which process the ends of the element cDNA and insert it into host DNA. The region of significant homology includes the conserved 'D,D(35)E' motif (residues D166, D240 and E276 in p480) of the 'core' catalytic domain of IN proteins (Khan *et al.*, 1991; Kulkosky *et al.*, 1992; van Gent *et al.*, 1992; Bushman *et al.*, 1993). The D,D(35)E motif has since been recognized in the transposases of other bacterial elements, including Tn*5090* (Radstrom *et al.*, 1994), Tn*7* (Bissonette and Roy, 1992), bacteriophage Mu (Baker and Luo, 1994), the IS*3* family (Fayet *et al.*, 1990) and the IS*4* family (Rezsohazy *et al.*, 1993). Most elements encoding a D,D(35)E transposase, including Tn*552*, have the terminal dinucleotides 5′-TG...CA-3′ (Figure 1).

p271 is a good candidate for an accessory transposition protein since it contains an NTP binding motif similar to those found in the Mu B, Tn*7* TnsC and Tn*5090* TniB (Radstrom *et al.*, 1994) proteins. The genes encoding these proteins are located downstream of the respective transposase genes (Figure 1). Mu B and TnsC are ATP dependent, non-specific DNA binding proteins with crucial roles in target DNA selection (transposition immunity; Adzuma and Mizuuchi, 1988, 1989; Gamas and Craig, 1992; Bainton *et al.*, 1993). Mu B also participates in the assembly of a stable synaptic complex (Surette and Chaconas, 1991; Surette *et al.*, 1991; Baker *et al.*, 1991). p271 has been overexpressed and substantially purified, but ATP binding or hydrolysis has not been detected (S.-J.Rowland, unpublished data).

BinL is a recombinase of the resolvase/invertase family; a possible function is the resolution of cointegrates formed during replicative transposition (Stark *et al.*, 1989; Grindley, 1994), although it is not known whether Tn*552* transposes replicatively (like phage Mu and Tn*3*), or by a conservative mechanism (like retroviruses and Tn*7*). BinL is 89% identical to the pI9789-encoded recombinase BinR, which catalyses both inversion and resolution reactions *in vivo* (Rowland and Dyke, 1988, 1990). However, *in vitro*, purified BinL and BinR have properties characteristic of transposon resolvases (S.-J.Rowland, in preparation).

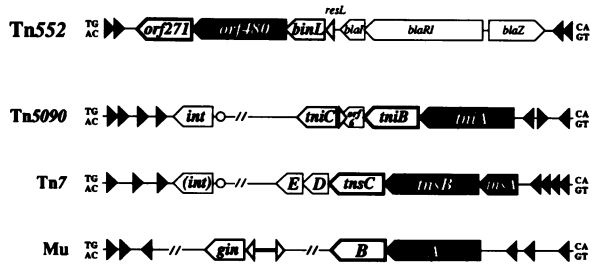

Fig. 1. Organization of transposition and recombination functions in Tn*552* (Rowland and Dyke, 1990; this work), Tn*5090* (Radstrom *et al.*, 1994), Tn*7* (Flores *et al.*, 1990; Sundstrom *et al.*, 1991) and bacteriophage Mu (Pato, 1989; Mizuuchi, 1992b) (diagrammatic: not to scale). For Tn*5090*, Tn*7* and Mu, only the genes encoding demonstrated or putative (*) transposition and site-specific recombination proteins are shown. The shading/outlining of genes indicates some degree of sequence homology between the products. *orf480*, *tniA**, *tnsB*, *tnsA* and the Mu *A* gene encode transposases; transposase binding sites at the ends of the elements are represented by arrowheads: the repeated sequences are 23 bp (Tn*552*; see Figure 5), 19 bp (Tn*5090*) and 22 bp (Tn*7* and Mu). *orf271**, *tniB**, *tnsC* and the Mu *B* gene encode ATP binding proteins. *binL*, *tniC** and *gin* encode site-specific recombinases of the resolvase/invertase family, and the *int* genes encode λ integrase-related recombinases (non-functional in Tn*7*); recombination sites are represented by arrowheads (*resL* upstream of *binL* and two *gix* sites upstream of *gin*; we note that there is a potential *res* site upstream of *tniC*), and small circles (upstream of *int*, representing the location of inserted gene cassettes). In Tn*552*, the *bla* genes specify inducible resistance to β-lactam antibiotics in *S.aureus*.

A comparison of Tn*552* with Tn*5090*, Tn*7* and phage Mu (Figure 1) suggests that its transposition functions are, in some respects, less complex. For example, Tn*552* has a similar arrangement of transposase binding sites at its left and right ends (this paper), whereas Tn*5090*, Tn*7* and Mu have distinctive, more elaborate left and right ends. In addition, Tn*552* has a preferred target site, but, unlike Tn*7* (Waddell and Craig, 1989), appears not to encode a separate protein to recognize this site. Like Tn*5090* (but not all integron-containing elements; Radstrom *et al.*, 1994), Tn*552* encodes a potential co-integrate resolvase; Tn*552* does not carry the λ-integrase-type recombinase and associated resistance gene cassettes characteristic of integrons (e.g. Collis *et al.*, 1993).

In vitro experiments have revealed fundamental similarities between the DNA strand transfer reactions catalysed by retroviral IN proteins and transposases from three bacterial elements (phage Mu, Tn*7* and Tn*10*; reviewed by Mizuuchi, 1992a,b). Proteins of the D,D(35)E family are nevertheless diverse in their primary structures and in their interactions with DNA and accessory proteins. The homology between p480, TniA of Tn*5090* and TnsB of Tn*7* (480, 559 and 702 amino acids, respectively) extends beyond the presumptive D,D(35)E catalytic domain, so that they can be aligned over the length of p480 (see Radstrom *et al.*, 1994). A potential DNA binding helix−turn−helix motif is located near the N-termini of these proteins (residues 36–55 in p480; Rowland and Dyke, 1990; Flores *et al.*, 1990), as in IS*3* family transposases (Prère *et al.*, 1990). In p480, this motif is part of a larger region (residues 8–62 of p480) with significant homology to several IS*3* family transposases, notably those of IS*2* and IS*426* (S.-J.Rowland *et al.*, unpublished).

In phage Mu transposition, it is clear that a single protein (Mu A) accomplishes all the essential reaction steps: Mu A recognizes the ends of the element, brings them together in a synapse and catalyses the strand cleavage and strand transfer reactions that join the ends to target DNA (Mizuuchi, 1992). Although the primary structure of p480 suggested that it might play a key role in Tn*552* transposition, this remained to be tested at the functional level. At present, there is no convenient assay for Tn*552* transposition in *S.aureus* and attempts to develop a 'mate-out' assay in *E.coli* (e.g. Rogers *et al.*, 1986) have not been successful (S.-J.Rowland, unpublished data). Therefore, as a first step in analysing the mechanism of Tn*552* transposition, p480 has been overexpressed, purified and characterized *in vitro*, and is shown to have activities consistent with its proposed function as a transposase.

Results

Overexpression and purification of Tn552 transposase

p480 and histidine-tagged p480 ('p480-His') were overexpressed in *E.coli* using a T7 RNA polymerase expression system (Studier *et al.*, 1990). In order to improve selection during the growth of cultures, the expression vectors were modified to confer kanamycin resistance. The vector pET-8c was further modified to fuse the histidine-tag to the N-terminus of p480 (see Materials and methods for details), facilitating its purification.

Like many other transposition proteins (e.g. Chalmers and Kleckner, 1994), p480 and p480-His had a strong tendency to aggregate; both were extremely insoluble when overexpressed under the culture conditions tested, and neither could be solubilized under non-denaturing conditions. A rapid method for the purification of p480 was developed that exploited this insolubility. An insoluble fraction highly enriched in p480 was produced and p480 was solubilized with 6 M guanidine hydrochloride (GuHCl). p480 was renatured by rapid dilution into a buffer containing 1 M NaCl and the non-denaturing detergent CHAPS; no other detergent tested was as effective, nor was slow or stepwise removal of the denaturant advantageous. The most important variable was the protein concentration, both before and after dilution, which if low enough allowed quantitative recovery of solubilized p480. Renatured p480 was further purified by gel filtration chromatography and concentrated by low salt precipitation; the final preparation was >90% pure when analysed by SDS−PAGE (Figure 2).

p480-His was purified by metal chelate chromatography in the presence of 6 M GuHCl, using Ni-NTA agarose resin, and by gel filtration chromatography following renaturation. Renatured p480-His also bound to the resin, demonstrating that the N-terminal histidine tag was accessible and providing a method to concentrate the protein. p480-His could not be renatured while bound to Ni-NTA agarose in a column since irreversible aggregation and precipitation occurred immediately upon elution. Although p480-His was relatively insoluble at low NaCl concentrations, the fraction remaining soluble at 20 mM NaCl was active in the binding and strand transfer assays described below and retained activity upon storage (data not shown). The molecular weight estimated by gel filtration chromatography was 50–52 kDa, indicating that

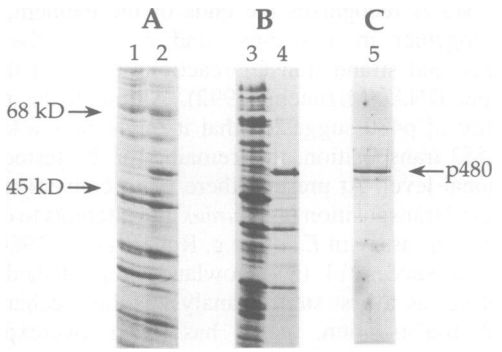

Fig. 2. SDS–polyacrylamide gels illustrating the overexpression and purification of p480. Gels A and B were stained with Coomassie Blue G250, and gel C with ammoniacal silver nitrate. p480 and the locations of size markers for gel A are indicated. Lanes 1 and 2: whole cell extracts of uninduced and induced cultures, respectively; lanes 3 and 4: supernatant and pellet fractions, respectively, of induced cells after lysis by sonication; lane 5: purified p480.

p480-His, with a predicted molecular weight of 57.8 kDa, was monomeric in solution under the conditions used here. The related Tn*7* protein, TnsB, has also been reported to be monomeric (Morrell, 1990; Arciszewska *et al.*, 1991). For both p480 and p480-His, specific DNA binding activity co-purified with monomer-sized protein at the gel filtration stage of purification.

p480 binds specifically to the ends of Tn552

The ends of Tn*552* are 80% identical inverted repeats (L1–116 and R1–121), each containing two adjacent 23 bp direct repeats (LI and LII; RI and RII) and an inverted repeat spanning ~50 bp (see Figure 5). The interactions of p480 and p480-His with L1–116 and R1–121 were analysed using a gel binding assay. Binding occurred specifically to DNA fragments containing both 23 bp repeats (L1–116, L1–70, R1–121 or R1–64; Figure 3A), producing in each case two retarded protein–DNA complexes (Figure 3B); the faster-migrating complex predominated at low protein concentrations. There was no significant difference in the affinity for the two ends (data not shown). A fragment containing one 23 bp repeat (LI; Figure 3A) produced a single retarded complex (Figure 3C). These data suggest that the 23 bp repeat contains the recognition sequence for p480. Interestingly, binding activity was obtained when partially-purified, denatured p480 (in 6 M GuHCl) was diluted directly into the assay mix (data not shown). Fractions containing proteolytic fragments of p480 or p480-His were also assayed (data not shown); several additional, faster-migrating protein–DNA complexes were observed, suggesting that one or more sub-fragments of p480 retain high-affinity sequence-specific DNA binding. A histidine-tagged deletion derivative of p480 lacking the N-terminal 148 amino acids has been overexpressed and substantially purified (S.-J.Rowland, unpublished data); this protein had neither DNA binding nor strand transfer activity, consistent with the proposed N-terminal location of the DNA binding domain.

The palindromic sequence between *binR* and *bin3* in pI9789 (Rowland and Dyke, 1989) contains a preferred integration site for Tn*552*. However, preliminary experiments have not demonstrated specific binding to this sequence by p480.

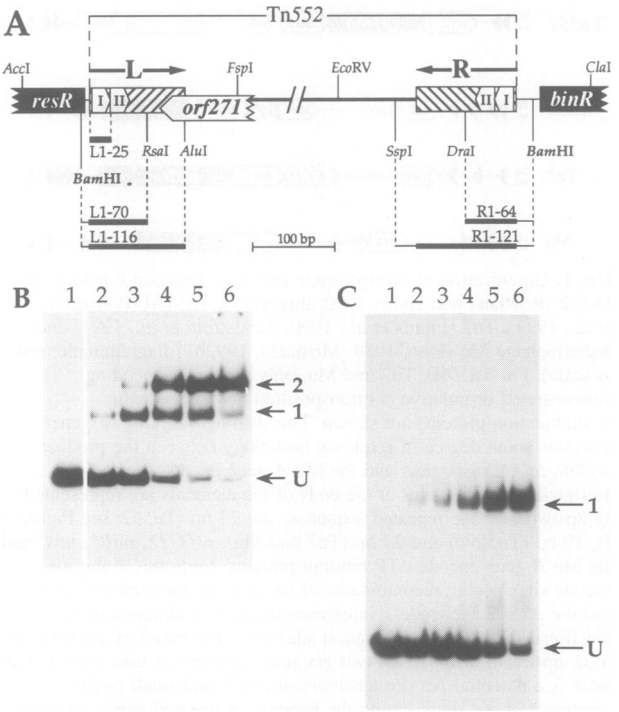

Fig. 3. (**A**) The ends of Tn*552* (located between *resR* and *binR* in pI9789; Rowland and Dyke, 1989) and the restriction fragments that were sub-cloned for this study (the *Bam*HI sites were created by site-directed mutagenesis). L and R are inverted repeats of 116 bp and 121 bp, respectively, each containing two 23 bp direct repeats (I and II; see Figure 5). The fragments are labelled according to the end sequences that they contain (numbering starts at the terminal nucleotides). Analysis of p480 binding to DNA fragments containing (**B**) L1–116 (142 bp) and (**C**) L1–25 (59 bp), by non-denaturing polyacrylamide gel electrophoresis. Protein–DNA complexes (1 and 2) and unbound DNA (U) are indicated. For each gel: lane 1, no p480; lanes 2–6, 100-, 30-, 10-, 3-fold diluted and undiluted p480 (4 μg/ml), respectively, was diluted into assay mixture.

Identification of p480 binding sites by DNase I footprinting

DNase I protection data for p480 binding to the ends of Tn*552* are shown in Figure 4 and summarized in Figure 5A. The footprints were extensive, covering bases L4–53 on the top strand and L1–52 on the bottom strand, and bases R4–54 on the top strand and R1–51 on the bottom strand. p480 therefore binds to the 23 bp repeats at the ends of Tn*552*. An isolated 23 bp motif was also protected from DNase I (data not shown). The individual repeats in each end were protected to similar extents, even at limiting protein concentrations, consistent with either highly cooperative binding (not supported by the binding studies described above), or non-cooperative binding with a similar affinity for each repeat. The phosphodiester bond between bases 7 and 8 on the bottom strand of each 23 bp repeat was accessible to DNase I, suggesting that p480 interacts equivalently with each repeat. However, striking patterns of DNase I hypersensitivity were observed at the 5′ ends of the element, immediately adjacent to the strongly protected regions. The bonds connecting the 3′ ends to flanking DNA, where p480 is expected to make

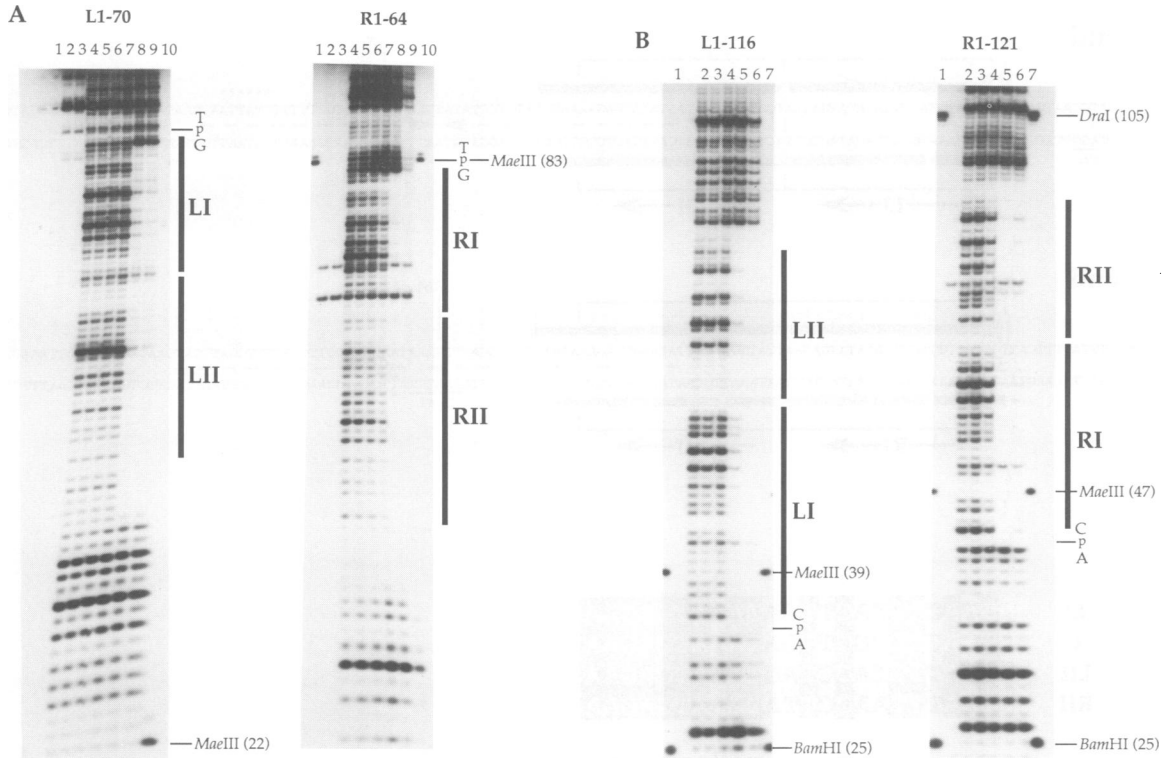

Fig. 4. DNase I footprinting of complexes formed between p480 and DNA fragments containing the left and right ends of Tn*552* (L1–116, L1–70, R1–121 and R1–64; Figures 3A and 5A); (**A**) and (**B**) represent analysis of the top and bottom strands (as shown in Figure 5A), respectively. The 23 bp repeats (LI, LII, RI and RII; Figure 5B), the terminal dinucleotides (5′-TpG; CpA-3′) and the sizes (nt) of the markers and the restriction enzymes used to generate them (see Figure 5A) are indicated. (A) (L1–70 and R1–64), lanes 1 and 10, markers; lane 2, no p480 or DNase I; lane 3, no DNase I; lane 4, no p480; lanes 5–9, 16-, 8-, 4-, 2-fold diluted and undiluted p480 (4 μg/ml), respectively, was diluted into assay mixture. (B) (L1–116 and R1–121), lanes 1 and 7, markers; lane 2, no p480; lanes 3–6, 30-, 10-, 3-fold diluted and undiluted p480 (4 μg/ml), respectively, was diluted into assay mixture.

cleavages (see below), were neither hypersensitive nor protected. p480-induced DNA bending at the 23 bp repeat LI was tested by the circular permutation method (using the vector pBend2; Kim *et al.*, 1989); a small, but significant p480-induced bend was observed (data not shown). The DNase I hypersensitivity could be a consequence of this distortion.

Higher resolution footprinting data are required to determine the possible significance of an inverted repeat of conserved bases within the 23 bp repeat (Figure 5B); a 4 bp inverted repeat provides important contacts within the 22 bp TnsB binding site (Arciszewska and Craig, 1991). It remains to be established whether p480 (or, indeed, TnsB; Arciszewska and Craig, 1991; Arciszewska *et al.*, 1991), binds to a single repeat as a monomer or a dimer, but it is notable that the Mu A protein binds as a monomer to a 22 bp motif (Kuo *et al.*, 1991).

p480 performs strand transfer in vitro

By analogy with characterized transposases (Mizuuchi, 1992a,b), Tn*552* transposase is expected to make site-specific endonucleolytic cleavages exposing the 3′-OH ends of the element, and to transfer these ends to 5′-phosphoryl groups 6 bp apart in opposite strands of the target DNA (Tn*552* is flanked by 6 bp duplications of target DNA; Rowland and Dyke, 1989). To date, donor strand cleavage by p480/p480-His has not been detected using either a linear DNA fragment or a supercoiled plasmid carrying a 'mini-Tn*552*' as a substrate. The requirement for cleavage was therefore bypassed by introducing a *Spe*I restriction site at each end of Tn*552*, so that digestion with *Spe*I created recessed, pre-cleaved 3′-OH ends with 4 nt 5′ overhangs (Figure 6A). A similar strategy has been used to uncouple strand cleavage and strand transfer in Mu, Tn*7* and AMV *in vitro* systems (Craigie and Mizuuchi, 1987; Bainton *et al.*, 1991; Fitzgerald *et al.*, 1992).

Strand transfer occurred in a simple buffer containing pre-cleaved end fragment, purified p480 or p480-His and Mn^{2+} (which could not be replaced by Mg^{2+}, Ca^{2+} or Zn^{2+}) (Figure 7). Since a divalent cation was not required for binding, it can be inferred that Mn^{2+} is a specific cofactor for the strand transfer reaction. Products were obtained in the absence of additional, non-specific DNA, reflecting the use of pre-cleaved end fragments as both substrates and targets. p480 and p480-His behaved similarly, therefore the N-terminal histidine-tag did not interfere significantly with activity. The reaction was optimal at 37°C and pH 7.5 (data not shown). Activity was not significantly affected by supplementing the reaction mixture (as specified in Materials and methods) with ATP, DMSO, BSA, CHAPS, spermidine or spermine, but ethylene glycol or yeast tRNA stimulated activity (data not shown). Higher glycerol concentrations increased the yield and also altered the distribution of individual products (Figure 7A, lanes 12–15), whereas strand transfer was significantly inhibited above 50 mM NaCl (Figure 7A; lanes 1–5). Inhibition of strand transfer by NaCl was

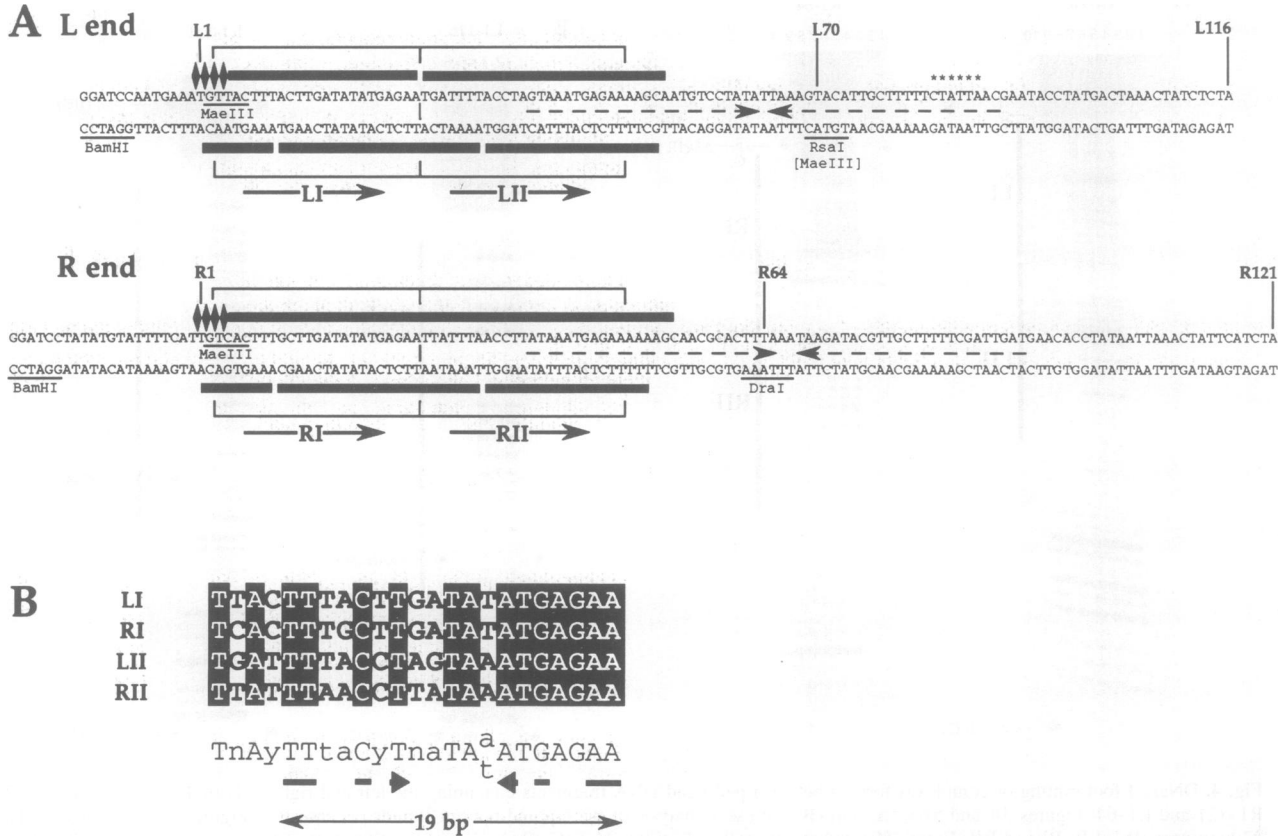

Fig. 5. (**A**) Summary of DNase I footprinting data for p480/p480-His binding to the ends of Tn*552*. The data are from Figure 4 and also from an analysis of DNA fragments with *Spe*I restriction sites overlapping the terminal base pairs and different flanking DNA sequences (not shown). The complete sequence of each end is shown (plus flanking sequence up to the *Bam*HI sites; see Figure 3A). Numbering starts at the terminal nucleotides. In each sequence, the inverted repeat (broken arrows) and the two 23 bp direct repeats (LI, LII; RI, RII; boxed) are indicated. The two stop codons at the end of *orf271*, which overlaps L1–116 (see Figure 3A), are marked with asterisks. Protected regions are represented by solid bars and sites of enhanced sensitivity are marked by diamonds. The restriction sites used to produce the marker fragments in Figure 4 are shown; the *Mae*III site overlapping bp L70 (in the fragment containing L1–70; Figure 3A) is a hybrid of the original *Rsa*I site and the *Hin*cII site in the cloning vector. (**B**) Alignment of the 23 bp repeats and a consensus sequence showing an imperfect inverted repeat of conserved bases.

reported for MoMLV, HIV-1 and visna virus integrases (Jonsson *et al.*, 1993; Vincent *et al.*, 1993; Katzman and Sudol, 1994) and was proposed to explain the accumulation of excised transposon fragments in a Tn*7 in vitro* system (Bainton *et al.*, 1991). Strand transfer was also inhibited by excess p480/p480-His (Figure 7A; lanes 7–11).

The strand transfer activity of p480/p480-His was unstable: the maximum yield of products was obtained within 30 s at 37°C and was not increased by a preincubation at 0°C; the failure of the reaction was not due to deterioration of the buffer (e.g. oxidation of the essential Mn^{2+}). Although most of the substrate remained unreacted, the reaction could not be rescued by adding fresh p480/p480-His. Furthermore, for a constant amount of added protein, it was beneficial to dilute the protein before adding it to the assay mix. These observations are consistent with non-productive aggregation of the added p480/p480-His, perhaps promoted by the conditions required for strand transfer activity (a low concentration of NaCl and a high concentration of Mn^{2+}). van Gent *et al.* (1992) reported that HIV-2 integrase could be significantly stabilized by binding to a solid support (thiopropyl-Sepharose). The effect of immobilizing p480-His, by preincubating with Ni-NTA agarose resin prior to initiating strand transfer, was therefore investigated (Figure 7B). The results suggest that resin-bound p480-His was inactive, but stable under the assay conditions, since active protein could be eluted by adding imidazole. It is possible that p480-His monomers were tethered to the resin in an inappropriate configuration, or too far apart to interact, for strand transfer.

The specificity of the strand transfer reaction was examined by labelling individual strands of the pre-cleaved substrate DNA fragment (as described by Craigie *et al.*, 1990; Bushman and Craigie, 1991). The results were as expected for ligation of the strand with the pre-cleaved 3′ end to target DNA (Figure 6B). Thus, when this strand (the 'transferred' strand) was 5′ end-labelled, products longer, as well as shorter, than the unreacted substrate strand were detected on denaturing polyacrylamide gels, whereas when the complementary ('non-transferred') strand was 5′ end-labelled, all the products were shorter (data not shown). In each case, the shorter strands presumably resulted from cleavage of the labelled strand when used as a target for strand transfer.

Evidence for the concerted integration of two pre-cleaved end fragments into a single target site was sought by including supercoiled plasmid DNA containing the palindrome from pI9789 as a target for strand transfer. The products were analysed by agarose gel electrophoresis (Figure 8; similar assays were described by Craigie *et al.*,

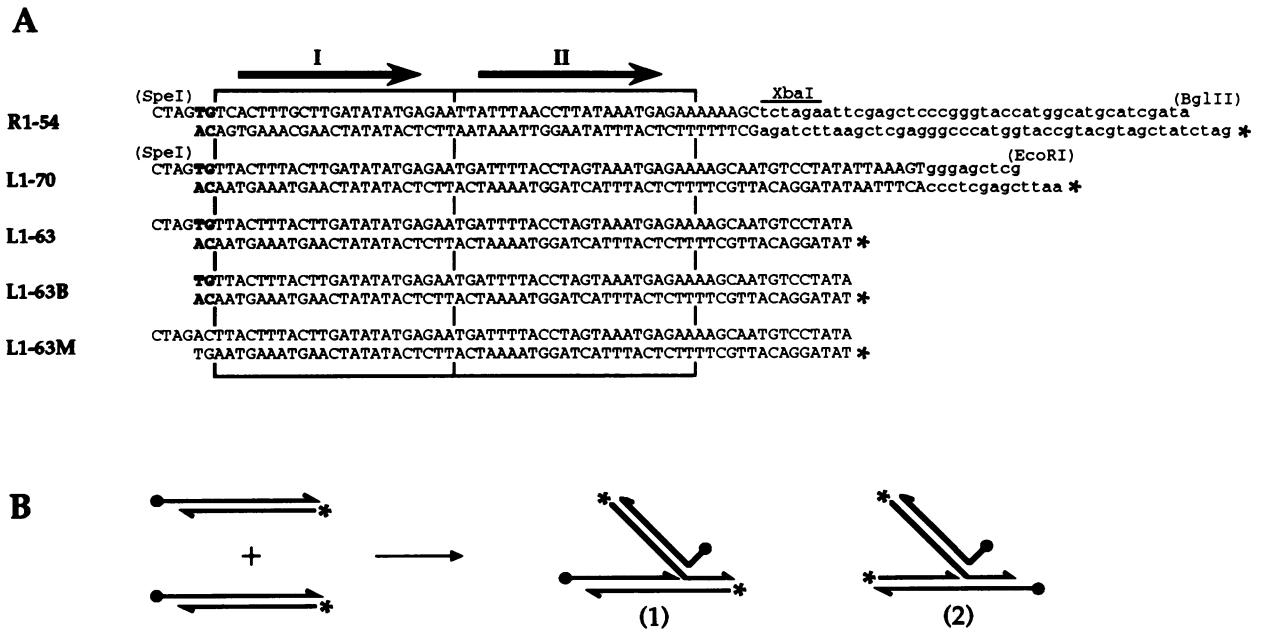

Fig. 6. (**A**) DNA fragments tested as strand transfer substrates, showing the 23 bp repeats (boxed) and the 5′ end labelling (*) of the transferred strand; the numbers in the fragment designations denote the end sequences contained (see Figure 5A). Non-transposon sequence (in the restriction fragments R1–54 and L1–70) is in lower case letters. L1–63, L1–63B and L1–63M were produced by annealing oligonucleotides. L1–63 has the 3′ end of the transferred strand exposed as if by *Spe*I digestion. L1–63B and L1–63M are identical to L1–63, except that L1–63B has a blunt end and L1–63M has a mutated terminal dinucleotide (CA to GT). (**B**) Diagram of the strand transfer assay: one pre-cleaved end fragment is shown integrating into another. The recessed pre-cleaved 3′-OH end is 5′ end-labelled (*), as in (A). Integration into the non-transferred strand (product 1) generates labelled strands longer than the substrate; integration into the transferred strand (product 2) generates labelled strands both longer and shorter than the substrate.

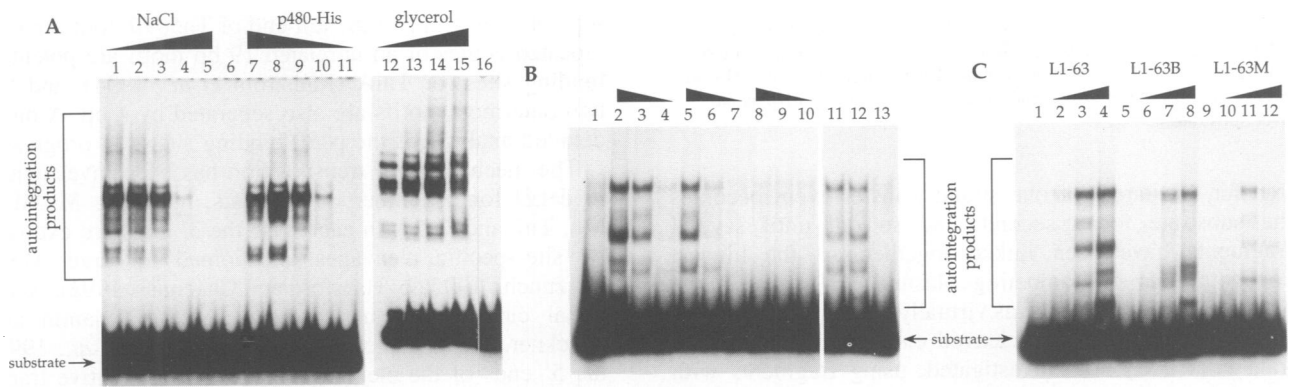

Fig. 7. Analysis of strand transfer products by non-denaturing polyacrylamide gel electrophoresis. (**A**) The effects of different concentrations of NaCl, p480-His and glycerol on the strand transfer activity of p480-His. Lanes 1–5: 10, 20, 50, 100 and 200 mM NaCl, respectively (and 20% glycerol; 0.2 μg/ml p480-His). Lanes 7–11: 1, 0.5, 0.2, 0.1 and 0.05 μg/ml p480-His, respectively (and 50 mM NaCl; 20% glycerol). Lanes 12–15: 5, 10, 20 and 40% glycerol, respectively (and 10 mM NaCl; 0.2 μg/ml p480-His). Lanes 6 and 16: no p480-His. The substrates (Figure 6A) were L1–70 (lanes 1–11) and R1–54 (lanes 12–16). (**B**) The effect of Ni-NTA agarose resin on strand transfer by p480-His (see Materials and methods for details). p480-His was incubated with equilibrated resin; an aliquot of this mixture ('preparation I') was supplemented with imidazole and incubated for a further 10 min., to elute bound p480-His ('preparation II'). Strand transfer was initiated by adding assay buffer containing L1–63 (Figure 6A). Lane 1, no p480-His; lanes 2–4, a control mixture of p480-His and resin equilibration buffer; lanes 5–7, preparation II; lanes 8–10, preparation I. In each set of three reactions the protein-resin mixtures were used undiluted, 3- and 10-fold diluted, respectively. Lane 13, preparation I; lane 12, preparation I supplemented with imidazole 5 min before initiating strand transfer; lane 11, preparation I supplemented with imidazole 5 min after initiating strand transfer. (**C**) The strand transfer activity of p480 with substrates L1–63, L1–63B and L1–63M (Figure 6A). Lane 1 (also 5 and 9), no p480; lanes 2–4 (also 6–8 and 10–12), 0.008, 0.04 and 0.2 μg/ml p480, respectively. Note: the NaCl concentration was 10 mM, except at the highest p480 concentration, when it was 25 mM.

1990 and Kulkosky *et al.*, 1992). Concerted integration would have generated linear plasmid molecules, but the only labelled plasmid species detected were relaxed circles, each tagged with one or more end-labelled end fragments; the same result was obtained using either a mixture of the substrates L1–70 and R1–54 (Figure 6A), or L1–70 alone. These data are consistent with 'single-ended' strand transfer events, in which joining of one pre-cleaved end is coupled to single-strand cleavage of the circular DNA target. Restriction analysis of the labelled products provided no evidence for preferential insertion into the palindrome (data not shown). A ladder of autointegration products was observed; this suggests that, due to aggregation of protein–DNA complexes competent for strand

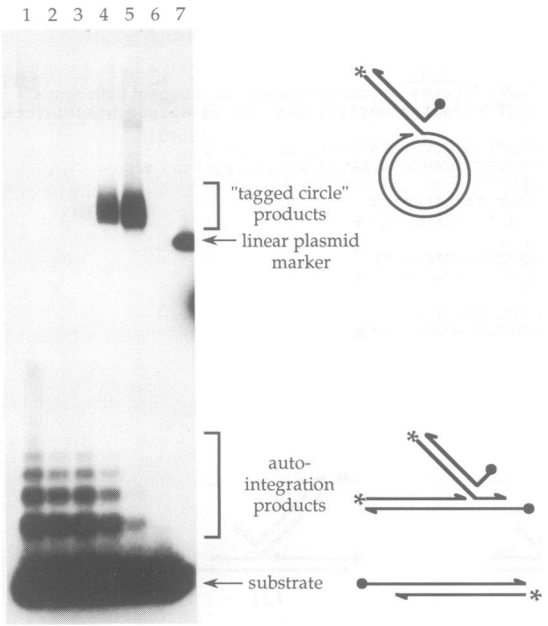

Fig. 8. Analysis of strand transfer products by non-denaturing agarose gel electrophoresis (1.2% gel), illustrating the effect of including supercoiled plasmid DNA as a target. The substrate and (the simplest) products are diagrammed; the integrating strand is 5′ end-labelled (*) as in Figure 6B. The substrate DNA was an equimolar mixture of L1–70 and R1–54 (Figure 6A), at a total concentration of ~0.005 pmol/μl. The 2641 bp plasmid was a recombinant of pMTL23 and the 161 bp *SspI*–*BclI* fragment containing the 67 bp palindromic sequence between *binR* and *bin3* in pI9789 (Rowland and Dyke, 1989). Lane 1, no plasmid DNA; lanes 2–5, 0.001, 0.01, 0.1 and 1 μg/ml plasmid DNA, respectively; lane 6, no p480 and lane 7, linear plasmid marker. The molar ratio of substrate to plasmid DNA ranged from a ~10-fold excess of substrate over plasmid, to a ~100-fold excess of plasmid over substrate.

transfer, the target of one strand transfer event becomes the substrate for a second, and so on, until several fragments have been linked together. At the highest concentrations of competing plasmid DNA target, the autointegration reaction was virtually abolished (Figure 8).

The nature of the pre-cleaved end required for strand transfer activity was investigated, using fragments with pre-cleaved 3′ ends that were either recessed, flush with the 5′ end, or recessed but with the terminal dinucleotide mutated from CA to GT (L1–63, L1–63B and L1–63M; Figure 6A). Both L1–63M and L1–63B were relatively poor strand transfer substrates (Figure 7C), demonstrating the importance of the terminal dinucleotide and the influence of flanking DNA, respectively. However, neither of these features was essential for substrate recognition, since p480-His had similar affinities for all three fragments in a gel binding assay (data not shown). Furthermore, a good strand transfer substrate (e.g. L1–70 or R1–54; Figure 6A) could be inactivated by end-filling with Klenow polymerase (data not shown). This indicates that strand transfer cannot proceed with a non-specific free 3′-OH that is displaced 4 nucleotides from the transposon end.

Discussion

The Tn*552*-encoded protein p480 and an N-terminally histidine-tagged version, p480-His, have been over-expressed and purified, both protocols involving denaturation and renaturation. Several retroviral IN proteins have also been purified using histidine tags (Bushman *et al.*, 1993; Jonsson *et al.*, 1993; Katzman and Sudol, 1994) and HIV IN has been renatured by a method very similar to that described here (Grandgenett and Goodarzi, 1994). The *in vitro* properties of p480 and p480-His are similar, and consistent with the proposal that p480 is a transposase.

Figure 1 compares the organization of transposition and site-specific recombination functions in Tn*552*, Tn*5090*, Tn*7* and phage Mu. p480 was shown to bind specifically to the two 23 bp repeats at each end of Tn*552*; the arrangement of the repeats means that bound molecules would be on the same face of the DNA helix. The regions protected from DNase I were extensive, but did not include the bonds where strand cleavage(s) would be made (Figure 5A). These features of binding are shared with TnsB (Arciszewska *et al.*, 1991; Arciszewska and Craig, 1991; Tang *et al.*, 1991), the Mu A protein (Zou *et al.*, 1991) and also γδ transposase (Wiater and Grindley, 1991) and IS*903* transposase (Derbyshire and Grindley, 1992). A further notable similarity between the DNase I footprints of p480 and TnsB is the hypersensitivity of several bonds near the 5′ termini (Figure 5A). The shortest strand transfer substrate tested contained 54 bp of one end (R1–54; Figure 6A); currently, there is no evidence that the inner half of the terminal inverted repeat has any function in binding or strand transfer.

As shown in Figure 5B, the p480 binding sites at each end of Tn*552* could be regarded as two 19 bp motifs separated by 4 bp. At the left end of Tn*5090*, four directly repeated copies of an unrelated 19 bp motif are potential binding sites for TniA (Radstrom *et al.*, 1994), and the two outermost motifs are also separated by 4 bp. A more detailed analysis of the p480 binding site is in progress.

The mechanism of transposition has been investigated in detail for a number of elements, including MoMLV, Mu, Tn*7* and Tn*10*; in each case the 3′ ends are exposed by site-specific cleavages and joined to target DNA (Mizuuchi, 1992a,b; Haniford and Chaconas, 1992). Additional cleavages, either at (e.g. Tn*10*; Benjamin and Kleckner, 1992) or near (e.g. Tn*7*; Bainton *et al.*, 1991) the 5′ ends of the element, ensure non-replicative transposition; replicative transposition requires that the 5′ ends remain joined to the donor replicon. Although *in vitro* conditions for donor strand cleavage by p480 were not established, p480 was active in strand transfer reactions with transposon end fragments having pre-cleaved 3′ termini. Concerted integration of two such fragments was not detected. It is possible that the conditions described here preclude formation of a productive synaptic complex between two ends; an interaction between the two ends, and in some cases the target DNA, is a prerequisite for donor cleavage in other elements (Bainton *et al.*, 1991, 1993; Surette *et al.*, 1991; Mizuuchi, 1992b; Murphy and Goff, 1992; Haniford and Kleckner, 1994).

The importance of the dinucleotide CA-3′ at the termini of Tn*552* was demonstrated by mutation, as it has been for other elements encoding a D,D(35)E transposase (including Tn*7*; P.Gary and N.L.Craig, personal communication). Although strand transfer was significantly inhibited by mutation from CA to GT (Figure 7C), binding by p480 was not detectably affected. The terminal base

pairs are therefore important for events subsequent to binding; their relative importance for strand cleavage and strand transfer remains to be determined. In other elements, mutations that preferentially inhibit either strand cleavage or strand transfer *in vitro* have been reported (LaFemina *et al.*, 1991; Surette *et al.*, 1991; Bushman and Craigie, 1992; Leavitt *et al.*, 1992; Haniford and Kleckner, 1994).

Strand transfer by p480 was relatively inefficient with a blunt, compared with a recessed, pre-cleaved end. In contrast, recessed and blunt pre-cleaved 3′ ends are used equally well by a Tn*7* *in vitro* system, even though a recessed end is the true intermediate (Bainton *et al.*, 1991). This difference could be consistent with the possibility that p480 makes cleavages only at the 3′ ends, and therefore does not normally deal with ends that have a double-strand break. Recent experiments suggest that TnsB and TnsA cleave at the 3′ ends and near the 5′ ends of Tn*7*, respectively (E.May, R.Sarnovsky and N.L.Craig, personal communication). Since p480 is homologous with TnsB, and since Tn*552* encodes a site-specific recombination system (*resL* and BinL; Figure 1) that could efficiently resolve cointegrates (S.-J.Rowland in preparation), it is reasonable to propose that Tn*552* transposes replicatively. The integron Tn*5090* also encodes a potential resolvase (TniC; Radstrom *et al.*, 1994), but this gene is absent from other integrons; it will be interesting to compare the modes of transposition of these elements with Tn*552*.

The divalent cation Mn^{2+} was essential for *in vitro* strand transfer by p480. In the case of Tn*7*, TnsA+TnsB+TnsC form a 'core recombination machine' that is activated by TnsD or TnsE (Bainton *et al.*, 1991; 1993). However, in the presence of Mn^{2+}, TnsA+TnsB alone can execute strand cleavage and joining reactions (M.Biery, M.Lopata and N.L.Craig, personal communication). Thus, the isolated transposase proteins of both Tn*552* and Tn*7* require Mn^{2+} for strand transfer, as do a number of purified retroviral integrases (Craigie *et al.*, 1990; Vincent *et al.*, 1993; Katzman and Sudol, 1994; Bushman and Wang, 1994). The cofactor *in vivo* is more likely to be Mg^{2+}, but the presumptive carboxylate ligands of the D,D(35)E motif (Kulkosky *et al.*, 1992) may bind Mn^{2+} more tightly, compensating for interactions that are inadequate or absent in the *in vitro* systems (Baker and Luo, 1994; Bushman and Wang, 1994).

The *in vitro* activities of p480 described in this paper are only a subset of those anticipated for a complete transposition reaction. It is hoped that further development of the system, for example by inclusion of p271 and perhaps unidentified host factors, will illuminate other aspects of Tn*552* transposition, such as donor strand cleavage, synapsis and concerted integration of the two ends and site-specific insertion into the palindromic sequence in pI9789.

Materials and methods

Enzymes

Restriction enzymes were from BRL, New England Biolabs and Boehringer Mannheim. T4 DNA ligase, Klenow fragment and T4 polynucleotide kinase were from BRL. Calf intestinal phosphatase (CIP) was from Boehringer Mannheim and mung bean nuclease from Promega. Sequenase version 2.0 DNA polymerase was from United States Biochemical Corp.

Nucleotides and oligonucleotides

Radiolabelled nucleotides were from ICN Flow at a specific activity of 3000 Ci/mmol, with the exception of [γ-^{32}P]ATP at 4500 Ci/mmol. Oligonucleotides were synthesized using an Applied Biosystems 391 DNA synthesizer and standard phosphoramidite chemistry. Oligonucleotides (except DNA sequencing primers) were purified on small sequencing gels and the bands were visualized using Stains-all (Aldrich Chemical Company). Annealing was performed by heating to 85°C in TE containing 0.1 M NaCl and cooling slowly to room temperature.

Site-directed mutagenesis

Site-directed mutagenesis was performed using Promega's Altered Sites *in vitro* Mutagenesis System. The absence of secondary mutations was verified by DNA sequencing.

Bacterial strains and plasmids

The expression vectors pET-3, pET-3a and pET-8c and the *Escherichia coli* strains BL21, BL21(DE3), BL21(DE3)[pLysS] and BL21(DE3)[pLysE] (Studier *et al.*, 1990) were donated by C.Preston (Institute of Virology, Glasgow). The ampicillin resistance gene of each vector was deleted as an *Ssp*I–*Dra*I restriction fragment and replaced (in the same orientation for transcription) by the kanamycin resistance gene (*Hin*cII fragment) from pUC4K (Pharmacia). The modified vectors were designated pKET-3, pKET-3a and pKET-8c. The 33 bp and 31 bp *Nhe*I–*Bam*HI fragments of pKET-3a and pKET-8c, respectively, were replaced by an *Nhe*I–*Bam*HI fragment (created by annealing two oligonucleotides) encoding a 'histidine-tag' (coding strand: 5′-GCTAGCCATCACCATCACCATCACAGCTCTGGTACCGGAGCTTTACGTACACGTGGATCC-3′; the *Nhe*I and *Bam*HI sites and the unique *Sna*BI fusion cloning site are underlined). The histidine-tag vectors were designated pKETH-3a and pKETH-8c. The two N-terminal amino acid residues of p480 (MK) were replaced by MASHHHHHHSSGTGALQ in p480-His.

The source of Tn*552* DNA was *S.aureus* plasmid pS1 (pI9789 containing Tn*552* inserted between *resR* and *binR*; Rowland and Dyke, 1989). In order to express p480, a *Bgl*II site was introduced by site-directed mutagenesis 62 bp upstream of *orf480* (base pairs 2396–2401 of Tn*552*, numbering from the left end); a second *Bgl*II site is located downstream of *orf480* (base pairs 708–713 of Tn*552*). The 1688 bp *Bgl*II–*Bgl*II fragment containing *orf480* was ligated to pKET-3 cut with *Bam*HI. In order to express p480-His, a *Bbs*I restriction site was introduced immediately upstream of *orf480*, so that digestion with (i) *Bbs*I and (ii) mung bean nuclease deleted the first 4 bp of *orf480*; the blunt-*Bgl*II fragment containing *orf480* was ligated to pKETH-8c cut with *Sna*BI and *Bam*HI.

Recombinants were screened in *E.coli* DS941 (*recF*, *lac*ZΔM15, *lac*I^q, Str^R) before transforming into *E.coli* BL21, BL21(DE3), BL21(DE3)[pLysS] and BL21(DE3)[pLysE]. Stocks of frozen cells ready for growth and induction were prepared as recommended by Studier *et al.* (1990).

DNA fragments were routinely subcloned in pTZ19R (Pharmacia) or pMTL23 (Chambers *et al.*, 1988).

Protein purification

Non-histidine tagged p480 was overexpressed in *E.coli* BL21(DE3) in the presence of pLysS. A 200 ml culture was grown directly from frozen stock cells in 2YT medium containing glucose (0.4%), kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml) to A_{600} 0.5–0.6, at which point IPTG was added to 0.4 mM and growth was continued for 3 h. The cells were chilled, harvested, washed in ice-cold buffer A [50 mM Tris–HCl (pH 8), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF (phenylmethylsulfonyl fluoride)] and resuspended in 10 ml buffer A. Lysis was achieved by three cycles of freezing in liquid nitrogen and thawing, with vigorous vortexing, and the viscosity was reduced by incubating with DNase I (10 μg/ml) and $MgCl_2$ (8 mM). Insoluble material, containing p480, was collected by centrifugation (50 000 *g*; 20 min.) and washed (using a Dounce homogenizer) with the following ice-cold buffers: (1) 10 ml buffer B (buffer A but 10 mM EDTA, 0.5% Triton X-100); (2) 10 ml buffer A; (3) 10 ml buffer C [4 M urea, 0.1 M Tris–HCl (pH 8), 1 mM DTT, 1 mM PMSF]; (4) 10 ml buffer A (twice); (5) 2 ml buffer D [50 mM Tris–HCl (pH 7.5), 6 M GuHCl (guanidine hydrochloride), 10 mM DTT, 0.1 mM EDTA] at 20°C. The final supernatant contained denatured p480, which was renatured by rapid dilution into 11 vol. ice-cold buffer E {55 mM Tris–HCl (pH 7.5), 1.1 M NaCl, 1.1 mM DTT, 0.11 mM EDTA, 11% glycerol, 5.5 mM CHAPS (3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 1 mM PMSF}. The remaining GuHCl was removed by dialysis against buffer F [50 mM Tris–HCl (pH 7.5), 1 M NaCl, 0.2 mM DTT, 0.1 mM EDTA, 10% glycerol, 2 mM CHAPS, 1 mM PMSF] at 4°C. The dialysate

was centrifuged at 50 000 *g* and aliquots of the supernatant were loaded onto a Superose 6 gel filtration column (Pharmacia) equilibrated with buffer G (buffer F without PMSF). Fractions containing p480 were pooled and dialysed against buffer H (buffer F but 50 mM NaCl). Precipitated p480 was completely re-solubilized in buffer G and re-centrifuged. The supernatant (containing 5–10 μg/ml p480 was diluted 1.8-fold with 100% glycerol and stored at −20°C.

Histidine-tagged p480 (p480-His) was overexpressed exactly as described for p480, except that buffer J [50 mM Tris−HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF] was used to wash and resuspend the cells for lysis. p480-His from a 100 ml culture was solubilized in 4 ml buffer K [50 mM Tris−HCl (pH 7.5), 6 M GuHCl, 10 mM β-mercaptoethanol, 5 mM imidazole], centrifuged (50 000 *g*; 20 min) and filtered (0.45 μm cellulose acetate; Costar). The filtrate was loaded at a flow rate of 3–4 column vol./h onto a 1 ml Ni-NTA agarose column (Qiagen, Hybaid) pre-equilibrated with buffer K at 20°C. The column was washed with 10 ml of each of the following buffers: (1) buffer K; (2) buffer K + 0.5% Triton X-100; (3) buffer K + 20% ethanol; (4) buffer K; (5) buffer K + 1 M NaCl. p480-His eluted in 2 ml of elution buffer (buffer K but with 50 mM imidazole) and was renatured by diluting in buffer E and dialysing against buffer F, exactly as described for p480. The dialysate was centrifuged (50 000 *g*; 30 min) and one aliquot of the supernatant (containing 50–100 μg/ml p480-His) was dialysed against buffer L [50 mM Tris−HCl (pH 7.5), 1 M NaCl, 2 mM CHAPS, 10% glycerol, 2 mM β-mercaptoethanol] to remove EDTA and DTT; this preparation was used, without further purification, in experiments requiring binding to Ni-NTA agarose. Remaining aliquots were loaded onto a Superose 12 gel filtration column (Pharmacia) equilibrated with buffer G and fractions containing p480-His (2–10 μg/ml) were collected. The column was calibrated with 25, 45 and 68 kDa protein markers (Combithek, Boehringer Mannheim). Fractions were diluted 1.8-fold with 100% glycerol and stored at −20°C.

The dilution buffers for p480 and p480-His were the buffers in which they had been prepared (or NaCl-free versions of these buffers for strand transfer assays), diluted 1.8-fold with 100% glycerol.

p480 and p480-His concentrations were estimated from the absorbances at 280 nm of the fractions obtained in the final gel filtration step.

Purification and labelling of DNA fragments

Restriction fragments for binding, footprinting and strand transfer experiments were purified by electrophoresis through 6% polyacrylamide gels. Gel slices were crushed and soaked in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and the solubilized DNA was filtered through 0.22 μm cellulose acetate ('Spin-X', Costar), extracted once with 1:1 phenol:chloroform and precipitated with ethanol.

Restriction fragments were labelled at their 3′ ends by end-filling with an appropriate [α-^{32}P]dNTP, using DNA polymerase I Klenow fragment. Fragments to be labelled at their 5′ ends were dephosphorylated using CIP, then phosphorylated using T4 polynucleotide kinase and [γ-^{32}P]ATP. Labelling reactions were terminated by extraction with 1:1 phenol:chloroform. Unincorporated nucleotides were removed by passage through a NucTrap column (Stratagene) and the DNA was precipitated with ethanol.

Restriction fragments with pre-cleaved ends for strand transfer experiments were excised from pMTL23- or pTZ19R-based recombinant plasmids by digestion with *Spe*I and *Eco*RI or *Bgl*II (Figure 6A); the *Spe*I sites were created by site-directed mutagenesis. The R1–54 sequence was made by annealing oligonucleotides. The strand with the pre-cleaved 3′ end (the 'transferred strand') was labelled at its 5′ end by phosphorylation at the *Eco*RI or *Bgl*II site, prior to digestion with *Spe*I; the 'non-transferred strand' was labelled at the *Spe*I site. Fragments L1–63, L1–63B and L1–63M (Figure 6A) were made by annealing oligonucleotides. The 5′ end-labelled strand was annealed with a 4-fold molar excess of the complementary, unlabelled strand. The fragment was passed through a NucTrap column and precipitated with ethanol. Strand transfer substrates were stored and diluted in 10 mM Tris−HCl (pH 7.5), 0.1 mM EDTA [10 mM Tris−HCl (pH 7.5), for reactions containing Ni-NTA agarose resin].

Gel binding assays

p480 or p480-His (1 μl) was mixed with labelled DNA fragment in 10 μl containing 10 mM Tris, 2 mM glycine, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol and 1–2 μg/ml carrier plasmid (pTZ19R) DNA, with 50–100 mM NaCl and 0.1 mM CHAPS contributed by the added protein fraction. After 10 min at room temperature (20°C), the entire reaction was loaded on a 6% polyacrylamide gel [acrylamide:bis-acrylamide 30:1; 50 mM Tris, 10 mM glycine, 0.1 mM EDTA (pH ~9.4)] that had been pre-run for 45 min at 20°C and 150 V (or at 4°C and 200 V). The gel was run for 2–3 h with constant recirculation of the buffer.

DNase I footprinting

Sequences from the left and right ends of Tn*552*, L1–116, L1–70, R1–121 and R1–64, were subcloned as *Bam*HI−*Alu*I, *Bam*HI−*Rsa*I, *Bam*HI−*Ssp*I and *Bam*HI−*Dra*I fragments, respectively, in pTZ19R cut with *Bam*HI and *Hin*cII (see Figures 3A and 5A). The *Eco*RI−*Pst*I fragments containing L1–116 and R1–121 were labelled at their *Eco*RI ends with Klenow fragment for bottom strand analysis (as shown in Figure 5A). The *Hin*dIII−*Sma*I fragments containing L1–70 and R1–64 were labelled at their *Hin*dIII ends with Klenow fragment for top strand analysis (as shown in Figure 5A).

p480/p480-His−DNA complexes were formed as described for gel binding assays, but in the presence of 10 mM $MgCl_2$ and in the absence of added carrier DNA, in a total volume of 3.5 μl (0.5 μl protein + 3 μl assay mixture). DNase I (1 μl of 1.5 μg/ml solution in 50% glycerol) was added and reactions were incubated for a further 10 min at 20°C before adding stop solution (4 μl formamide-dye containing 25 mM EDTA) and heating to 80°C. A 2–3 μl sample was analysed on a 6% sequencing gel, alongside markers generated by digesting aliquots of the fragment under investigation with appropriate restriction enzymes.

Strand transfer reactions

Strand transfer reactions were initiated by adding 1 vol. of p480 or p480-His (0.01–0.1 pmol; usually in 1 μl) to 10 vol. (usually 10 μl) of pre-warmed assay buffer containing labelled DNA (0.01–0.2 pmol). The final reaction contained 50 mM Tris−HCl (pH 7.5), 10 mM $MnCl_2$, 0.1 mM EDTA, 1 mM DTT, 20% glycerol, 0.1 mM CHAPS and 5–50 mM NaCl, the latter two reagents contributed by the added protein preparation. The glycerol concentration was reduced to 10% with the first two of the following reagents tested as supplements to the reaction: ethylene glycol (10%), dimethylsulfoxide (DMSO; 10%), CHAPS (10 mM), yeast tRNA (100 μg/ml) or bovine serum albumin (BSA; 100 μg/ml). When p480/p480-His was diluted in NaCl-free dilution buffer (in order to reduce the final concentration of NaCl in strand transfer reactions) dilutions were always prepared fresh. Reactions were routinely incubated for 10–15 min at 37°C and stopped by incubating for 20–30 min at 37°C with 0.5 vol. of gel loading buffer containing 60 mM EDTA, 0.3% SDS and 0.9 mg/ml protease K. The reaction products were analysed on 6–8% non-denaturing polyacrylamide gels using TBE buffer. Products for analysis on 6% sequencing gels were precipitated with ethanol and resuspended in formamide-dye loading mix.

In order to analyse the effect of Ni-NTA agarose resin on strand transfer, 40 μl p480-His (50–100 μg/ml in buffer L) and 60 μl of 25% resin [equilibrated in 50 mM Tris−HCl (pH 7.5), 2 mM CHAPS, 2 mM β-mercaptoethanol] were incubated at 0°C for 2 h with frequent mixing. Reactions were initiated by adding 100 μl of pre-warmed assay mixture to 10 μl of protein−resin mixture. The final reaction contained 50 mM Tris−HCl (pH 7.5), 10 mM $MnCl_2$, 2 mM β-mercaptoethanol, 20% glycerol, 0.1 mM CHAPS and 25 mM NaCl. p480-His was prevented from binding to the resin, or eluted from the resin by adding imidazole to a final concentration of 50 mM. Protease K stop mix (as above) was added after 1 h and reaction tubes containing resin were centrifuged briefly before removing samples for analysis.

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