Complete transposition requires four active monomers in the Mu transposase tetramer

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A tetramer of Mu transposase (MuA) cleaves and joins multiple DNA strands to promote transposition. Derivatives of MuA altered at two acidic residues that are conserved among transposases and retroviral integrases form tetramers but are defective in both cleavage and joining. These mutant proteins were used to analyze the contribution of individual monomers to the activity of the tetramer. The performance of different protein combinations demonstrates that not all monomers need to be catalytically competent for the complex to promote an individual cleavage or joining reaction. Furthermore, the results indicate that each pair of essential residues is probably donated to the active complex by a single monomer. Although stable, tetramers composed of a mixture of mutant and wild-type MuA generate products cleaved at only one end and with only one end joined to the target DNA. The abundance of these abortive products and the ratios of the two proteins in complexes stalled at different steps indicate that the complete reaction requires the activity of all four monomers. Thus, each subunit of MuA appears to use the conserved acidic amino acids to promote one DNA cleavage or one DNA joining reaction.

[Key Words: Mu transposase; transposition; protein multimers; genetic recombination]

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Transposition is the type of genetic recombination that moves a mobile DNA element from one site to another in the DNA of a host organism. The reaction proceeds through a series of DNA cleavage and joining reactions. Donor DNA cleavage separates the element from the host DNA at the old location while DNA strand transfer covalently joins the element DNA to the DNA at the new target site. Retroviruses and long terminal repeat (LTR) retrotransposons use a very similar mechanism to integrate a reverse-transcribed copy of their genomes into the DNA of the host cell. In most systems a single protein, the transposase or integrase, is responsible for both DNA cleavage and strand transfer. For phage Mu, the transposase is the MuA protein [MuA].

The chemistry of transposition has been examined in some detail (for review, see Mizuuchi 1992a,b). Donor DNA cleavage uncoverts a 3'-OH at each end of the transposon DNA. In some elements, including Mu and retroviruses, only one strand is cleaved [Craigie and Mizuuchi 1987; Surette et al. 1987; Fujiwara and Mizuuchi 1988; Brown et al. 1989; Craigie et al. 1990], whereas in others, such as Tn10, Tn7, and P elements, donor cleavage involves double-stranded breaks [Bainton et al. 1991; Benjamin and Kleckner 1992; Kaufman and Rio 1992]. In the subsequent strand transfer step, the two 3'-OH ends of the donor DNA are covalently joined to two 5' phosphates, staggered by a few base pairs, in the two DNA strands at the target site. Strand transfer by the Mu transposase [Mizuuchi and Adzuma 1991] and the human immunodeficiency virus [HIV] integrase [Engelman et al. 1991] results in inversion of a chiral phosphorothioate at the site of insertion in the target DNA. Similarly, during donor cleavage by the HIV integrase, the chirality of a phosphorothioate at the cleavage site is inverted [Engelman et al. 1991]. These results support the view that both cleavage and strand transfer proceed by a one-step phosphor transfer mechanism and are consistent with models in which the two reactions are catalyzed by a common active site [Engelman et al. 1991; Mizuuchi 1992b].

The similarities in the mechanism of transposition used by many elements extends to resemblance in the proteins that catalyze the reactions. Retroviral integrases have three highly conserved acidic amino acids termed the D-D-35-E motif, as the second aspartate (D) and the glutamate (E) are separated by 35 residues; these residues of the HIV and Rous sarcoma virus (RSV) integrases are important for both cleavage and strand transfer in vitro [Drelich et al. 1992; Engelman and Craigie 1992; Kulkosky et al. 1992; Lefemina et al. 1992; van Gent et al. 1992; Leavitt et al. 1993]. We have recently identified acidic residues in MuA that are required for both DNA cleavage and strand transfer [Baker and Luo 1994]. The similar effect of mutations, as well as amino acid se-
quence similarity, argue that the essential acidic amino acids of MuA (D269 and E392) are analogs of those of the retroviral integrases [Baker and Luo 1994]. Convincing D-D-35-E motifs have been detected in many transposition proteins, including those from Tn7, Tn552, IS3, and mariner elements (Fayet et al. 1990; Rowland and Dyke 1990; Robertson 1993; Baker and Luo 1994; Rådström et al. 1994) and a related motif has been identified in the proteins encoded by excising elements from ciliated protozoa [Doak et al. 1994]. Thus, MuA appears to be a member of a large protein family that utilizes a similar constellation of residues to promote genetic recombination. The essential acidic amino acids have been suggested to form part of the active site for both cleavage and strand transfer by coordinating divalent cations [Mg$^{2+}$ or Mn$^{2+}$] (Engelman and Craigie 1992; Kulksky et al. 1992, Bushman et al. 1993). Some activity is restored to the D269N and E392Q MuA derivatives by addition of high concentrations of Mn$^{2+}$ in support of this suggestion [Baker and Luo 1994].

Successful transposition critically depends on coordination of donor cleavage and strand transfer on the two ends of the element. This coordination appears to be mediated by a multimeric complex of the transposase. Transposition of Mu, Tn10, and Tn7 all involve higher-order complexes between the transposases and the donor and target DNAs [Surette et al. 1987; Haniford et al. 1991; Mizuuchi et al. 1992; Bainton et al. 1993]. Retroviral integration also involves a multimeric complex of the integrase protein [Jones et al. 1992; Engelman et al. 1993; van Gent et al. 1993b]. Characterization of these complexes is most advanced for Mu, where it has been demonstrated that the transposition complex consists of a tetramer of 75-kD MuA monomers bound simultaneously to the two ends of the Mu DNA [Lavoie et al. 1991; Mizuuchi et al. 1992]. This tetramer pairs the two DNA ends and engages the DNA cleavage sites [Lavoie et al. 1991; Mizuuchi et al. 1991, 1992; Baker et al. 1993]. Formation of the complex involves several MuA-binding sites on a supercoiled donor DNA and the host DNA-binding proteins HU and IHF [Surette and Chaconas 1989, 1992; Baker and Mizuuchi 1992; Mizuuchi, et al. 1992]. Assembly appears to activate the catalytic activity of MuA, and once formed, the constituent monomers do not exchange with those in solution [Lavoie et al. 1991; Surette et al. 1991; Mizuuchi et al. 1992]. Three stable MuA–DNA complexes have been characterized: The stable synaptic complex (SSC, or type 0 transpososome), which assembles on the donor DNA prior to cleavage, the cleaved donor complex (CDC, or type 1 transpososome), and the strand transfer complex (STC, or type 2 transpososome). Transposition involves a second phage-encoded protein, MuB. MuB interacts with the MuA–donor DNA complexes and stimulates cleavage and strand transfer by MuA [Baker et al. 1991; Surette and Chaconas 1991; Surette et al. 1991].

The knowledge that Mu transposition is promoted by a stable tetramer of MuA allows for a level of analysis of the transposition mechanism not yet possible in other systems. In this report we address how the four sets of essential acidic amino acids of the MuA tetramer contribute to the activity of the complex. The activities of the mixed tetramers containing wild-type MuA and mutant proteins altered at the essential acidic amino acids indicate that normal transposition requires catalytic activity of all four monomers. Thus, these data support models in which the acidic amino acids participate in both cleavage and strand transfer but different monomers carry out the two chemical steps.

Results

Mutant and wild-type MuA form mixed complexes that have partial activity.

To start to address how the four sets of essential acidic amino acids in the MuA tetramer contribute to the cleavage and strand transfer activity of the complex, the impact of the D269N and E392Q proteins on reactions containing wild-type MuA (WTMuA) was investigated. These proteins, altered at the conserved acidic amino acids, assemble with the donor DNA into stable complexes but are severely defective in both cleavage and strand transfer [Baker and Luo 1994]. Two types of donor DNA were used to assay the protein mixtures: a circular plasmid containing two intact Mu DNA ends, and precleaved linear DNA, in which the sites of donor DNA cleavage have already been uncovered by digestion with HindIII. With the circular substrate, MuA nicks the DNA at two specific sites and then joins these 3′-OH ends to the target DNA by strand transfer. With the precleaved DNA, MuA carries out only strand transfer.

Cleavage of the circular substrate, and strand transfer of both types of donor DNA, were steeply dependent on the concentration of WTMuA; little product was observed with <0.5 pmole of MuA, whereas full activity was seen with 1.3 pmole. In reactions containing WTMuA with circular DNA, addition of E392Q [1.3 pmole] had two principal effects [Fig. 1]: (1) Detectable donor cleavage occurred at WTMuA concentrations too low to support the reaction on their own; and (2) at higher levels of WTMuA, E392Q inhibited strand transfer, and the cleaved donor DNA accumulated. Addition of E392Q also affected strand transfer of precleaved donor DNA. E392Q stimulated strand transfer in the presence of low levels of WTMuA. Furthermore, the mutant protein changed the distribution of the strand transfer products; a single band with a migration distinct from the products formed by WTMuA was the principal product when both proteins were present. The following experiment addresses the possibility that this product resulted from strand transfer of only one of the two Mu DNA ends into the target DNA.

To probe the structure of the strand transfer products formed by the mixtures of WTMuA and an inactive mutant protein (here, D269N, E392Q, and D269N have qualitatively similar affects), restriction fragments carrying only the right end of the Mu DNA were used as the donor DNA. With this type of substrate, MuA efficiently pairs two fragments, and inserts them into a target site
is used alone, most of the nicked DNA is cleaved at both the left and right ends [Surette et al. 1991; Mizuuchi et al. 1992]. In contrast, when E392Q or D269N is present in addition to WTMuA, a substantial amount of the nicked donor DNA remains uncleaved at one of the Mu DNA ends [data not shown]. For example, in the presence of 0.13 or 0.43 pmole of WTMuA and 1.1 pmole of
D269N, about half of the nicked DNA was cut at the left end and half was cut at the right end (values between 47% and 53%). Thus, under these conditions, most of the cleaved donor DNA appears nicked at only one of the two Mu DNA ends, indicating that the mutant protein impedes cleavage when incorporated at certain positions in the tetramer.

The contribution of the acidic amino acids of MuA to the activities of the tetramer was investigated further by the assaying of both the double mutant protein and mixtures of the two mutant proteins. MuA mutated simultaneously at both essential amino acids (the D269N/E392Q double mutant) had an impact on cleavage and strand transfer very similar to the single substitution proteins when mixed with WTMuA [Fig. 3]. Therefore, in the context of the tetramer, monomers with either one or two altered acidic amino acids behave similarly. In contrast, mixing the D269N and E392Q proteins did not yield any detectable activity with either the circular or precleaved donor substrate [data not shown]. Taken together, these results indicate that D269 and E392 are probably donated to the active complex together by a single monomer, rather than D269 and E392 from different monomers working together in the final complex.

How many monomers need the essential acidic amino acids for the complete reaction?

Possible models

The activities of mixtures of different forms of MuA indicate that proteins lacking an essential acidic amino acid form mixed tetramers with WTMuA readily and that at least some of these complexes are able to promote an isolated cleavage or strand transfer event. However, these mixtures frequently generate aberrant products, indicating that incorporation of the mutant protein into the tetramer disrupts the normal sequence of chemical reactions. Therefore, we set out to address how many monomers in tetramer need D269 and E392 for the complex to form the normal strand transfer intermediate.

Formation of the strand transfer intermediate requires two DNA cleavages and two strand transfer reactions, one each on the left and right ends of the Mu DNA. In principle, one, two, three, or all four monomers might need to be active for the tetramer to catalyze these reactions. However, considering the symmetry of the reactions, and the fact that MuA is a tetramer, it seems most probable that either two or four catalytically active monomers are required. Furthermore, the characterization of the incomplete reaction products presented above argues against models involving one or three active monomers. If only one WTMuA monomer were necessary for the complete reaction, no accumulation of incomplete products by the mixed tetramers would be expected in contrast to the high frequency of aberrant products observed [unless the mixed complexes were unstable, which is tested below]. Likewise, three subunit models stipulate that either one active monomer promotes cleavage of both ends of the Mu DNA [with the other two promoting strand transfer] or that one active monomer promotes strand transfer of both ends (with the other two required for cleavage). If one active monomer cleaves both ends, donor DNA cleaved at just one end would not be an expected product, in contrast to our findings. Similarly, we found that the mixed complexes promote single-end strand transfer of a precleaved DNA at high frequency, a reaction that would not be expected if one active monomer promoted strand transfer of both ends. Thus, different monomers appear responsible for the chemical reactions on the two Mu DNA ends, indicating that an even number of active subunits participate in the complete transposition reaction.

We therefore considered in detail models of the tetramer that involve two or four monomers participating in catalysis [Fig. 4]. In Figure 4, the monomers are labeled La and Lb if they interact with the left end of the Mu DNA and Ra and Rb if they interact with the right end. In the four-subunit model, each monomer needs the essential acidic amino acids to perform one function, that is, cleavage or strand transfer of one end. The La monomer must be wild type for the complex to cleave the left end, whereas the Ra monomer must be wild type for cleavage of the right end; in addition, the Lb and Rb monomers also must be wild type for these cleaved ends to complete strand transfer. In contrast, in the two-subunit model the same monomer donates the acidic amino acids for both cleavage and strand transfer of one end of the Mu DNA [the La and Ra monomers in Fig. 4; there is another type of two-active-monomer arrangement that will be discussed further below].

There are 16 possible configurations of two types of monomers in the tetramer [Fig. 4, bottom]. [Shaded circles represent mutant MuA, and unshaded circles repre-
Figure 4. Model with all the types of mixed tetramers arranged in the two- and four-subunit models. Two possible arrangements of the core domains of MuA and the substrate DNAs are shown at the top. In the four-subunit model, the four copies of domain II in the MuA tetramer are shown with each subunit interacting with one of the four phosphodiester bonds in the donor and target DNAs that participate in the chemical steps (*). The 3'-OH ends generated by the La and Ra subunits during cleavage could attack the phosphodiesters activated by Lb and Rb during strand transfer, therefore all four monomers would need D269 and E392 for the complete strand transfer intermediate to be generated. In the two-subunit model, both the donor and target DNA are activated by the D269 and E392 residues in the La and Ra monomers; thus, Rb and Lb are not required to be wild-type monomers for the formation of the complete strand transfer intermediate. [Bottom] The mutant monomers are represented by the shaded in circles and WTMuA is shown by unshaded circles. The 16 arrangements of the tetramer were organized for both models on the basis of the products expected after reaction with a circular donor DNA substrate.

sent WTMuA. These 16 tetramer arrangements were organized on the basis of the products they would be expected to generate after a complete reaction with a circular donor DNA using either the two- or four-subunit model (Fig. 4, bottom). A major difference between the two models is that the four-subunit arrangement predicts a class of complexes that are stalled after cleavage (assuming that cleavage is essentially irreversible), unable to carry out strand transfer for lack of a second active monomer in the correct position. However, in the two-subunit model, all of the complexes that cleave the DNA are predicted to complete strand transfer of their cleaved end. Inspection of the products formed by mixed complexes in Figures 1 and 3 reveals that in contrast to the situation with WTMuA alone and in apparent support of the four-subunit model, much of the donor DNA acted on by the mixed complexes appears stalled after cleavage. The reasons for this incomplete strand transfer are explored in the following section.

Mixed tetramers are stable and some complexes slowly promote single-end strand transfer

One possible explanation for inefficient strand transfer by the mixed tetramers is that cleaved donor complexes containing the mutant protein fall apart before completing strand transfer. Two potential sources of instability are the presence of the mutant protein and that only one of the Mu DNA ends is cleaved in most circumstances.

To directly observe transposition complexes containing E392Q, the protein was radiolabeled with [35S]cysteine and [35S]methionine. Reactions utilizing the purified labeled protein mixed with unlabeled WTMuA were subjected to electrophoresis on agarose gels both after addition of SDS to observe the DNA and without SDS to observe the MuA–DNA complexes [Fig. 5A]. Nearly all of the cleaved DNA was found in the stable cleaved donor complex (CDC) rather than as free nicked DNA. Autoradiography revealed E392Q in this complex as well as in the stable complex on the uncleaved donor DNA (SSC) and the strand transfer complex (STC). These data demonstrate that a majority of the cleaved complexes are stable for at least 1 hr. The complexes were also stable to heat denaturation, requiring incubation at temperatures higher than 60°C for 10 min to destroy them. They were, however, less thermostable than the CDC containing all WTMuA [data not shown].

Another possible explanation for incomplete strand transfer by the mixed tetramers is that strand transfer of one cleaved end might be slow or impossible if the other Mu DNA is uncleaved. Strand transfer by the mixed complexes was therefore compared with reactions containing WTMuA and a donor plasmid deleted for the cleavage site on the right end (ARCS plasmid) as another condition that may give rise to single-end strand transfer products [Fig. 5B]. Although strand transfer with WTMuA on a normal donor DNA is usually complete within 10 min, a predominant strand transfer product...
Mu transposition requires four active monomers

Figure 5. [A] Mixed tetramer complexes are stable. Reactions were done under standard conditions with either 35S-labeled E392Q alone or a mixture of 35S-labeled E392Q and WTMuA. Samples were removed for electrophoresis at the times shown at the top and run on agarose gels either with or without addition of SDS to the samples. Competitor DNA was added prior to electrophoresis as described in Materials and methods. The protein–DNA complexes are labeled as follows: (SSC) The stable synaptic complex, the MuA complex on the uncleaved donor DNA; (CDC) the cleaved donor complex; (STC) the strand transfer complex. The SSC migrates just slower than the supercoiled donor DNA (Do), whereas the CDC migrates in a manner similar to the supercoiled target DNA (To). The level of WTMuA present was 0.46 pmole; the concentration of the labeled E392Q protein was not determined carefully (for quantitative experiments, see Fig. 6). [B] Time course of single-end strand transfer. Reactions were done under standard reaction conditions and contained either a mixture of WTMuA (0.46 pmole) and E392Q (1.3 pmole) and the donor plasmid pSG1 or WTMuA (1.3 pmole) and the donor plasmid ARCS. The different DNA species are labeled at left, as described in the legend to Fig. 1 and the single-end strand transfer product is labeled with an asterisk (*). Changing the nucleotide sequence at one cleavage site does not completely block cleavage and strand transfer of this end (Surette et al. 1991); therefore, appearance of the normal double-end strand transfer products (marked with an asterisk in Fig. 5B) accumulated between 20 and 180 min in both experimental reactions. This product is not generally seen with WTMuA on a normal donor DNA (e.g., see Fig. 3A, lane 2). The mobility of the product suggested it may be the single-end strand transfer product. Double-end strand transfer products migrate as a family of bands because different numbers of superhelical turns in the donor DNA are trapped during strand transfer; covalent joining of only one end fails to trap these supercoils and therefore would yield a single product band.

The structure of the new strand transfer product (*) made by the E392Q/WTMuA mixtures was probed by digestion with restriction enzymes and two-dimensional agarose gel electrophoresis (data not shown). This analysis demonstrated that the DNA species consisted of the donor plasmid joined to the target DNA at only one end and that the left and right ends were joined at similar frequencies. In most of this class of products made by E392Q/WTMuA mixtures, the unjoined end was not cleaved (M. Mizuuchi, pers. comm.). The kinetics of reactions promoted by the E392Q/WTMuA mixture was also measured after addition of competitor DNA at 20 min. The rate and extent of strand transfer were unaffected by the competitor DNA, indicating that this single-end strand transfer product resulted from activity of stable complexes assembled within the first 20 min (data not shown). Accumulation of the single-end strand transfer product was essentially complete within 2 hr. Thus, neither the instability of the WTMuA/E392Q-cleaved complexes nor an indirect barrier to strand transfer of a cleaved end when the other end is uncleaved explain why the mixed tetramers fail to complete the normal sequence of cleavage and strand transfer reactions. In the following sections, the abundance of the different DNA products and the ratios of the mutant and wild-type proteins in complexes stalled at different steps are quantitated and compared with those predicted by the models.

The pattern of DNA products supports the four-subunit model

Four fates of the donor DNA can be directly quantitated from agarose gels: the unreacted DNA; the cleaved donor DNA; the single-end strand transfer product; and the double-end strand transfer products. The probability of forming each of these four products was calculated for ratios of E392Q/WTMuA of 9:1, 3:1, and 1:1 by use of the two- and four-subunit models outlined in Figure 4, and the predicted values were compared with those determined from quantitation of the reaction products observed on agarose gels (Table 1). The assumptions in the calculations were that [1] MuA is not limiting in the reactions, and [2] the tetramers form without bias to the position of the mutant and wild-type monomers. The observations that the left and right ends are left uncleaved by the mixed tetramers with similar frequency,
Table 1. Fate of donor DNA in reactions containing different ratios of WTMuA and E392Q compared with predictions

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Unreacted</th>
<th>Cleaved</th>
<th>Single-end transfer</th>
<th>Double-end transfer</th>
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<tr>
<td>9:1</td>
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<td></td>
</tr>
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<td>predicted 2 subunits</td>
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<td>18.0</td>
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<td>15.4</td>
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<tr>
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<tr>
<td>observed</td>
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<td>18.1 ± 7.8</td>
<td>6.5 ± 2.6</td>
<td>N.D.</td>
</tr>
<tr>
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<td></td>
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<tr>
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<td>6.3</td>
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<td>17.9 ± 4.6</td>
<td>2.8 ± 4.9</td>
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</table>

The predicted values calculated for the four- and two-subunit models are for the subunit arrangements shown in Fig. 4. The three-subunit arrangements are as follows: Case 3-1: La [or Ra] must be wild type for cleavage of both ends, whereas the activity of Ra [or La] has no effect; Lb must be wild type for strand transfer of the left end, and Rb must be wild type for strand transfer of the right end. Case 3-2: Either La or Ra must be wild type for both ends to be cleaved; Lb must be wild type for strand transfer of the left end and Rb must be wild type for strand transfer of the right end. Case 3-3: La must be wild type for cleavage of the left end. Ra must be wild type for cleavage followed by strand transfer than for cleavage alone, again in agreement with the four-subunit model.

The distribution of DNA products was also compared with the predictions of models in which three active subunits are required for transposition. Three specific three-subunit models are outlined in the footnote to Table 1. These models all predict substantially less cleaved DNA and more double-end strand transfer product than observed [see especially the 1:1 ratio]. Thus, in addition to finding that the mixed tetramers promote both single-end cleavage and single-end strand transfer of a pre-cleaved DNA, which argues against the three-subunit models [see above], this quantitative analysis also favors the four-subunit over the three-subunit arrangement.

Finally, an additional type of two-subunit model should be mentioned. An example of this type of model is that the Ra monomer could both cleave of the right end and promote strand transfer of the left end, whereas the La monomer cleaves the left end and promotes strand transfer of the right end. This type of model is also disqualified by the observed product distribution; in this case, the only abortive products predicted to accumulate are the two single-end cleavage products. If an active monomer were available to promote strand transfer of one cleaved end, it could also cleave the other end and pair-wise cleavage and strand transfer would be expected to be completed.

and that the two ends participate in single-end strand transfer approximately equally [data not shown], indicate that E392Q does not exhibit a strong assembly bias. Thus, random assembly of E392Q within the tetramer appears to be a reasonable approximation.

The observed distributions of DNA products supported the four-subunit model. At all three protein ratios a substantial amount of the donor DNA was cleaved rather than in the strand transfer product; the observed cleaved DNA agreed with the predictions of the four-subunit model within the error of the measurements [average error was ±6%]. These data clearly favor the four-subunit model over the two-subunit model, which predicts complete conversion of the cleaved donor DNA to strand transfer products [see Fig. 4 and Table 1]. Furthermore, a larger fraction of the donor DNA was converted to the strand transfer product when more WTMuA was present. Even if one disregards the unreacted DNA and considers only the efficiency of conversion of the cleaved DNA to the strand transfer products, more of the cleaved DNA was converted at the 1:1 ratio than in the 3:1 and 9:1 cases (37.5%, 24.2%, and 25.3% respectively). These data indicate that more WTMuA monomers are needed for cleavage followed by strand transfer than for cleavage alone, again in agreement with the four-subunit model.

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The contribution of mutant and wild-type monomers in the stalled transposition complexes also supports the four-subunit model

The distribution of mutant and WTMuA in the stable MuA–DNA complexes was determined at the three different E392Q/WTMuA ratios. Either 35S-labeled E392Q or 35S-labeled WTMuA was used in parallel reactions with the other protein unlabeled. The reaction products were electrophoresed both after addition of SDS to observe the DNA (Fig. 6, top) and without SDS to observe the MuA–DNA complexes (Fig. 6, bottom). A 40-fold excess of a competitor DNA oligonucleotide containing MuA-binding sites was added prior to running the gel; therefore, the protein in the complex bands reflects stably incorporated MuA. The ratio of E392Q/WTMuA in the complexes formed on the unreacted DNA, the cleaved DNA, and the strand transfer products was quantitated for the experiment shown in Figure 6 (Table 2).

The results of calculating the ratios of mutant and wild-type protein in the complexes can be summarized as follows. First, as the input ratio of E392Q to wild-type protein was increased to nine, the E392Q/WTMuA ratio in the cleaved donor complexes approached three. These data indicate that tetramers consisting of one wild-type and three mutant monomers can cleave the donor DNA at one end. Second, at both the 3:1 and 9:1 ratios of E392Q/WTMuA, where almost all of the strand transfer product is joined at only one end (see above), nearly equal amounts of the two proteins were found in the strand transfer complexes. Thus, complexes that promote cleavage and single-end strand transfer appear to consist of two mutant and two wild-type monomers. A logical extension of these observations is that pair-wise cleavage and strand transfer of the two Mu DNA ends requires four active monomers of MuA.

Discussion

Complete transposition requires four active monomers of MuA

Catalytically compromised MuA derivatives altered at the acidic amino acids conserved among many transposases and retroviral integrases were used to probe the role of individual monomers in the transposition complex. Tetramers containing the mutant and wild-type proteins assemble and are active but frequently generate incomplete recombination products. Formation of the different classes of abortive products argues that four active monomers participate in generating the complete strand transfer intermediate. Several observations support this conclusion. [1] Pair-wise strand transfer of two Mu end fragments by mutant/wild-type protein mixtures requires more WTMuA than does single-end strand

<table>
<thead>
<tr>
<th>E392Q/WTMuA</th>
<th>Time [hr]</th>
<th>Unreacted</th>
<th>Cleaved</th>
<th>Strand transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>2</td>
<td>2.2</td>
<td>1.0</td>
<td>0.66</td>
</tr>
<tr>
<td>1:1</td>
<td>3</td>
<td>2.2</td>
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<td>0.68</td>
</tr>
<tr>
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<td>2</td>
<td>4.7</td>
<td>1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>3:1</td>
<td>3</td>
<td>4.9</td>
<td>1.6</td>
<td>1.1</td>
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<tr>
<td>9:1</td>
<td>2</td>
<td>9.3</td>
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<tr>
<td>9:1</td>
<td>3</td>
<td>9.6</td>
<td>2.8</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The relative amounts of the mutant and wild-type proteins in the different stable complexes were determined by exposure of the dried agarose gel shown in Fig. 6A to a PhosphorImager screen for several days and determination of the intensities in the various complex bands. The background was determined by quantitation of the intensity of the whole lane, and the emergence of the complex peaks above this background was used to calculate the values. This type of background measurement was necessary as the background varied between different lanes and within a lane, depending on how much labeled protein was present. These values were then corrected for the difference in specific radioactivity of the two protein preparations, and the value for the mutant protein was divided by that of the wild-type protein. The experiment was repeated several times with similar results.

Figure 6. Composition of the transposition complexes formed at different ratios of E392Q and WTMuA. Reactions were done in parallel with either WTMuA or E392Q labeled with 35S and the other MuA derivative unlabeled as shown at the top. Three ratios of E392Q and WTMuA were investigated. All reactions contained 1.3 pmole of E392Q. At the 1:1 ratio, reactions also included 1.3 pmole of WTMuA, whereas the 3:1 ratio included 0.44 pmole and the 9:1 ratio included 0.15 pmole of WTMuA. The specific radioactivity of WTMuA was 1.58-fold higher than that of E392Q. In contrast to the DNA products, the single-end and double-end strand transfer complexes are not well separated during electrophoresis.
MuA-574 mixed with WTMuA on a supercoiled donor DNA is also similar to that of the point mutants: MuA-574 stimulates donor cleavage in the presence of low levels of WTMuA but inhibits strand transfer, resulting in the accumulation of the cleaved donor complex (Baker et al. 1993). Therefore, like the experiments presented here, the activity of mixtures of MuA-574 and WTMuA indicate that two monomers are principally responsible for cleavage and two different monomers are responsible for strand transfer.

Distinctions in the behavior of MuA-574 and D269N and E392Q also provide a hint about the possible roles of these regions. In contrast to the point mutations, MuA-574 does not form tetramers in the presence of Mu end DNA. Mixed tetramers with MuA-574 and WTMuA form, but MuA-574 appears to avoid (during assembly) the positions in the tetramer that cleave the donor DNA. As a result, most of the MuA-574/WTMuA-cleaved complexes are cleaved at both ends but unable to promote strand transfer. These results suggested that domain IIIA (which is missing from MuA-574) is involved in contacting the cleavage sites on the donor DNA during tetramer assembly and the site of insertion on the target DNA during strand transfer (Baker et al. 1993). In contrast, the mutants altered at the essential acidic amino acids do not appear to be severely defective in contacting the cleavage site or the target DNA, as inferred from their ability to assemble and the activity of the mixed complexes. Thus, the D269N and E392Q proteins are probably defective in a function distinct from that defective in MuA-574, and this function is needed at a later stage of the reaction.

In addition to implicating all four monomers in catalysis, the mixing experiments provide some additional clues about the organization of the active transposase complex. When mixed with WTMuA, the E392Q/D269N double mutant has similar activity to the E392Q or D269N single mutant proteins. Furthermore, mixtures of the two single substitution mutant proteins do not have detectable activity. These results argue against arrangements in which D269 from one monomer acts together with E392 of another monomer in the active complex. In this type of arrangement, the double mutant protein would be expected to be more defective than the single mutants in mixtures with WTMuA [inactivating two catalytic centers], whereas the two single substitution proteins, together, might form a partially active complex. By similar logic, the mixing of proteins mutated at residues of the yeast 2μ plasmid’s site-specific recombinase (FLP) essential for catalysis [the active site Tyr and an Arg, His, Arg triad] indicates that the tyrosine and the triad are donated from different monomers to form one active site (Chen et al. 1992, 1993). This type of fractional active site may be common to the Int family of site-specific recombinases (Han et al. 1993) and ensures that the protein is not catalytically active until the complex is assembled. MuA and HIV integrase, like FLP, may be activated by multimerization, however, assembly of the essential acidic amino acids from different monomers does not seem to be the basis of this activation. As
with MuA, mixing two mutant HIV integrase proteins carrying changes in two different residues of the D-D-35-E triad does not generate an active complex [van Gent et al. 1993b]. However, assembly of a catalytic center from different monomers is still an attractive possibility with the transposases and integrases. Certain pairs of inactive integrase proteins with alterations in different domains have nearly wild-type activity when mixed [Engelman et al. 1993; van Gent et al. 1993b]. Furthermore, mixtures of MuAl-574 and E392Q have strand transfer activity, indicating that although both proteins are inactive, they appear to form catalytically competent mixed tetramers (T. Baker, unpbl.).

**Comparison of the Mu transposase to other recombinase complexes**

The emerging relationships between both the mechanism of transposition and the transposition proteins encoded by different elements make it tempting to speculate about the organization of other transposases based on the results with MuA. Although the multimeric state of HIV integrase is not known, kinetic data suggest that the active form is a dimer [Jones et al. 1992] and both dimers and tetramers have been detected by protein–protein cross-linking [Engelman et al. 1993]. In vitro, HIV integrase promotes almost exclusively single-end cleavage and strand transfer [Engelman et al. 1993; van Gent et al. 1993b], in contrast to WTMuA, which nearly always promotes cleavage and strand transfer of a pair of ends together [Craigie and Mizuuchi 1985]. If an integrase dimer promotes the reactions on a single end, perhaps a tetramer promotes the two cleavages and two-strand transfer reactions necessary for viral integration with the essential acidic amino acids from each monomer participating. In contrast to Mu and HIV, some transposable elements, such as Tn10, Tn7 and P elements, cleave both strands of the element–host DNA junction prior to strand transfer of the cleaved 3′ ends into the target DNA [Bainton et al. 1991; Benjamin and Kleckner 1992; Kaufman and Rio 1992]. Transposition of these elements involves a total of six DNA phosphoryl transfer reactions, four cleavages, and two strand transfers. Thus, a more complex arrangement of the active multimer may be required. Perhaps the D-D-35-E motif proteins are flexible enough to assemble into a complex that can promote all of these steps. For example, Tn7 encodes two proteins with D-35-E motifs, TnSB and TnSA. Both proteins are required for donor cleavage and strand transfer, but altering the acidic amino acids in TnSA blocks one set of donor DNA cleavages, without affecting cleavage or strand transfer of the other strands (N. Craig, pers. comm.). Two alternative arrangements for the transposes subunits are attractive: (1) The active form may be a hexamer with one subunit promoting each reaction; or (2) the active form may be a tetramer, with one dimer promoting two sets of reactions.

Multimeric protein–DNA complexes promote many important biological reactions, including initiation of transcription, DNA replication, and genetic recombina-

**Materials and methods**

**DNA**

The target DNA was φX174 RFI [BRL]. The circular donor DNA was pSG1 for most experiments [Baker and Luo 1994]. The pre-cleaved donor DNA was made by linearization of pKN37 [identical to pMK426 between the two Mu DNA ends] with HindIII as described previously [Craigie and Mizuuchi 1987]. The donor DNA fragments used in Figure 2 were made by digestion of pKN37 with HindIII and BamHI. The ARCS plasmid is a derivative of pSG1, which carries the following modification. In pSG1, the sequence from the R1 MuA-binding site to the cleavage site is R1...5′-CTTCA*TATGAAATTC, whereas in ARCS the sequence is R1...5′-TAAGG*AAATTC. The EcoRI site used for cloning the synthetic fragments into pMK589 (which lacks the Mu right end but carries the left end) is shown in boldface type. The natural MuA cleavage site in pSG1 is shown by the asterisk, and the analogous position is marked in the ARCS sequence. ARCS has one other difference from pSG1. The R3 MuA-binding site actually has the sequence of the L3 repeat. This change has little effect on the donor DNA activity of the plasmid in the absence of the change at the cleavage site (T. Baker, unpbl.).
Proteins

MuB protein was purified as described by Chaconas et al. (1985), with the additional step described by Adzuma and Mizuuchi (1991) to remove aggregated protein. HU protein was purified from the overproducing strain of R. McMacken (Johns Hopkins University, Baltimore, MD) by the method of Dixon and Kornberg (1984) with an additional step of Mono S chromatography in 20 mM NaPO₄ (pH 4.8), eluted with a gradient of 0–500 mM NaCl. MuA protein, D296N, E392Q, and D269N/E392Q were purified as described in Baker et al. (1993). The protein concentration of MuA preparations was determined spectrophotometrically with the value of ε₂₈₀ = 1.58 for 1 mg/ml. The concentration of the ³⁵S-labeled preparations was measured by use of the Coomassie brilliant blue-dye binding method (Bio-Rad) with purified WTMuA as a standard.

A plasmid expressing the double mutant [D269N/E392Q] was constructed by fragment swapping with the two single mutants [D269N and E392Q] cloned into the T7 RNA polymerase expression vector pMK591 (Baker and Luo 1994). Both plasmids that contain the single mutant were subjected to digestion by BamHI and BssHI. The 947-bp BamHI-BssHI fragment that carries E392Q was swapped into the vector that contains the D269N mutation. The presence of both mutations in the products was confirmed by sequencing with the Sequenase version 2 kit (U.S. Biochemical).

Both the wild-type MuA and the E392Q mutant were radio-labeled in vivo, and the labeled proteins were then purified according to the method described in Baker et al. (1993), except that the source of ³⁵S used was the EXPRESS³⁵S Protein Labeling Mix, from Du Pont. To label 100 ml of cell culture, 2 mCi products was quantitated from a scanning negative of the ethidium bromide-stained gels essentially as described previously (Mizuuchi et al. 1992) except an LKB 2202 Ultrascan Laser Densitometer with a Hewlett Packard 3390A Integrator was used to collect the data. Each lane of the negative was scanned at two or three positions, and the values were averaged.

The percent distribution of ³⁵S-labeled E392Q and WTMuA in the stable MuA donor DNA complexes was determined by drying the agarose gels and exposing them to a Phosphorimager screen for several days. Band intensities were measured by a Molecular Dynamics Phosphorimager. The relative specific radioactivity of the two protein preparations was determined by scintillation counting.

Calculation of predicted DNA products

The probability of forming each of the 16 types of tetramers shown in Figure 4 was calculated for the different input ratios of mutant to WTMuA of 1:1, 3:1, and 9:1. In each case the probability (P) that a given monomer was mutant or wild type was determined from the input ratio of the two proteins. The following values were used: for the 1:1 ratio, Pmut = 0.5 and Pwt = 0.5; for the 3:1 ratio, Pmut = 0.75 and Pwt = 0.25; and for the 9:1 ratio, Pmut = 0.9 and Pwt = 0.1. The probability of forming each of the types of tetramers was then calculated. For example, the probability of forming the all wild-type tetramer is (Pwt)⁴, or (0.25)⁴ = 0.0039 at the input protein ratio of 3:1, whereas the probability of a mixed tetramer containing two mutant and two wild-type monomers was [Pmut]²[Pwt]² or (0.75)²(0.25)² = 0.035. The tetramers were arranged in the two ways shown in Figure 4 for the basis of the type of product they would be expected to generate after a complete reaction with the circular donor DNA. The probabilities of forming each of the types of tetramers in the no-reaction, cleaved, single-end strand transfer, and complete reaction classes were calculated, and the values were summed and multiplied by 100 to give the percent of the total donor DNA expected to be in each class.

Measuring the efficiency of cleavage of each Mu DNA end

The efficiency of cleavage of the left and right ends of the Mu DNA by mixtures of MuA and D269N was measured as follows. Standard transposition reactions were prepared except that ATP, MuB, and target DNA were omitted. The reaction mixtures were stopped as described above, run on a 0.8% low-melting-temperature agarose gel, and the nicked DNA band was cut out of the gel. The DNA was recovered from the gel by use of β-agarase (New England Biolabs) and ethanol precipitation.

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Complete transposition requires four active monomers in the mu transposase tetramer.

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