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Role of instability in the cis action of the insertion sequence IS*903* transposase

(transposition/cis-acting proteins/ *lon* gene product)

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ABSTRACT **An unusual subset of DNA-binding proteins, termed cis-acting proteins, has been shown to act preferentially at their site of synthesis; the transposases of several bacterial insertion sequences (ISs) fall into this class. The transposase of IS*903* exhibits a strong preference for action in cis: complementation of defective transposons in trans occurs at less than 1%. Furthermore, transposition mediated by transposase acting in cis is extremely sensitive to the distance between the 3′ end of the transposase gene and the nearest transposon inverted repeat; we find that an insertion of 1 kilobase of DNA reduces transposition to 1–2% of control levels. Here we show that there is a strong correlation between the stability of transposase and its ability to act in trans. We found that the wild-type transposase is a very unstable protein with a physical half-life of about 3 min. However, a transposase–β-galactosidase fusion protein has a much greater half-life and can act equally well in cis or in trans. In addition, the native transposase is stabilized in *lon*⁻ strains of *Escherichia coli*, and, in these protease-deficient strains, trans action of transposase is increased 10- to 100-fold. These results suggest that instability of the IS*903* transposase is a major determinant of its cis action and that the La protease, product of the *lon* gene, is an important determinant of transposase instability.**

The cis–trans complementation assay is the classical genetic test used to assign mutations to separate genes (1). This assumes that gene products are diffusible and so can act in trans. However, there are several examples of genes whose products do not complement efficiently in trans, and these are called cis-acting proteins. The cis-acting proteins have been divided into three groups based on their function (2). These are: (*i*) the transposition proteins (transposases) of several bacterial insertion sequences (ISs); (*ii*) the *A* gene proteins of single-stranded DNA phages, which make the origin-specific nick during phage replication; and (*iii*) certain regulatory proteins such as the antitermination protein, Q, of bacteriophage λ and the D-serine deaminase activator protein of *Escherichia coli*. Based on known properties of some of these proteins, mechanisms have been proposed to account for their cis preference. For example, it has been suggested that the cell membrane acts as a trap for the phage A protein so that those molecules that do not immediately find their site of action (in the *A* gene) are rapidly prevented from doing so (3). Similarly, it has been proposed that specific action, in trans, of phage λ Q protein is inhibited by interactions with the vast excess of nonspecific nucleic acid and that this may be compounded by metabolic instability of the protein (4). These models have not yet been tested and so remain speculative.

The transposases of IS*903*, IS*10*, IS*1*, and IS*50* have all been shown to act preferentially in cis (5–9). For IS*903*, complementation in trans is poor (5), and even action in cis is sensitive to the proximity of the transposase gene to its inverted repeats. When analyzing model transposons with one wild-type end and one mutant end, we found that transposition was very sensitive to the location of the wild-type end relative to the transposase gene; efficient transposition was dependent on the wild-type end being adjacent to the 3′ end of the transposase gene (6). In this paper we describe a more thorough examination of this location effect; furthermore, we show that cis preference can be correlated with transposase instability.

MATERIALS AND METHODS

Bacterial Strains. RR1023, an *E. coli recA56* strain carrying the F derivative pOX38, was used as the donor strain in bacterial matings (10). The recipient was NG135 (F⁻, *recA56*, *strA*). M5219 carries the *c*I857 allele of phage λ and was used for heat inductions of phage λ P_L-promoter vectors (11). *lon* strains and their isogenic parents were the gift of S. Gottesman (National Institutes of Health) and were either (*i*) derivatives of MC4100 (ref. 12)—SG20250 (Δ*lac lon*⁺), SG1041 (Δ*lac lon-100*), and JT4000 (Δ*lac lon-510*) or (*ii*) derivatives of SA500—SG13008 (*lon*⁺) and SG13009 (*lon-100*) (ref. 13). In mating-out assays from these strains, pOX38 was used as the conjugative plasmid and 14R525 (nalidixic acid resistant) was the recipient.

Plasmids. pKD100 is a pBR322 derivative encoding resistance to chloramphenicol, ampicillin, and kanamycin. It also carries (*i*) the IS*903* transposase gene under control of the *lac* UV5 promoter and (*ii*) two IS*903* inverted repeats. The model transposon contained within these inverted repeats includes the entire plasmid except the kanamycin-resistance gene (Fig. 1 and ref. 6). pKD158 is a transposase-deficient derivative of pKD100 (6); it contains a frame-shift mutation close to the 5′ end of the transposase gene. pKD162 is a derivative of the expression vector pAS1 (14) containing the transposase gene under control of the phage λ P_L promoter; a strong transcription terminator within the transposase gene (ref. 5; T. A. Weinert and N.D.F.G., unpublished results) has been removed by oligonucleotide-directed mutagenesis to improve expression. pKD71 contains a fusion of the transposase and *lacZ* genes (joined in frame by a short collagen gene linker) cloned into pAS1 and under control of the phage λ P_L promoter. A collagen-*lacZ* gene construct (15) was fused to the transposase gene after insertion of an in-frame *Bam*HI linker at the transposase gene terminal codon. The tripartite gene fusion was subcloned from pKD71 into either pKD100 (to give pKD189) or pACYC184 (ref. 16; to give pKD195) for *in vivo* transposition assays; in all cases the transcriptional and translational initiation signals for the transposase and transposase–β-galactosidase fusion proteins are identical. pKD192 is a derivative of pKD100 that contains an untrans-

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Abbreviation: IS, insertion sequence.

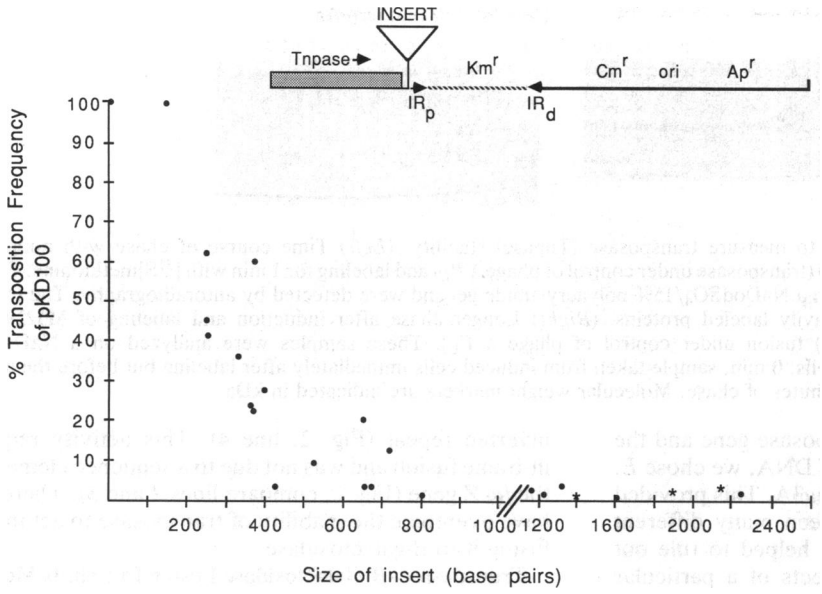

FIG. 1. Effect on transposition of increasing the distance between the 3′ end of the transposase gene and the proximal inverted repeat. Asterisks denote inserts that were used for further experiments discussed in the results. (*Inset*) pKD100 and the site of insertion of chromosomal DNA. IR_p and IR_d are the transposon proximal and distal inverted repeats, with respect to the transposase gene (Tnpase). The points of the arrowheads are the outside ends of the transposon; thus, in pKD100 and its derivatives, the transposon consists of the entire plasmid except the kanamycin gene, which is outside of the inverted repeats. Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Ap^r, ampicillin resistance; ori, plasmid origin of replication.

lated *lacZ* gene inserted into the unique *Bam*HI site of pKD100, placing it 17 base pairs (bp) downstream of the transposase gene (Fig. 2). pKD168 has the transposase gene and promoter from pKD100 inserted into the *Eco*RI site of pACYC184. pCJ136 is a pACYC184 derivative carrying the phage λ *c*I857 gene. This was used to regulate P_L expression from pKD162 in the *lon* strains and was a gift of C. Joyce.

Techniques. Standard molecular and genetic techniques were used (17). Mating assays were carried out as described (6), and frequencies presented are the average of at least three experiments. The sizes of chromosomal DNA fragments inserted into pKD100 were estimated by comparison with known standards after digestion with *Sau*3A and agarose gel electrophoresis.

Pulse–chase experiments for examining protein stability were carried out by diluting overnight cultures of appropriate plasmid-containing strains into fresh M9 minimal media with Casamino acids and growing at 30°C to an $OD_{600} \approx 0.3$. The cells were then washed and resuspended in minimal medium containing 2% methionine assay mix (Difco) and grown for a further 30 min. The cells were then quickly shifted to 42°C to inactivate phage λ repressor and induce P_L-mediated expression. After 30 min of induction, cells were labeled for 1 min with 10 μCi (370 kBq) of [^{35}S]methionine per 100 μl of cells (Amersham; specific activity, 1426 Ci/mmol) before chasing with excess nonradioactive methionine. Samples were taken at appropriate times into $NaDodSO_4$ sample buffer and frozen on dry ice. They were then boiled for 2 min before separation of the products on $NaDodSO_4$/polyacrylamide gels.

The half-lives of transposase and its derivatives were determined by densitometric scanning and integration of autoradiograms on an LKB Ultroscan laser densitometer. In each case the transposase band was normalized to a stable host protein.

RESULTS

Frequency of IS*903* Transposition Is Sensitive to the Distance Between the Transposase Gene and its Nearest Inverted Repeat. Our prototype transposon in pKD100 (Fig. 1; ref. 6) has 29 bp between the 3′ end of the transposase gene and the proximal inverted repeat. In addition to showing the importance of this proximity, our earlier studies had shown that it was the 3′ end of the gene that must be proximal to the inverted repeat (6). We have investigated this location effect further by measuring the effect on transposition of insertions of DNA into the unique *Bam*HI site of pKD100, which is

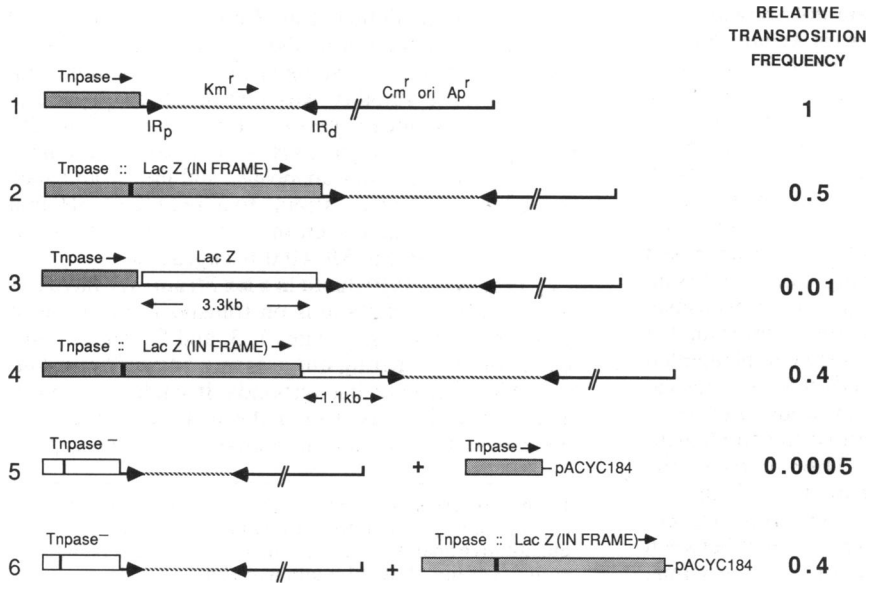

FIG. 2. A transposase–β-galactosidase gene fusion can act in trans. Transposition frequencies are relative to that of pKD100 (line 1), which was 1.6×10^{-4}. Plasmids used were pKD189 (line 2), pKD192 (line 3), pKD198 (line 4), pKD158 and pKD168 (line 5), and pKD158 and pKD195 (line 6). The pACYC184-derived plasmids, pKD168 and pKD195, are present at about 50% of the copy number of the remaining pBR322-based plasmids. Abbreviations are as in the legend to Fig. 1.

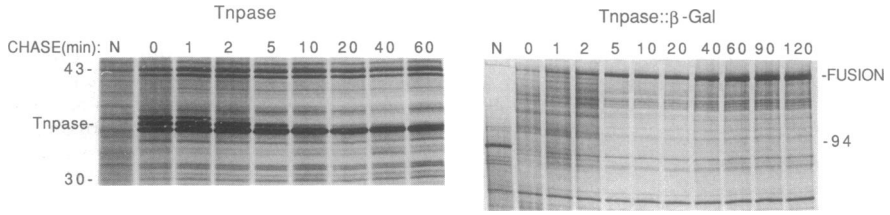

FIG. 3. A pulse–chase experiment used to measure transposase (Tnpase) stability. (*Left*) Time course of chase with nonradioactive methionine after induction of M5219 (pKD162) (transposase under control of phage λ P_L) and labeling for 1 min with [^{35}S]methionine. The labeled proteins were separated by electrophoresis on a NaDodSO$_4$/15% polyacrylamide gel and were detected by autoradiography. The transposase protein is the middle band of the three heavily labeled proteins. (*Right*) Longer chase after induction and labeling of M5219 (pKD71) [transposase–β-galactosidase (Tnpase::β-Gal) fusion under control of phage λ P_L]. These samples were analyzed on a NaDodSO$_4$/8% polyacrylamide gel. Lanes: N, noninduced cells; 0 min, sample taken from induced cells immediately after labeling but before the addition of excess nonradioactive methionine; 1–120, minutes of chase. Molecular weight markers are indicated in kDa.

located between the 3′ end of the transposase gene and the proximal inverted repeat. As a source of DNA, we chose *E. coli* chromosomal DNA digested with *Sau*3A. This provided us with the potential to clone and screen many different inserts of a similar size range and thus helped to rule out anomalous results due to sequence effects of a particular fragment. The digested chromosomal DNA was size-fractionated on an agarose gel before cloning the individual pools into the linearized vector DNA. Several clones from each size pool were then picked and assayed for transposition by a mating-out assay (Fig. 1).

Action of the IS*903* transposase was clearly very sensitive to the length of inserted DNA as determined by this assay. An inverse relationship was seen between the transposition frequency and the distance between the 3′ end of the transposase gene and the proximal inverted repeat. Once this distance reached about 1000 bp, the transposition frequency leveled off at about 1% of the control level.

The general trend of reduced transposition as a function of insert size was apparent with many independent data points derived from different inserted sequences. This suggests that the length of the insert (and not other insert characteristics) was the critical determinant. Two alternative possibilities—that the inserts caused a reduction either in activity of transposon ends or in levels of active transposase—were ruled out by the following experiments with a representative set of three clones (marked with an asterisk in Fig. 1). First, each of the transposons in this set showed equal and full transposition activity when the transposase–β-galactosidase fusion protein (see below) was provided in trans (data not shown). Second, the level of transposase activity provided by each of the set in a complementation assay was indistinguishable from that provided by pKD100 (data not shown).

Transposase Fusion Protein Overcomes cis Action. As part of our ongoing efforts to obtain active IS*903* transposase protein, we constructed an in-frame fusion between the transposase gene and *lacZ*. The fusion was made between the transposase terminal codon and the codon corresponding to the amino terminus of a collagen–β-galactosidase fusion construct (15). The transposition frequency from this construct was compared with that of pKD100 by using a mating-out assay (Fig. 2, compare lines 1 and 2). The two transposed at almost identical frequencies, showing that the fusion protein was almost as active as the native transposase. Furthermore, the fusion protein was as active in trans (and at a distance) as it was in cis. The fusion protein complemented transposition from pKD158 (a transposase-defective derivative of pKD100) at levels similar to transposition in cis (Fig. 2, compare lines 2 and 6). This is in contrast to complementation from a pACYC184 derivative, pKD168, carrying the transposase gene alone (Fig. 2, compare lines 5 and 6). Transposition mediated by the fusion protein was also insensitive to the insertion of a 1.1-kilobase (kb) segment between the gene encoding the fusion protein and the most proximal inverted repeat (Fig. 2, line 4). This activity required an in-frame fusion and was not due to a sequence element within the *lacZ* gene (Fig. 2, compare lines 2 and 3). Therefore, we have overcome the inability of transposase to act in trans by fusing it to β-galactosidase.

Transposase–β-Galactosidase Fusion Protein Is More Stable than Transposase. We suggested previously that transposase was functionally unstable during the period between its synthesis and the first binding step (6). The ability of the fusion protein to act in trans suggested that this instability was overcome. Therefore, we compared the half-life of the two proteins (Fig. 3). The half-life of transposase was very short, about 3 min, and the protein was not detectable above background after 40 min. This is consistent with our inability to accumulate transposase protein from a variety of expression systems (unpublished results). In contrast, the transposase–β-galactosidase fusion protein had a much longer half-life and was clearly still present after a 120-min chase. This result is consistent with the cis action of IS*903* transposase being mediated by protein instability.

We have noticed that the fusion protein has much less β-galactosidase activity *in vivo* or *in vitro* than equivalent amounts of (nonfusion) β-galactosidase. This, along with the fact that we did not see any accumulation of β-galactosidase-size proteins in our gels, suggests that trans action is a property of the fusion protein and is not due to breakdown of the fusion into its component parts.

Protease-Deficient Strains Improve Transposase Stability. *E. coli* deficient in the protease La and the heat shock σ factor (HtpR) have been shown to improve protein stability (18, 19). We found that in such a strain the half-life of transposase is dramatically increased to >60 min (data not shown). Unfortunately, strains deficient in HtpR are inviable at 37°C, and so we have limited our analysis to the effect of different *lon* alleles on transposase stability and transposition frequency.

Transposase stability was measured in isogenic pairs of strains by inducing expression of transposase from the phage λ P_L promoter and labeling with [^{35}S]methionine. Fig. 4 shows the increase in transposase stability from a half-life of about 3 min in the *lon*$^+$ strain* to a half life of >60 min in the *lon*$^-$ strain. We have seen this effect with both *lon-100* and *lon-510* alleles in the MC4100 background.

cis Preference Is Reduced in a *lon* Strain. We have examined the effect of *lon* mutations on transposition, using the constructs shown in Fig. 2 (lines 1, 3, and 5). Table 1 shows the results of these mating-out assays using different *lon* alleles and different genetic backgrounds. In each case transposition in trans was increased (and the distance effect in cis was reduced) in the *lon*$^-$ strain relative to the wild-type isogenic

*In this experiment a minor portion of transposase (about 18%) appears to be resistant to degradation in the *lon*$^+$ strain. However, quantitative analysis of the gel indicates that the remainder decayed with a half-life of about 3 min as before.

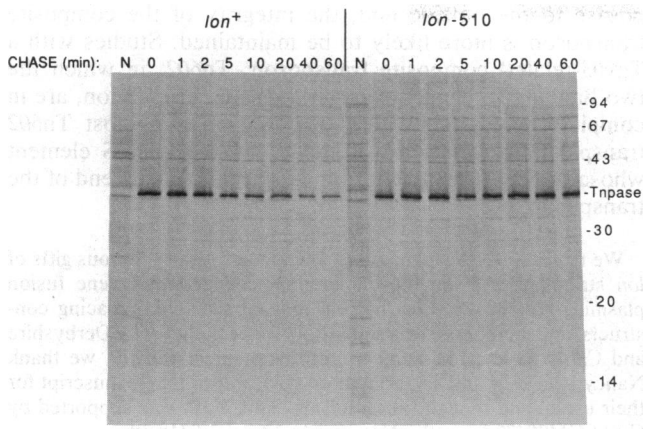

FIG. 4. A pulse–chase experiment comparing the transposase (Tnpase) half-life in a *lon*$^+$ and a *lon*$^-$ strain. Inductions were carried out in the *lon*$^+$ strain SG20250 and the *lon*$^-$ strain JT4000 carrying both pKD162 (transposase under control of phage λ P_L) and pCJ136 (pACYC184 carrying the *c*I857 gene). Lanes: N, noninduced cells; 0 min, sample taken from induced cells immediately after labeling but before the chase; 1–60, minutes of chase. Labeled proteins were separated on a 15% denaturing polyacrylamide gel and detected by autoradiography.

strain. In the SA500 background, trans action was increased >100-fold, and in the MC4100 background complementation in trans was increased 10-fold. Thus again we observed a direct correlation between transposase stability and reduction of cis preference. We assume the differences between the efficiency of rescue seen in the two different backgrounds (MC4100 and SA500) reflect the presence or absence of other factors that limit cis action and transposition. Indeed, the level of transposition from pKD100 in MC4100 was 7.7 times higher, suggesting that there are intrinsic differences between the two genetic backgrounds. However, the relative level of transposition from pKD100 (i.e., in cis) was the same in *lon*$^+$ and *lon*$^-$ derivatives of either genetic background, suggesting that the transposase gene and inverted repeat are in their optimum location and that transposition is now limited by other factors such as transposase expression.

Transposition promoted by the transposase–β-galactosidase fusion protein both in cis and in trans was insensitive to the activity of the *lon* gene, occurring at about 50% of the frequency of transposition from pKD100 (data not shown). Thus, as expected, the stable fusion protein bypassed the role of *lon* in allowing action of transposase in trans.

DISCUSSION

We have confirmed and extended our earlier observation that the transposase of IS*903* is cis-acting; complementation of defective transposons in trans is very inefficient, and transposition is highly sensitive to the distance between the 3′ end of the transposase gene and the most proximal inverted repeat. The transposase of IS*10* is also cis-acting, and limited data indicate that it too is sensitive to the distance between the transposase gene and the transposon (7). However, in the case of IS*10*, the effects of short distances (0–1 kb; the critical region for IS*903*) were not studied, so our results are not directly comparable.

cis action of IS*903* transposase could be overcome either by a transposase–β-galactosidase fusion or by using a strain of *E. coli* deficient in the *lon* gene product, La. We showed not only that cis action was reduced in these two situations but also that transposase stability was increased. Thus, in two different circumstances, we have observed a direct correlation between increased transposase stability and loss of cis preference. Therefore, we would argue that the instability of IS*903* transposase is a direct determinant of its cis action and that this instability is regulated at least partially by the La protease. One prediction of our hypothesis is that trans-acting mutants of transposase should be more stable. These mutants might also provide valuable insight into the site of action of the La protease.

Interestingly, the Q protein of phage λ is also both unstable and cis-acting (4, 20). A potential mechanism for Q protein cis action was based on this instability, but no direct evidence has been presented to support it. However, instability alone is not sufficient to explain cis action, since there are clear examples of unstable nucleic acid binding proteins that do act in trans and whose sites of action are distant from their genes—for example, the N and CII proteins of phage λ (21, 22).

In the simplest model, instability of a protein could give rise to cis action only if the following three conditions are fulfilled. (*i*) The time required to find a distant site must be greater than the time required to find a site adjacent to its gene. (*ii*) The time taken to find a distant site must be long relative to the half-life of the protein. (*iii*) The quantity of available active protein must be limiting so that the assayed activities (cis and trans) will be sensitive to differences in local concentration. Presumably, the IS*903* transposase fulfills all three conditions. These conditions could be facilitated by other properties of the protein or its gene expression. For example, the time taken to find a distant site could be increased by the protein spending extended periods in un-

Table 1. Transposition frequencies of IS*903* derivatives in *lon*$^{+/-}$ strains

	Relative transposition frequencies				
	MC4100 background			SA500 background	
Location of transposase gene*	*lon*$^+$	*lon-510*	*lon-100*	*lon*$^+$	*lon-100*
cis: transposase adjacent to inverted repeat	1	0.93	1.5	1	2.1
cis: transposase 3.3 kb away from inverted repeat	0.01	0.06	0.05	0.03	0.7
trans: transposase on different plasmid to transposon	0.003	0.05	0.03	0.007	1.1

The transposition frequencies from mating-out assays are relative to pKD100 in the wild-type background; for MC4100 this was 2.7×10^{-4}, and for SA500 this was 3.5×10^{-5}.

*The location of the transposase gene was varied by using the following plasmids: pKD100 was used for line 1, where the 3′ end of the transposase gene is 29 bp from the nearest inverted repeat; pKD192 was used to place the 3′ end of the transposase gene 3.3 kb from the nearest inverted repeat (this construct contains the *lacZ* gene inserted between the transposase gene and the proximal inverted repeat, see Fig. 2, line 3); pKD168, a pACYC184 derivative, was used for line 3 to supply transposase in trans to a defective transposon construct, pKD158 (see Fig. 2, line 5).

productive complexes with nonspecific DNA (4) in which the protein is still susceptible to degradation.

The acquisition of trans activity by the transposase–β-galactosidase fusion protein could result in principle from the failure to fulfill any of the three conditions listed above. In other words, the time required to find a distant site could be reduced, or its half-life could be increased, or it could be made in significantly larger amounts. We favor the second explanation because the increased stability of the fusion protein is readily observed. However, we cannot eliminate the additional possibility that altered properties of the fusion protein might speed up its search for a site of action in trans (for example, it might be less efficiently sequestered by interactions with nonspecific DNA). The third possibility, increased synthesis of the fusion protein, is less likely because both native transposase and the fusion protein are made by using the same transcriptional and translational initiation signals.

We have previously suggested that the IS*903* transposase interacts with the IS ends in a sequential manner, first with the closest end and then, as a transposase-end complex, with the second, more distant, end (6). As shown in the experiments described above (Fig. 1), transposition frequency is sensitive to the distance between the transposase gene and the nearest end, suggesting that protein degradation is significant during this initial search. However, transposase instability may not be a significant factor while the initial transposase-end complex searches for the second end. If it were, one would predict that the transposon length (i.e., distance to the second inverted repeat) would also have a dramatic effect on transposition. This is not the case; increase in transposon length has only a small effect on IS*903* transposition (23). This suggests either that the specific complex between transposase and one end is functionally more stable than transposase alone or that the search for the second end is rapid relative to the initial search.

The evolution of cis-acting transposases may be of particular relevance for transposons (such as IS*10*; ref. 24) that transpose by a cut-and-paste mechanism in which excision of the element from the donor site leaves a broken chromosome. In such a case, the cis preference of transposase ensures that a silent copy of the element in the target genome will not be trans-activated to give a potentially lethal excision. Furthermore, the combination of a cis-acting transposase and the regulation of transposition by *dam* methylation (as seen with IS*10*, IS*50*, and IS*903*; refs. 25 and 26) will favor transposition of an element that has just been duplicated. It is notable that transposons of the Tn*3* family, which move by a replicative mechanism not involving lethal chromosome breaks, have trans-acting transposases (27).

A second consequence of the extreme cis action of the IS*903* transposase may be to increase the maintenance of the composite transposon Tn*903*. In contrast to composite transposons such as Tn*10* and Tn*5*, the transposase gene of IS*903* is oriented such that the 3′ end of the gene is at the outside end of the composite transposon. This ensures that the transposase interacts first with an outside end of the element (because of cis action); the complex can then recognize the inside end of the same IS or the other extreme outside end of the composite transposon, thus giving rise to transposition of either the IS element or the composite transposon. If the transposase interacted first with the inside ends of the composite transposon, then only inverse transpositions or IS transpositions would be seen. Thus, by directing the transposase to the outside end, the integrity of the composite transposon is more likely to be maintained. Studies with a Tn*903*-related composite transposon, Tn*602*, in which the two flanking IS elements are in the same orientation, are in complete agreement with this proposal (28); most Tn*602* transposition events are promoted by the one IS element whose transposase gene terminates at the outside end of the transposon.

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