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The phage Mu transpososome core: DNA requirements for assembly and function

Harri Savilahti, Phoebe A.Rice and Kiyoshi Mizuuchi[1]

Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 5, Room 237, Bethesda, MD 20892, USA

[1]Corresponding author

The two chemical steps of phage Mu transpositional recombination, donor DNA cleavage and strand transfer, take place within higher order protein–DNA complexes called transpososomes. At the core of these complexes is a tetramer of MuA (the transposase), bound to the two ends of the Mu genome. While transpososome assembly normally requires a number of cofactors, under certain conditions only MuA and a short DNA fragment are required. DNA requirements for this process, as well as the stability and activity of the ensuing complexes, were established. The divalent cation normally required for assembly of the stable complex could be omitted if the substrate was prenicked, if the flanking DNA was very short or if the two flanking strands were non-complementary. The presence of a single nucleotide beyond the Mu genome end on the non-cut strand was critical for transpososome stability. Donor cleavage additionally required at least two flanking nucleotides on the strand to be cleaved. The flanking DNA double helix was destabilized, implying distortion of the DNA near the active site. Although donor cleavage required Mg^{2+}, strand transfer took place in the presence of Ca^{2+} as well, suggesting a conformational difference in the active site for the two chemical steps.

Keywords: DNA transposition/protein–DNA complex/site-specific recombination

Introduction

A large number of mobile genetic elements translocate among sites on the chromosome of their host organisms by a special class of DNA recombination called transposition (for reviews see Haniford and Chaconas, 1992; Mizuuchi, 1992). These elements include both prokaryotic and eukaryotic transposons (Berg and Howe, 1989; Kaufman and Rio, 1992; van Luenen *et al.*, 1994). Retrotransposons and retroviruses integrate their DNA into the host chromosome by a closely related mechanism (for reviews see Bingham and Zachar, 1989; Boeke, 1989; Brown, 1990; Goff, 1992).

Critical to transpositional recombination are the two chemical reactions carried out by the element-encoded transposase or integrase protein. First, the transposon/flanking host DNA boundary is cut at the 3′-ends of the element, exposing the 3′-OH ends (Mizuuchi, 1984; Craigie and Mizuuchi, 1985; Fujiwara and Mizuuchi, 1988; Brown *et al.*, 1989; Roth *et al.*, 1989). Second, the donor 3′-ends are joined to a target DNA by a pair of strand transfer reactions, generating a branched or gapped DNA intermediate, which is subsequently resolved by host proteins. Prior to strand transfer some elements also cut the other strand at or near the ends of the transposon, resulting in excision of a linear transposon fragment from the host chromosome (Bainton *et al.*, 1991; Haniford *et al.*, 1991; Benjamin and Kleckner, 1992; Kaufman and Rio, 1992; van Luenen *et al.*, 1994). Some elements bypass the donor DNA cleavage step, also known as 3′ processing, because of the lack of extra nucleotides at the element DNA ends (Eichinger and Boeke, 1990).

Phage Mu transposition is one of the most thoroughly studied examples of transpositional recombination (Haniford and Chaconas, 1992; Mizuuchi, 1992). While a monomeric form of the Mu transposase (MuA) exhibits sequence-specific DNA binding activities (Craigie *et al.*, 1984; Kuo *et al.*, 1991), it fails to mediate either of the two chemical steps. Instead, these steps take place within the context of stable higher order protein–DNA complexes called Mu transpososomes (Craigie and Mizuuchi, 1987; Surette *et al.*, 1987; Mizuuchi *et al.*, 1992). Within a Mu transpososome four MuA monomers tightly hold the two ends of the Mu genome together (Craigie and Mizuuchi, 1987; Surette *et al.*, 1987; Lavoie *et al.*, 1991; Baker and Mizuuchi, 1992; Baker *et al.*, 1993). Each of the Mu ends carries, in different arrangements, three copies of the end-type MuA binding sites, which are named, in order from the end-most site, R1, R2 and R3 and L1, L2 and L3, for the right and left ends respectively (Craigie *et al.*, 1984). Within the transpososome, however, only three of the binding sites, R1, R2 and L1, are tightly bound by the MuA tetramer (Lavoie *et al.*, 1991; Mizuuchi *et al.*, 1991, 1992). This basic structure of the transpososome, referred to as the transpososome core, is maintained throughout the two chemical steps of transpositional recombination.

Three forms of Mu transpososomes with increasing stability have been identified, based on the chemical steps their DNA components have undergone (Figure 1). The first complex on the reaction pathway, the stable synaptic complex (the SSC, also called the type 0 complex), contains donor DNA that has not yet been cleaved. The SSC accumulates when MuA carries an active site mutation (Baker and Luo, 1994) or when Mg^{2+} is replaced with Ca^{2+} (Mizuuchi *et al.*, 1992); Ca^{2+} promotes assembly of the transpososome, but cannot function as the cofactor for the donor DNA cleavage reaction. In the presence of Mg^{2+} or Mn^{2+}, cleavage at the Mu DNA ends generates the cleaved donor complex [the CDC, also called the type I complex (Craigie and Mizuuchi, 1987; Surette *et al.*, 1987)]. Next, the 3′-ends of the Mu DNA are covalently

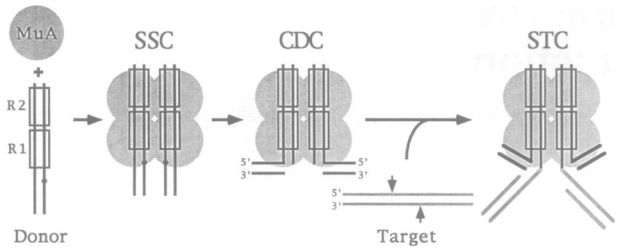

Fig. 1. Outline of Mu transpositional recombination. Three types of transpososomes corresponding to the three stages of the reaction (SSC, stable synaptic complex; CDC, cleaved donor complex; STC, strand transfer complex) are depicted. The transpososomes are shown with two copies of the Mu R end fragment, a configuration studied in this report, although natural Mu transpososomes contain one copy each of the left and right ends of the Mu genome. The R1 and R2 Mu end binding sites are shown as rectangles. MuA is depicted as a tetramer of shaded circles and target DNA is shown in gray. The small arrows on the target DNA indicate the 5 bp staggered locations for strand transfer on the two strands. The dots in the SSC indicate the Mu end cleavage sites.

connected to the two strands of a target DNA at 5 bp staggered positions by a coupled cleavage/joining reaction. This reaction produces the strand transfer complex (the STC, also called the type II complex), which contains the branched strand transfer product DNA (Craigie and Mizuuchi, 1985; Surette *et al.*, 1987). Efficient strand transfer, especially to intermolecular target sites, requires activation of the CDC and delivery of a target DNA to the transpososome by a second transposon-encoded protein, MuB (Maxwell *et al.*, 1987; Baker *et al.*, 1991). MuB is an ATP-dependent DNA binding protein involved in selection of proper target DNA sites (Adzuma and Mizuuchi, 1988, 1989, 1991).

Mu transpososome assembly is controlled by several cofactors: IHF and HU host proteins, an enhancer-like DNA sequence element present within the Mu DNA (the internal activating sequence or IAS), those MuA binding sites at the Mu ends that are not tightly bound in the assembled complex and a specific donor DNA topology (Craigie *et al.*, 1985; Craigie and Mizuuchi, 1986; Leung *et al.*, 1989; Mizuuchi and Mizuuchi, 1989; Surette and Chaconas, 1989; Surette *et al.*, 1989; Allison and Chaconas, 1992). These extensive cofactor requirements make it difficult to disect the separate contributions of the protein and DNA components to the assembly, stability and functionality of the Mu transpososome. Previously we found that modification of the reaction buffer, especially inclusion of dimethylsulfoxide (DMSO), alleviates the cofactor requirements for transpososome assembly (Craigie and Mizuuchi, 1986; Mizuuchi and Mizuuchi, 1989; Baker and Mizuuchi, 1992). We also noted that utilization of pre-cut donor DNA eliminates many of these requirements (Craigie and Mizuuchi, 1987; Mizuuchi and Mizuuchi, 1989). The normal transposition reaction takes place most efficiently with one left and one right end of the Mu DNA. With pre-cut donor DNA, however, strand transfer is most efficient when a pair of Mu right ends is used (Craigie and Mizuuchi, 1987; Namgoong *et al.*, 1994) and the ensuing transpososomes contain two copies of Mu right ends in which the R1 and R2 sites are stably bound by MuA tetramer (Mizuuchi and Adzuma, 1991; Mizuuchi *et al.*, 1991). Thus the asymmetric left/right donor end combination is not essential for transpososome assembly and activity.

In this study we used modified permissive reaction conditions and short Mu right end DNA fragments as the substrates for transpososome assembly. Donor DNAs with different lengths and configurations of the flanking segment outside the Mu end were compared for their ability to participate in the three reactions central to Mu transposition: transpososome assembly, donor DNA cleavage and DNA strand transfer. These studies provided insights into important transposase–substrate DNA interactions around the active site. In addition, we discovered a differential cation selectivity for the cofactor of the two chemical steps and uncovered a dramatic effect of the size of the participating DNA molecules on assembly efficiency and activity of the transpososome.

Results

Mu end DNA fragments can be assembled with MuA to form all three types of Mu transpososomes

To determine whether Mu transpososomes can be assembled with a short Mu end DNA fragment we prepared a 66 bp substrate DNA carrying a 50 bp sequence derived from the right end of the Mu genome. This segment includes the R1 and R2 MuA binding sites and 16 bp of flanking DNA (Figure 2A). In order to detect assembled complexes the substrate was labeled with ^{32}P and incubated with MuA in the presence or absence of divalent metal ions. Samples were taken at intervals, subjected to native agarose gel electrophoresis and gels analyzed by autoradiography (Figure 2B). When Mg^{2+} was included in the reaction two bands of protein–DNA complexes were observed. When Ca^{2+} was substituted for Mg^{2+} only the slower migrating band appeared and its intensity was considerably weaker than the corresponding band made in the presence of Mg^{2+}. In the absence of a divalent metal ion bands corresponding to the protein–DNA complexes were almost undetectable.

The above results suggested that a divalent metal ion was essential for formation of the observed complexes. Another possibility remained, however: complexes might form even in the absence of a metal ion, but are unstable during gel electrophoresis. To test this possibility reactions were carried out in two stages: a pre-incubation step, either in the presence of Ca^{2+} or without a metal ion, was followed by a chase reaction in which Mg^{2+} was added together with excess competitor DNA carrying two MuA binding sites, which prevented additional complex formation (Figure 2C). Prior to the chase limited amounts of the slower migrating complex were made (lanes 1 and 5). In contrast, with only a few minutes of Mg^{2+} chase a large increase in both slower and faster migrating complexes was observed (lanes 2–4 and 6–8). In a control reaction without pre-incubation the presence of competitor DNA during the reaction with Mg^{2+} prevented complex formation (lane 9). We conclude that competitor DNA-resistant complexes were formed during pre-incubation with Ca^{2+} or without a metal ion, but that most of these complexes did not withstand electrophoresis. Thus detection of these complexes by electrophoresis depends not only on their assembly, but also on their stability. Therefore, in this

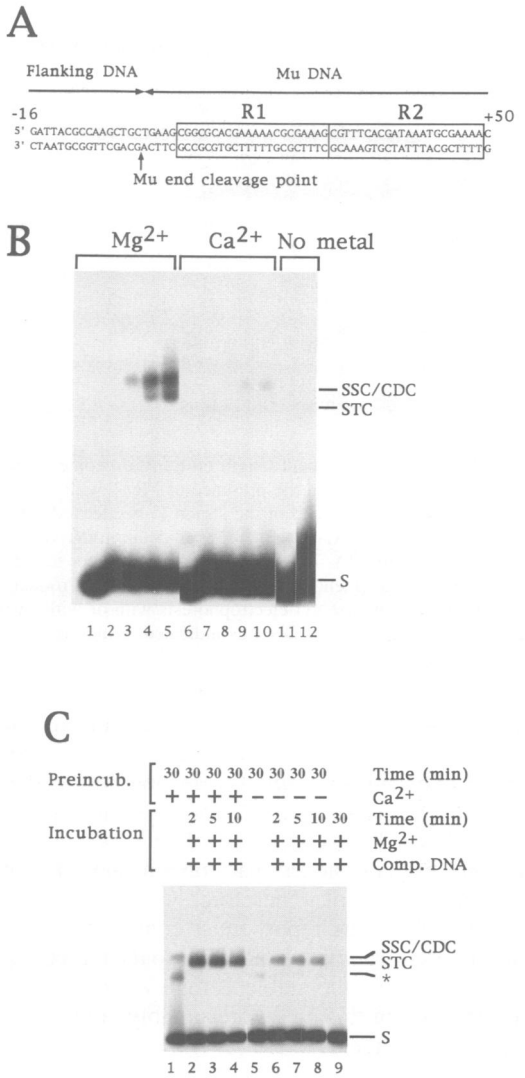

Fig. 2. (**A**) Sequence of the 66 bp Mu donor DNA fragment used in this study. It contains the first 50 bp of the right end of the Mu genome, including the R1 and R2 MuA binding sites (rectangles) and 16 bp of flanking host DNA sequence that has previously been shown to support efficient donor DNA cleavage in plasmid substrates (Wu and Chaconas, 1992). The flanking DNA is long enough to include all the nucleotides that may interact with MuA within a transpososome, as judged from previous footprinting experiments (Lavoie *et al.*, 1991; Mizuuchi *et al.*, 1991, 1992). (**B**) Time course of protein–DNA complex formation using the substrate shown in (A) radioactively labeled at the 5′-end of the bottom strand. MuA was incubated in the presence of Mg^{2+}, Ca^{2+} or in the absence of metal ions. Products were analyzed by native agarose gel electrophoresis (6 h, 2.7 V/cm, 4°C) without addition of competitor DNA. Samples prior to addition of MuA are shown in lanes 1, 6 and 11. Time points: 0 min, lanes 2 and 7; 10 min, lanes 3 and 8; 30 min, lanes 4 and 9; 60 min, lanes 5, 10 and 12. Positions of the transpososomes (SSC, stable synaptic complex; CDC, cleaved donor complex; STC, strand transfer complex) and the substrate (S) are indicated on the right. (**C**) Complex formation in the absence of Mg^{2+}. Reactions were carried out in two stages: a 30 min pre-incubation with Ca^{2+} or without a metal ion as indicated, after which competitor DNA and Mg^{2+} were added and the reaction was stopped at different time points. Lanes 1 and 5, pre-incubation only; lanes 2–4 and 6–8, time course after the chase; lane 9, reaction in the presence of Mg^{2+} and competitor DNA without pre-incubation. The radioactive band below the transpososomes which is marked with an asterisk is competitor sensitive and most likely represents MuA bound to a single Mu end fragment (see also Figure 5).

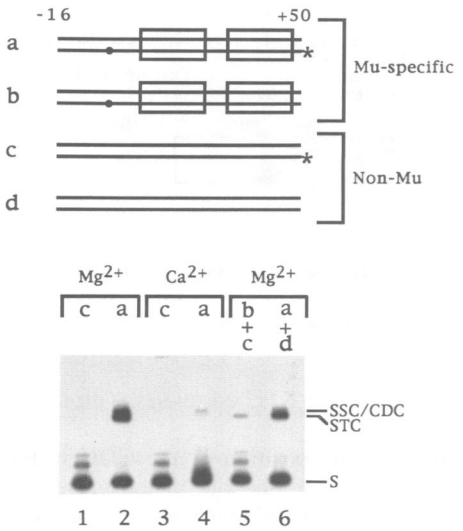

Fig. 3. Strand transfer complex formation with Mu-specific donor and non-Mu target DNA fragments. Strand transfer reactions were carried out in the presence of Mg^{2+} or Ca^{2+} using donor and target fragments, individually or combined as indicated (substrates a–d). The locations of radioactive label are indicated by asterisks. Reaction products were analyzed by native agarose gel electrophoresis in the presence of excess competitor DNA. Band positions are labeled as in Figure 2.

paper we operationally use the term 'stable complex' to refer to complexes which are not only competitor DNA-resistant, but also withstand the electrophoresis conditions used.

The protein–DNA complexes described in this paper possess all the basic attributes of Mu transpososomes characterized previously using larger DNA substrates.

The slower migrating complex observed in the presence of Mg^{2+}, which co-migrates in the gel with the complex formed in the presence of Ca^{2+}, presumably includes the SSC. This earliest form of the Mu transpososome has been shown to assemble in the presence of Ca^{2+}. Donor DNA cleavage, however, requires either Mg^{2+} or Mn^{2+} (Baker *et al.*, 1991; Mizuuchi *et al.*, 1992). In the above experiment, as expected, donor DNA cleavage did not occur in the presence of Ca^{2+} (data not shown).

The faster migrating stable complex contains the STC. We suspected that in the reactions containing Mg^{2+} the Mu end DNA fragments might also be used as a target, producing the STC. To test this possibility reactions were assembled in the presence of an additional 66 bp DNA fragment, the sequence of which was unrelated to that of the Mu ends (we shall call this the target fragment) (Figure 3). In the presence of Mg^{2+}, when the donor fragment was labeled, stable complexes with two distinct gel mobilities were observed, irrespective of the presence of the target fragment (lanes 2 and 6). However, only the faster migrating complex was detected by autoradiography when the labeled target fragment was combined with unlabeled donor fragment, demonstrating that it contains the STC (lane 5). As expected, no stable complexes were detected when only the target fragment was present in the reaction (lanes 1 and 3). When Ca^{2+} was used in the reaction with the labeled donor DNA only the slower migrating complex (the SSC) was observed (lane 4). As demonstrated below, formation of the faster migrating complex with this donor

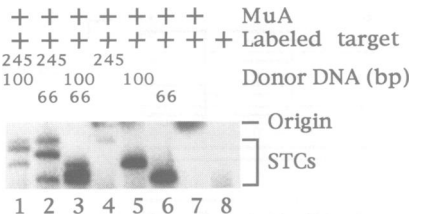

Fig. 4. The transpososome core contains two donor DNA fragments. Strand transfer reactions were assembled with unlabeled donor DNAs of different lengths and a labeled target DNA fragment. Complexes were analyzed by native agarose gel electrophoresis (see Materials and methods for details).

DNA assigned to the STC correlates with strand transfer activity.

To determine the position of the CDC in the agarose gel, reactions were carried out in the presence of Mg^{2+} and the two bands containing transpososomes were excised from a gel similar to that shown in Figure 2; the DNA contents of the bands were analyzed by urea–polyacrylamide gel electrophoresis (data not shown). Although some cross-contamination was unavoidable due to the close proximity of the two bands, it was clear that the slower migrating band contained both uncleaved donor DNA and donor DNA cleaved at the end of the Mu sequence. In contrast, the faster migrating band contained strand transfer products. In addition, cleaved donor DNA, but very little strand transfer product, was observed early in the reaction time course, when only a trace amount of the faster migrating complex was detectable (see Figure 2B). Thus the CDC co-migrates with the SSC under the electrophoresis conditions used.

Previous studies have shown that a Mu transpososome contains four MuA molecules (Lavoie *et al.*, 1991; Baker and Mizuuchi, 1992; Baker *et al.*, 1993). To confirm that the stable complexes formed with the 66 bp substrate DNA share this common architecture we analyzed the oligomeric state of MuA by protein cross-linking, essentially as previously described (Lavoie *et al.*, 1991). Samples containing all three forms of Mu transpososome were made in the presence of Mg^{2+} using ^{35}S-labeled MuA treated with the homobifunctional cross-linking agent dithio-*bis*(succinimidyl propionate) (DSP) and analyzed by agarose–polyacrylamide composite gel electrophoresis in the presence of SDS with or without partial reversal of the cross-linking. As has been observed previously with Mu transpososomes made with a variety of substrates (Lavoie *et al.*, 1991; Baker and Mizuuchi, 1992), cross-linking produced a slowly migrating oligomeric form of MuA, which upon partial reversal of cross-linking yielded two additional bands between itself and the monomer position (data not shown). Thus the stable complexes studied here, like conventional Mu transpososomes, contain a tetrameric form of MuA.

To determine the number of Mu end fragments contained within the transpososome, complexes were prepared using pairs of different length Mu donor DNA fragments. To avoid complications due to the gel mobility differences among the different forms of complexes we used unlabeled donor DNA fragments with ^{32}P-labeled target DNA. Thus only strand transfer complexes were visible in this experiment. When only one length of donor fragment was used a single STC band was observed (Figure 4, lanes 4–6).

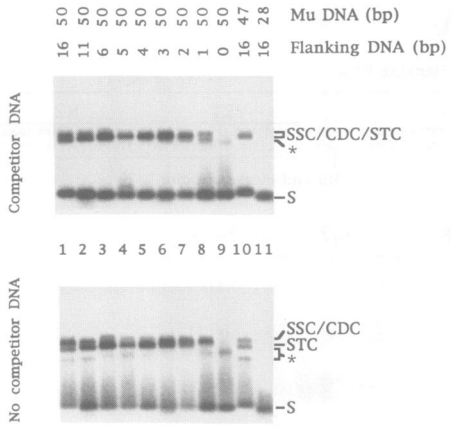

Fig. 5. Effect of donor fragment length on transpososome assembly. Reactions were carried out in the presence of Mg^{2+} with donor fragments labeled at the 5′-end of the strand to be cleaved. These fully double-stranded donor DNA fragments were deletion derivatives of that depicted in Figure 2A. The numbers at the top refer to the length (bp) of the Mu end segment and the flanking segment. Products were analyzed by native agarose gel electrophoresis with or without prior addition of excess competitor DNA as indicated. Band positions are labeled as in Figure 2.

However, when donor fragments of two different lengths were mixed STCs with three different gel mobilities were detected: two with mobilities corresponding to the STCs made with unmixed fragments and one with intermediate mobility (lanes 1–3). The band of intermediate mobility must represent complexes that contain one long and one short donor fragment. Therefore, we conclude that the STCs studied in this report must contain two copies of the donor DNA fragment in addition to the target fragment. By analogy, all of the stable complexes detected by gel electrophoresis in this study presumably contain a pair of donor DNA fragments.

Influence of the donor DNA fragment length

To investigate the effect of donor DNA length on the behavior of the transpososome we prepared Mu end DNA fragments further resected from the 66 bp fragment used in the previous experiments. Formation of transpososomes with these fragments in the presence of Mg^{2+} was assayed by native agarose gel electrophoresis. Gels were run with or without addition of excess competitor DNA following formation of the complexes, in order to distinguish transpososomes from competitor DNA-sensitive complexes (Figure 5). Efficient complex formation required both the R1 and R2 MuA binding sites: while deletion of a small part of R2 had only minor effects (lane 10), a larger deletion that eliminated R2 abolished stable complex formation (lane 11). The length of the outside flanking sequence was not critical; even the presence of 1 bp beyond the Mu end was sufficient for stable complex formation (lane 8). If the entire outside sequence was removed, however, only a small amount of stable complexes was detectable (lane 9). The complexes that migrated in the gel at the positions we assigned for transpososomes were all resistant to the competitor DNA (compare the two panels). When the samples were analyzed without addition of the competitor DNA an additional protein–DNA complex which migrated faster than the transpososomes was observed; the quantity of this

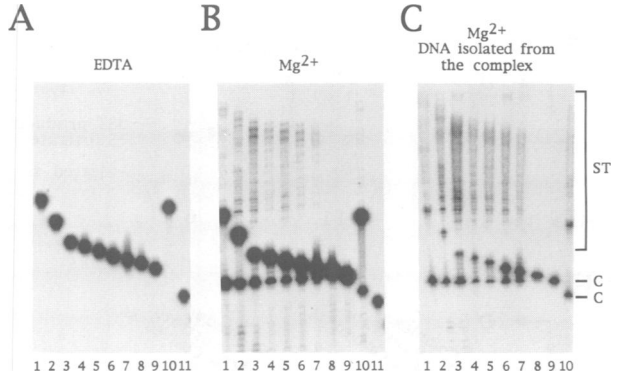

Fig. 6. Effect of donor fragment length on cleavage and strand transfer. The same donor DNA fragments as in Figure 5 were used (lanes are labeled accordingly). Products were analyzed on an 8% urea–polyacrylamide gel and visualized by autoradiography. (**A**) Reactions without metal ions (in the presence of 10 mM EDTA). (**B**) Reactions with Mg^{2+}. (**C**) As (B) except that the DNA samples were isolated from the transpososome bands in a native agarose gel similar to that shown in the upper panel of Figure 5. Band positions: C, donor DNA cleavage product; ST, strand transfer products.

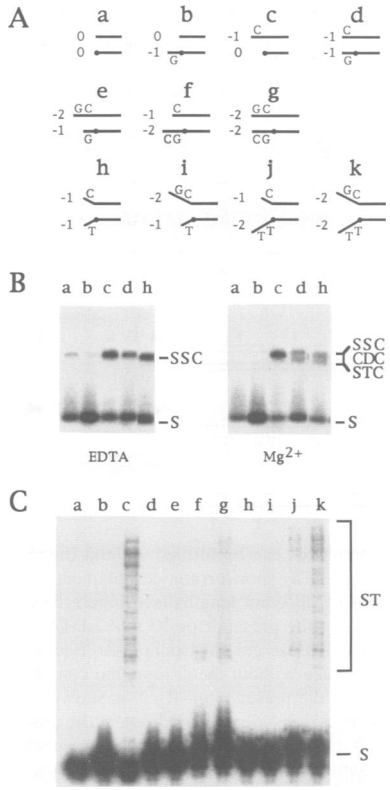

Fig. 7. Critical residues in the flanking DNA for stable complex formation and donor DNA cleavage. (**A**) Substrates (a–k) contained 50 bp of the Mu end sequence (see Figure 2A) in addition to the flanking nucleotides depicted. The numbers on the left indicate the lengths (nt) of each flanking strand. Note that substrates h–k contained non-complementary flanking nucleotides. The dots in substrates indicate the donor cleavage sites. (**B**) Transpososome formation with the substrates shown in (A). Reactions were carried out in the presence of 10 mM Mg^{2+} or 10 mM EDTA and products were analyzed by native agarose gel electrophoresis. Note that while substrates d and h cannot accomplish donor cleavage or strand transfer (C), the SSC formed with those substrates carrying a very short flanking segment apparently can bind a target DNA fragment stably enough to generate complexes that migrate at the position of the STC. Such complexes were most easily detectable when the reactions contained Mg^{2+} and also an excess of potential target DNA, such as the competitor DNA (see also Figure 5, lane 8). (**C**) Samples of the above reactions with Mg^{2+} were analyzed for strand transfer by electrophoresis on an 8% urea–polyacrylamide gel. S, substrate; ST, strand transfer products.

competitor-sensitive complex varied among experiments and this complex was not characterized further.

Next we examined the influence of donor DNA length on the chemical steps of transposition. Reactions were assembled with the above substrates in the absence of a metal ion or in the presence of Ca^{2+} or Mg^{2+}; DNA products were analyzed by urea–polyacrylamide gel electrophoresis. In addition, to confirm the association of the DNA products with the transpososomes the complexes made in the presence of Mg^{2+} were first separated by native agarose gel electrophoresis, after which DNA was isolated from the complex bands and analyzed (Figure 6). Neither donor cleavage nor strand transfer was detected in reactions in the absence of a metal ion (Figure 6A) or in the presence of Ca^{2+} (data not shown). In general, in the presence of Mg^{2+} donor cleavage and strand transfer took place in parallel with stable complex formation (compare Figure 6B with Figure 5). The only exception was the donor fragment with a 1 bp flanking sequence, which was not cleaved (nor did it undergo strand transfer), despite forming stable complexes (lane 8). Essentially all of the DNA cleaved at the Mu end and all the strand transfer products were associated with the complexes and not found as free DNA (data not shown), indicating that they existed as the CDC and the STC respectively (Figure 6C). A limited amount of uncleaved donor DNA was also evident in the complexes (Figure 6C). It was most likely derived from the SSC and possibly from complexes that contained one cleaved and one uncleaved donor fragment.

The donor DNA fragments of various lengths were also tested for their strand transfer activity using ϕX174 RFI DNA as the target in the presence or absence of MuB and ATP. The strand transfer activity with this target, irrespective of the presence or absence of MuB, generally paralleled that observed above when short DNA fragments were used as targets in the absence of MuB (data not shown). Thus transpososomes with short donor DNA fragments are capable of utilizing intermolecular target DNA, irrespective of its size and without the help of MuB and ATP. The only notable difference was that the donor fragment which lacked flanking DNA accomplished strand transfer as efficiently as those with 2 bp or longer flanking segments. We currently do not know the reasons for this different behavior; supercoiling, size and concentration of the target DNA are the only obvious differences.

To investigate further the relative importance of the length of the two strands of the flanking segment for complex formation and for the chemical steps the 11 substrates shown in Figure 7 were prepared and tested. For stable complex formation the first flanking nucleotide on the non-cleaved strand is critical, while that on the strand to be cleaved is not (Figure 7B, substrates a–c). The donor DNA fragments with a single nucleotide 5′ flanking segment were able to assemble stable complexes even in the absence of any divalent metal ion (Figure 7B left). For the cleavage reaction, however, the second flanking nucleotide on the strand to be cleaved is important, while that on the non-cleaved strand is not (Figure 7C,

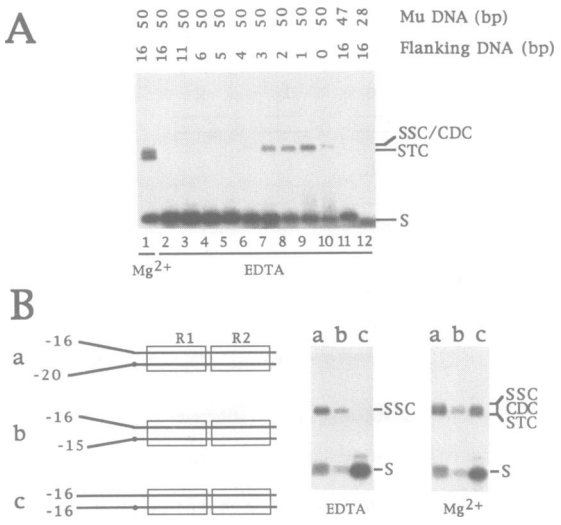

Fig. 8. Stable complexes can be formed without metal ions if the flanking DNA segment is short or non-complementary. (**A**) Reactions with donor DNAs of different lengths (see Figure 5) were assembled without metal ions in the presence of 10 mM EDTA. Products were analyzed by native agarose gel electrophoresis. Because the chemical steps cannot take place without metal ions, the complexes detected are SSCs. A reaction in the presence of Mg^{2+} is shown in lane 1 for comparison. (**B**) Reactions were carried out in the absence or presence of metal ions (10 mM EDTA or 10 mM Mg^{2+} respectively). The donor DNA contained non-complementary (substrates a and b) or complementary (substrate c) flanking sequences as depicted. The numbers on the left correspond to the length (nt) of the flanking segment on each strand. The dots in substrates indicate the donor cleavage sites. Band positions are labeled as in Figure 2.

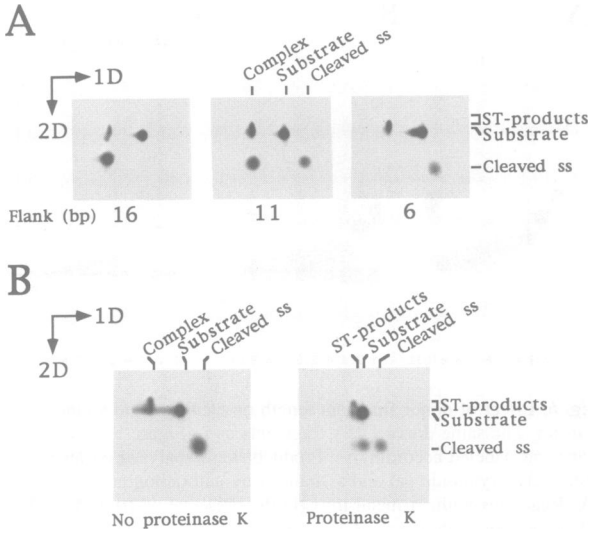

Fig. 9. (**A**) The fate of the cleaved flanking strand is influenced by its length. Reactions in the presence of Mg^{2+} were carried out using donor fragments containing different lengths of flanking DNA. The label was at the 3′-ends of the strand to be cleaved (see Materials and methods). Products were analyzed by two-dimensional gel electrophoresis without addition of competitor DNA. The first dimension, under non-denaturing conditions, separated the complexes from free substrate DNA and from cleaved flanking strand that was released from the complex. Electrophoresis in the second dimension was performed under alkaline conditions to separate all the DNA strands. The length of the flanking segment (bp) is indicated below each gel. The identity of cleavage products was confirmed by urea–polyacrylamide gel electrophoresis of the DNA isolated from bands on an agarose gel (data not shown). (**B**) The cleaved flanking strand dissociates from the transpososome more readily than from protein-free DNA. Transpososomes with 11 bp flanking DNA were made by a 1 h incubation in the presence of Mg^{2+}. The reaction mixture was then divided into two halves: one half was incubated for 4 h with proteinase K to digest MuA, while the other half was incubated in the same buffer without proteinase K. An unlabeled competitor DNA for the cleaved flanking strand was then added to both reactions and the incubation was continued for another hour. Two-dimensional gel electrophoresis was carried out as for (A).

substrates d–f). Although the cleaved donor DNA is not clearly resolved from substrate in the figure shown, a shorter exposure of a gel that was electrophoresed longer (not shown) confirmed the precise correspondence between presence or absence of the strand transfer products and the presence or absence of donor DNA cleavage. Whether the flanking nucleotides were base paired (substrates d–g) or not (substrates h–k) did not influence the outcome of the experiment with these substrates carrying very short flanking segments.

The cleaved flanking duplex is destabilized in the complex

In the absence of a divalent metal ion donor DNA fragments with flanking segments of 3 bp or less could form stable complexes efficiently (Figure 8A). Interestingly, the donor fragment with no flanking segment appeared to assemble a stable complex more efficiently in the absence of a divalent cation than in its presence (lane 10; see also Figure 7B, substrate a). The length *per se* of the two strands in the flanking segment is not the reason some substrates fail to form a stable complex in the absence of a divalent cation, since donor fragments with longer flanking segments on both strands can form a stable complex regardless of the divalent cation, as long as the two flanking strands are non-complementary (Figure 8B, substrates a and b). As expected, urea–polyacrylamide gel electrophoresis of the products of the above reactions without a divalent cation showed no donor DNA cleavage or strand transfer with any of the donor fragments tested (Figure 6A and data not shown).

The above findings raised the possibility that the flanking DNA within the synaptic complex prior to donor cleavage may not be in a duplex configuration. If this were the case, upon cleavage the flanking strands might easily separate from one another. This would not result in release of the cleaved flanking strand from the normal CDC, because there exist long flanking segments beyond the synaptic complex. However, when the complexes are assembled with substrates containing short flanking segments this destabilization might be detectable as preferential release of the cleaved flanking strand from the CDC and STC compared with the same DNA without bound protein.

Since the stability of the cleaved flanking strand was expected to depend on its length, we first prepared a set of donor DNA fragments with different flanking segment lengths, assembled transpososomes with them in the presence of Mg^{2+} and then monitored release of the flanking strand from the complex following donor cleavage (Figure 9A). Two-dimensional agarose gel electrophoresis was used for this analysis. The first, non-denaturing, dimension separated complex-bound DNA from unbound DNA, thus after the second dimension in alkali the distribution of the cleaved strand between the complex-bound and unbound fractions could be determined. The 16 nt cleaved flanking strand was stably retained within the complex (left).

Approximately half of the 11 nt cleaved flanking strand was retained within the complex, while the other half was released (middle). Cleaved strands of 6 nt or shorter were all released from the complex (right and data not shown).

To determine whether interactions within the complex were promoting or inhibiting release of the cleaved strand we compared the stability of the nicked duplex before and after removal of the bound protein (Figure 9B). Stable complexes were formed with donor DNA containing an 11 bp flanking segment in the presence of Mg^{2+} to allow donor DNA cleavage and strand transfer. Half of the sample was treated with proteinase K to digest MuA, while the other half was incubated without proteinase K. An excess of unlabeled single-stranded competitor DNA carrying the same sequence as the cleaved strand was then added in order to detect possible reversible interaction between the complex and the cleaved strand. After an additional incubation samples were analyzed by two-dimensional agarose gel electrophoresis to measure the amount of the cleaved strand that remained stably associated with its original DNA partner. In the sample, in which MuA was digested by proteinase K, ~50% of the cleaved strand remained associated with its original DNA partner (right). Thus no further release of the cleaved strand occurred during incubation with competitor DNA after proteinase K treatment. In contrast, when the complex was not treated with proteinase K essentially all of the cleaved strand was released from the complex during incubation with the single-stranded competitor (left). This result shows that the 11 nt cleaved strand is destabilized within the complex as compared with protein-free DNA. A parallel experiment using a donor DNA with a 16 bp flanking segment showed that the additional 5 nt prevented dissociation of the cleaved strand from the complex, even in the presence of a competitor (data not shown). These results suggest that deformation of the flanking segment is confined to several nucleotides adjacent to the cleavage site.

Pre-nicked donor DNA: relaxed requirements for complex assembly and strand transfer

The effects of a pre-existing nick at the MuA cleavage site on requirements for stable complex formation and strand transfer were also investigated, using a fully double-stranded nicked donor DNA with a 16 bp flanking segment. This pre-nicked DNA differed from pre-cleaved donor DNA previously studied (Craigie and Mizuuchi, 1987) in that it also contained the cleaved flanking strand. With this substrate, as with the unnicked substrates having very short or non-complementary flanking sequences, stable complex formed efficiently in the absence of a divalent metal ion (data not shown).

Although Ca^{2+} cannot support the donor cleavage reaction, it can effectively promote strand transfer when the donor cleavage step is by-passed (Figure 10). In the presence of either Mg^{2+} or Ca^{2+} complexes made with unlabeled pre-nicked donor DNA can stably incorporate a labeled target DNA (Figure 10B, lanes 5 and 6). This is in contrast to the results with uncleaved donor DNA (lanes 1–3), where labeled target was found in the complexes only in the presence of Mg^{2+}. Analysis of the reaction products by urea–polyacrylamide gel electrophoresis (Figure 10C) confirmed the presence of strand

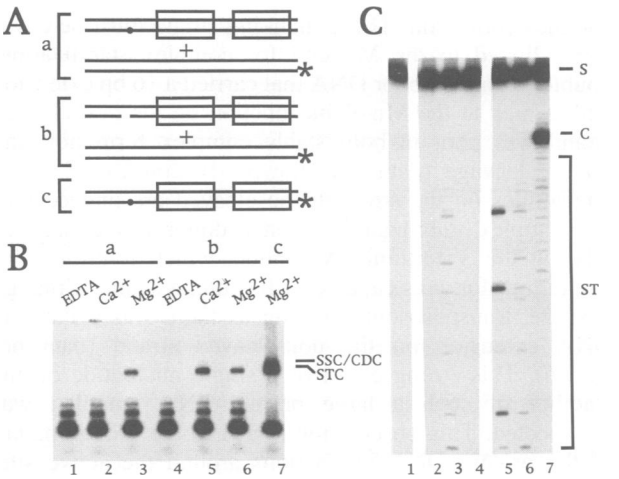

Fig. 10. Requirements for strand transfer with uncleaved and pre-cleaved substrates. The donor fragment (66 bp) contained the sequence shown in Figure 2A with or without a nick at the MuA cleavage site. The target fragment (66 bp) is described in Materials and methods. Band positions are labeled as in Figures 2 and 6. (**A**) Uncleaved or pre-cleaved donor DNA was combined with labeled target DNA (substrate sets a and b). The reaction was also carried out with labeled donor DNA only (substrate c). The label was located on the 5′-end in each substrate, as depicted. The symbols are as in Figure 3. (**B**) Reactions were carried out in the presence of 10 mM Mg^{2+}, 10 mM Ca^{2+} or 10 mM EDTA and the products were analyzed by native agarose gel electrophoresis. (**C**) Products of the reactions in (B) were electrophoresed on an 8% urea–polyacrylamide gel.

transfer products when either Ca^{2+} or Mg^{2+} was used in the reaction (lanes 5 and 6). Essentially the same results were obtained with pre-cut donor DNA that did not contain the cleaved flanking strand (data not shown).

Discussion

Transpositional recombination of phage Mu takes place within the context of protein–DNA complexes called transpososomes. Formation of the Mu transpososome under physiological conditions requires *cis*-acting DNA sequences, a specific donor DNA topology and a variety of accessory proteins (Mizuuchi, 1992). These requirements make it difficult to study the basic protein–DNA interactions that influence the stability and reactivity of the Mu transpososome core. This obstacle has been overcome by using modified reaction conditions that allow assembly of the active MuA tetramer without donor DNA superhelicity or the accessory protein and DNA sequence factors. The two buffer components used in this study that contribute to relaxation of the cofactor requirements, non-denaturing detergent and DMSO, can stimulate transpososome assembly individually, although assembly is more efficient when both are present (data not shown).

The results presented here reveal important protein–DNA interactions near the Mu donor DNA end, as the length and configuration of the flanking non-Mu segment have a strong influence on efficiency of formation and stability of the transpososome. There must be a critical contact between the MuA protein and the first flanking nucleotide adjacent to the Mu end on the 5′ flanking strand. While a donor fragment that lacked flanking DNA did not form a stable complex efficiently, addition of a single flanking nucleotide was sufficient to support stable

complex formation. The 5′ flanking strand must be covalently linked to the Mu end for complex stabilization: double-stranded donor DNA that carried a 16 bp extension with a nick at the Mu–flank junction on the non-cleaved strand was poor in both stable complex formation and donor cleavage (data not shown). In contrast, flanking nucleotides on the strand to be cleaved are unnecessary for complex stabilization. Even a donor DNA that was missing the 3′-terminal A residue, which is critical for strand transfer, was capable of forming a stable, although inactive, transpososome as long as there was a flanking DNA extension on the non-cleaved strand (data not shown). This strong effect of a single nucleotide on the stability of such a large protein–DNA complex was unexpected. This observation points to the critical nature of the protein–DNA interactions around the active site, not only for catalysis, but also for the overall architectural integrity of the transpososome.

Within the transpososome several nucleotides of flanking DNA adjacent to the Mu end appear to be unpaired. Although stable complex formation normally requires a divalent metal ion, certain donor DNAs escape this requirement: those with very short (1–3 bp), unpaired or single-stranded flanking regions and those that are prenicked. Considered together, these results suggest that the stabilizing contacts between the MuA tetramer and the flanking nucleotides outside the Mu 5′-end require deformation of these nucleotides away from their B-form double helical configuration. Accelerated release of the cleaved flanking strand strongly supports this conclusion. Deformation of Mu end DNA within the complex has previously been suggested based on hydrogen peroxide hypersensitivity (Lavoie *et al.*, 1991; B.D.Lavoie and G.Chaconas, personal communication). In support of our conclusion several nucleotides of the non-cleaved flanking DNA adjacent to the Mu end in the CDC were found to be sensitive to single-strand-specific probes (Z.Wang, S.Y.Namgoong, X.Zhang and R.Harshey, personal communication). Binding of the Tn*5* transposase dimer to the Tn*5* ends appears to be enhanced by prior hydroxyl radical cleavage of the DNA at any of several positions adjacent to the Tn*5* end, again suggesting deformation of the DNA helix at this location (R.Jilk, D.York and W.Reznikoff, personal communication).

Deformation of the flanking DNA segment in normal donor substrates most likely contributes to the high activation energy for SSC formation. Those donor DNAs that did not require a divalent cation for complex stability also formed stable complexes on ice (data not shown). This indicates that a relatively lower activation energy is required for transpososome core assembly when the donor DNA carries a readily deformable flanking segment compared with normal donor substrates, as the latter require incubation at >20°C (data not shown; Mizuuchi *et al.*, 1992). Divalent metal ions could either directly aid flanking DNA distortion or add stability elsewhere in the complex, helping the transition through the energy barrier.

Additional protein–DNA interactions to those required for stable complex assembly are required for the chemical steps of transpositional recombination. Although a donor with 1 bp of flanking DNA efficiently assembled a stable complex, a second flanking nucleotide at the 3′-end was required for donor cleavage. Thus there appear to be critical contacts between the second nucleotide and MuA protein that are important for chemical activation of the scissile bond. In contrast, after cleavage, flanking 3′ nucleotides were not required for strand transfer, but the A residue at the 3′-end of the Mu sequence was essential (data not shown).

Previously, a consensus sequence, NPyG/CPuN, was found to be preferentially used as the target site for Mu transposition (Mizuuchi and Mizuuchi, 1993). The same target sequence preference was also detected in this study; we found this bias accentuated when the strand transfer reaction was carried out in the presence of Ca^{2+} instead of Mg^{2+} (see Figure 10C). Mu donor DNA that carried the preferred target sequence adjacent to the Mu ends, resulting from usage of the consensus target sequence in the previous round of transposition, appeared to transpose more efficiently than other donor DNA (Z.Wu and G.Chaconas, personal communication). We believe physical contacts between the MuA tetramer and a few nucleotides adjacent to the Mu end reported here form the basis for this sequence preference. The fact that the same sequence is preferred both as a target site and as the flanking DNA of a Mu donor suggests that the same part of the MuA protein, perhaps on different protomers within the MuA tetramer, interacts with both the target site and the donor flanks.

Effects of donor DNA end structure near the donor DNA processing site have also been studied for the closely related HIV integration reaction. The natural substrate for HIV integrase has double-stranded flush ends 2 bp beyond the donor DNA cleavage site. As in the Mu case, substrate DNA with a frayed end is processed more efficiently than normal substrate (P.Brown, personal communication). Extension of the double-stranded flanking DNA beyond 6 bp inhibits cleavage, while the reaction is more tolerant of single-stranded 3′ extensions (Vink *et al.*, 1991; Bushman and Craigie, 1992). A longer flanking DNA extension can be tolerated if the two strands are non-complementary adjacent to the viral DNA cleavage site (P.Brown, personal communication). In contrast to MuA, HIV integrase cleaves donor DNA with its 5′-end shortened by 2 nt (such that after cleavage a flush-ended product is made) somewhat more efficiently than the natural substrate, but the resulting DNA is less efficient in the subsequent strand transfer reaction (Vink *et al.*, 1991; Bushman and Craigie, 1992). Thus while some aspects of substrate preference for the HIV reaction are distinct from those of the Mu reaction, many similarities between the two reactions are apparent.

The two chemical steps of Mu transpositional recombination have different divalent cation selectivity. A divalent cation is essential for both donor DNA cleavage and strand transfer. While Mg^{2+} or Mn^{2+} can support both donor DNA cleavage and strand transfer, Ca^{2+} can support only strand transfer. This relaxed metal ion requirement for the strand transfer step does not depend on the presence of DMSO and detergent (M.Mizuuchi and K.Mizuuchi, unpublished results). Different cation selectivity for the two chemical steps may appear to suggest that two different active sites on MuA catalyze the two reactions. However, this possibility is inconsistent with the behavior of mutant MuA proteins with defective active sites. These mutant proteins are equally defective for both of the two

chemical reactions. In addition, mixed MuA tetramers containing wild-type protein and any of several active site mutant proteins appear to stall at either chemical step, at one or both ends, depending on the specific location of the mutant protein within the tetramer (Baker and Luo, 1994; Baker *et al.*, 1994). Further, the crystal structure of the core domain of MuA shows that these residues are clustered around a single active site (Rice and Mizuuchi, 1995). Therefore, it appears that the same active site is used by different MuA protomers within the complex to catalyze the two reaction steps. The differential metal ion selectivity must reflect either conformational differences between the active sites responsible for the two steps or a difference in the way the two distinct nucleophiles for the two steps are activated within the active site pocket.

The overall size of the multiple DNA fragments which participate in complex assembly appears to have a significant effect on reaction efficiency. This is most noticeable in the efficient intermolecular strand transfer of transpososomes made with short donor DNA fragments; both intermolecular target DNA binding and strand transfer take place within a few minutes under the conditions used in this study (data not shown). In comparison, in reactions that involve circular plasmid donor and target DNA molecules intermolecular strand transfer is very inefficient and only slow intramolecular strand transfer can be seen in the absence of MuB. While DMSO and Triton X-100 accelerate intramolecular strand transfer by pre-formed SSCs, these buffer additives do not allow intermolecular strand transfer with a several kilobase pair circular donor (M.Mizuuchi and K.Mizuuchi, unpublished observation). Shortening the flanking donor DNA regions (to 16 bp or less) in the CDC appears, at least in part, to help intermolecular target DNA capture without MuB.

It was previously noted that short linear Mu end DNA is more efficient than longer DNA for mediating MuA tetramerization (Baker and Mizuuchi, 1992). Similarly, when the IAS is supplied *in trans* as a cofactor for SSC assembly it is more effective when carried on a short fragment or near the end of a long fragment than when internally located on a long fragment (Surette and Chaconas, 1992). It may be that electrostatic repulsion increases the activation energy for assembly when longer DNA that is not essential for the reaction is attached (on both sides) to the DNA sequence element. Thus one important function of MuB (and MuA) may be target and donor DNA charge neutralization and/or charge shielding. This type of DNA size effect may be general to other DNA reactions as well; smaller DNA molecules may be more efficient in reaction steps that require close association of multiple DNA segments. The DNA size effect discussed here contrasts with that seen with some sequence-specific DNA binding proteins which do not demand stable association of multiple DNA segments. In some of these cases proteins appear to find a specific target more quickly when it is carried on a longer DNA fragment (Winter *et al.*, 1981).

Materials and methods

Proteins, nucleotides and reagents

MuA was overexpressed and purified as described (Baker *et al.*, 1993). ^{35}S-Labeled MuA, purified essentially as the unlabeled protein, was a gift of M.Mizuuchi. MuB was purified as described (Chaconas *et al.*, 1985), with additional steps (Adzuma and Mizuuchi, 1991) to remove aggregated protein. Sequenase (version 2.0) was purchased from United States Biochemical Corp., proteinase K from BRL, terminal deoxynucleotidyl transferase (TdT) and T4 polynucleotide kinase (PNK) from Pharmacia and bovine serum albumin (BSA) from Sigma. [α-^{32}P]dNTPs (3000 Ci/mmol), [γ-^{32}P]ATP (6000 Ci/mmol) and [α-^{32}P]cordycepin-TP (5000 Ci/mmol) were purchased from New England Nuclear. DSP was from Pierce and DMSO from Aldrich. Four types of agarose from FMC Bioproducts were used; Metaphor, NuSieve-GTG, SeaKem-HGT and SeaPlaque.

DNA

Oligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer and purified by urea–polyacrylamide gels electrophoresis prior to use (Sambrook *et al.*, 1989). Single strands were labeled either on the 3′-end with TdT and [α-^{32}P]cordycepin-TP or on the 5′-end with PNK and [γ-^{32}P]ATP (Sambrook *et al.*, 1989). Proteins and unincorporated nucleotides were then removed by sequential phenol and chloroform extractions, followed by passage through a spin column (G25 or G50; Boehringer Mannheim) and ethanol precipitation. Strands were annealed in TEN buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA, 50 mM NaCl) by boiling for 2 min, followed by slow cooling to room temperature.

For the experiments shown in Figure 9 the double-stranded substrates were labeled at their flanking DNA 3′-ends as follows. Oligonucleotide pairs with flanking sequences that were 1 nt shorter on the 3′-ends were annealed in 1× Sequenase buffer (40 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl). The recessed 3′-ends were filled in with the appropriate [α-^{32}P]dNTP (80 μCi) and Sequenase (1.5 U) for 10 min at 37°C in 1× Sequenase buffer containing 10 mM dithiothreitol. The corresponding unlabeled dNTP was then added to a final concentration of 50 μM and incubation was continued for 5 min, after which the enzyme and unincorporated nucleotides were removed as above. After ethanol precipitation the labeled duplexes were resuspended in TEN buffer.

The sequence of the 66 bp double-stranded Mu end oligonucleotide is shown in Figure 2A; when indicated, 3′ and 5′ deletion variants were used. A substrate that contained a nick at the Mu end cleavage position was made by annealing the appropriate three single-stranded oligonucleotides. The 5′-end of the flanking strand at the nick was left unphosphorylated and thus this substrate differs from the natural substrate for strand transfer made by MuA which contains a 5′-phoshate at the nick. A 66 bp target DNA was prepared by annealing the two oligonucleotides MM450 (5′-TGTAAGCCGAGCTATAGAAATTGG-CGAGTTAGGTCGAACCATCCAGGCCGTTAGACAATGGGACTG-3′) and MM449 (5′-CAGTCCCATTGTCTAACGGCCTGGATGGTTC-GACCTAACTCGCCAATTTCTATAGCTCGGCTTACA-3′). The label on this substrate was placed either at the 3′- or 5′-end of MM449. Frayed DNA molecules (substrates a and b shown in Figure 7B) were prepared by annealing the oligonucleotide 5′-GTTTTCGCATTTATCG-TGAAACGCTTTCGCGTTTTTCGTGCGCCGCTTCATCCGATCTA-GCTCGTGGACG-3′ (substrate a) or its 3′ deletion derivative (substrate b) with the top strand shown in Figure 2A.

The longer Mu donor DNA fragments (100 and 245 bp) used in the experiment shown in Figure 4 were obtained by *Eco*RI digestion of plasmid pMK426 (Craigie and Mizuuchi, 1987). They were purified by electrophoresis on a 5% polyacrylamide–TBE gel and isolated by the 'crush and soak' method (Sambrook *et al.*, 1989). The competitor DNA containing two strong MuA binding sites has been described previously (Mizuuchi *et al.*, 1991). The 16 nt single-stranded competitor DNA used in the experiment of Figure 9 had the 3′ 16 nt sequence of the bottom strand shown in Figure 2A. Phage φX174 RFI DNA was purchased from BRL.

Transposition reactions

Standard reactions (25 μl) contained 50 nM ^{32}P-labeled Mu end DNA fragment, 116 nM MuA, 25 mM Tris–HCl, pH 8, 25 μg/ml BSA, 15% (w/v) glycerol, 15% (v/v) DMSO, 0.1% (w/v) Triton X-100, 156 mM NaCl and 10 mM $MgCl_2$. In some reactions $MgCl_2$ was substituted with other reagents, as noted. Standard reactions did not contain additional DNA fragments, but the donor DNA fragments also functioned as target DNA if conditions allowed strand transfer. Reactions were carried out at 30°C for 1 h and stopped by freezing in liquid nitrogen. When a 66 bp non-Mu DNA fragment (25 nM) was used as target the reactions also contained 25 nM Mu donor DNA fragment. Reactions with

ϕX174RFI DNA as target (10 μg/ml) were done with or without 600 nM MuB and 2 mM ATP.

To detect stable protein–DNA complexes samples were electrophoresed for 1.5 h at 5.3 V/cm, unless otherwise noted, on a native 2% Metaphor agarose gel at 4°C in 1× TAE buffer (40 mM Tris–acetate, pH 7.8, 8 mM sodium acetate, 1 mM EDTA) with buffer circulation. Competitor DNA (at an ~20-fold molar excess) was added to the samples prior to electrophoresis to trap loosely bound MuA, unless otherwise noted. Ficoll 400 (0.2 vol, 25%; Pharmacia) was added to the samples for gel loading. The gel was dried onto DEAE paper (DE81, Whatman) and the bands visualized by autoradiography using a Fuji BAS 2000 PhosphorImager.

DNA products were usually analyzed by denaturing polyacrylamide gel electrophoresis (Sambrook *et al.*, 1989); an equal volume of a loading dye (95% formamide, 10 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol) was added to the samples for gel loading. When ϕX174 RFI DNA was used as the target the reaction products were analyzed by electrophoresis on a 0.9% SeaKem-HGT agarose gel in 1× TAE buffer. In this case 0.2 vol loading dye (0.1% bromophenol blue, 2.5% SDS, 50 mM EDTA, 25% Ficoll 400) was used to dissociate proteins.

Two-dimensional gel electrophoresis was performed with a 3% Metaphor agarose gel. The first dimension was electrophoresed under native conditions (see above) for 1.5 h at 5.3 V/cm. The gel was then soaked in an alkaline running buffer (30 mM NaOH, 1 mM EDTA) for 2 h at room temperature. The second dimension was electrophoresed for 1.5 h at 2.7 V/cm in an alkaline running buffer at room temperature with buffer circulation. The gel was neutralized in 1× TAE buffer for 15 min, rinsed with water and dried onto DEAE paper for autoradiography.

Number of donor DNA fragments in the stable complex

Reactions were assembled essentially as the standard reactions; 106 nM MuA, 54 nM total Mu end fragment and 54 nM 5′-labeled 66 bp non-Mu target DNA were used. Reactions were incubated for 45 min at 30°C prior to addition of excess competitor DNA. Protein–DNA complexes were separated on a gel containing 6% NuSieve-GTG agarose, 0.5% SeaKem-HGT agarose and 80 μg/ml BSA in 1× TAE. Inclusion of BSA in the gel markedly sharpened the complex bands compared with the gel without BSA. Electrophoresis was at 4°C for 4.5 h at 5.3 V/cm in 1× TAE buffer containing 80 μg/ml BSA.

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