Supplementary data, Rowley et al,

DNA binding affinities of the hyperactive mutants

The hyperactive mutant proteins L460P, Y475H, E449G and E449K were used in DNA binding affinity assays with all the *att* sites (Fig. S1). None of the mutants bound to any of the *att* sites with significantly lower affinity that wild type integrase. Indeed the most defective integrase analysed here, Y475H, had a higher affinity to all the *att* sites than the wt integrase. L460P and E449G had similar binding affinities to each other and the affinities of E449K resembled those for the wt integrase. Loss of function phenotypes of Y475H and L460P are not due to low affinity DNA binding.

Qualitative differences in binding were observed between the mutants and the wt integrase. E449G consistently had a faster mobilities with *attP* and *attB* and L460P had slower mobilities with *attL* and *attR* compared to the wt. Different mobilities could be indicative of different integrase or DNA conformations.

Rate of DNA cleavage and recombination in excision by E449K using linear substrates

The rate of excision by E449K was assayed using a radio-labelled DNA fragment encoding *attL* and a longer unlabelled fragment encoding *attR*. the reactions were stopped after increasing incubation times, treated with subtilisin to remove integrase and then the DNAs separated by PAGE. The radioactivity in each band was quantified and used to calculate the proportions of product and cleaved substrate (Figure S2). The rate of DNA cleavage and product formation in excision is much slower than the equivalent reactions during integration by E449K. The level of cleaved substrate is about three times the amount of product. Possibly the low rate of

product formation may be a consequence of a higher rate of *attP x attB* synapsis and cleavage compared to that with *attL x attR*.

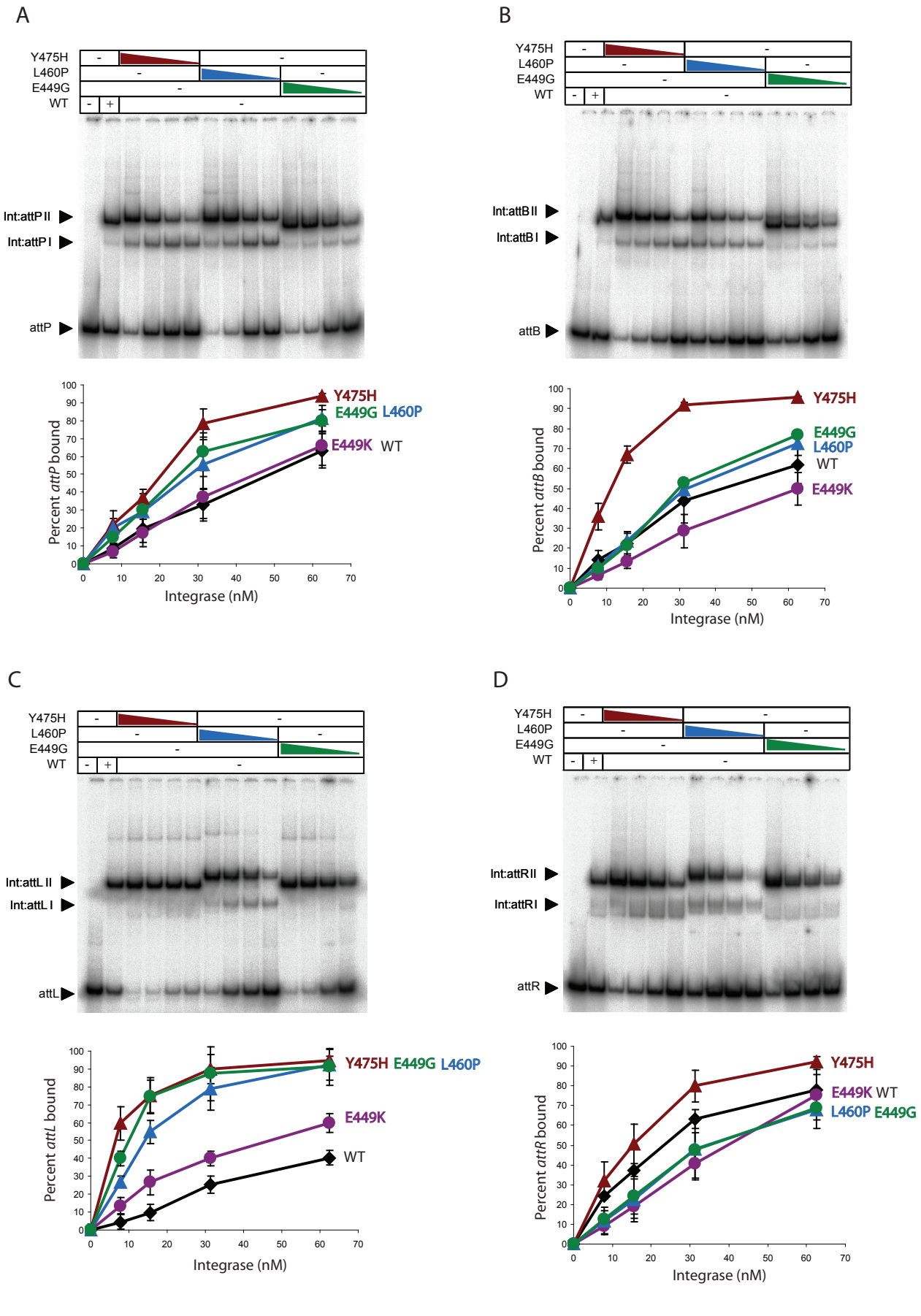
A
Y475H
L460P
E449G
WT
Int:attP II
Int:attP I
attP
Percent attP bound
Integrase (nM)
Y475H
E449G L460P
E449K WT
B
Int:attB II
Int:attB I
attB
Percent attB bound
Integrase (nM)
Y475H
E449G
L460P
WT
E449K
C
Int:attL II
Int:attL I
attL
Percent attL bound
Integrase (nM)
Y475H E449G L460P
E449K
WT
D
Int:attR II
Int:attR I
attR
Percent attR bound
Integrase (nM)
Y475H
E449K WT
L460P E449G

Figure S1

Figure S1. DNA binding affinities of the hyperactive mutants. Integrases Y475H, L460P, E449G were incubated with radio-labelled fragments encoding *attP* (panel A), *attB* (panel B), *attL* (panel C) and *attR* (Panel D). *attB* (106 bp), *attP* (98 bp), *attL* (90 bp) and *attR* (88 bp) fragments were purified from pRT602 (SalI/BglII), pRT702 (HindIII/BglII), pRT600702 (BglII/SphI) and pRT600702 (HindIII), respectively. The DNA fragments (~ 1 pmol) were labelled with α-^{32}P-dCTP by polymerisation of the recessed 3' ends by DNA polymerase I, Klenow Fragment (New England Biolabs) (Sambrook and Russell, 2001). Excess α-^{32}P-dCTP was removed using *Micro*CLEAN (Microzone Ltd) according to the supplier's instructions. DNA binding was performed using 1.5 nM of ^{32}P labelled *att* site DNA with integrase (0, 63, 31, 16, 8 nM) in binding buffer (Smith et al., 2004). Samples were incubated for 30 mins at 30^{o}C and the products analysed by electrophoresis in 5% polyacrylamide gels, which were dried down and exposed to Fuji phosphorimager screens and the images analysed using AIDA software (Raytest). For each binding experiment controls using wt integrase (31 nM) and the free probe were also added (2 left hand lanes).

The percent bound (average and standard errors) from at least three different experiments is shown below the phosphorimages for the representative binding experiments. Data is also included here for the percent bound by E449K.

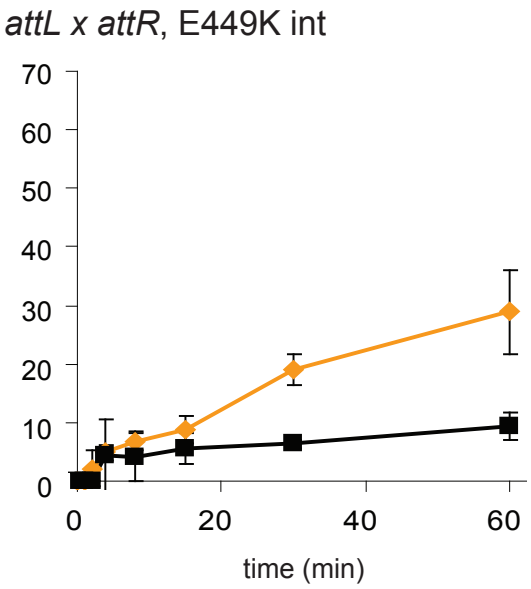

Figure S2

Figure S2. Rate of DNA cleavage and recombination in excision by E449K

Time course of *attL x attR* recombination using linear DNA fragments. E449K integrase (733 nM) was incubated with radio-labelled *attL* (90 bp; 1.5 nM) prepared by digestion of pRT600702 (BglII/SphI) and unlabelled *attR* (13 nM) for the indicated times. The reactions were stopped by heat treatment (80^{o}C, 10 mins.) and subtilisin (Sigma) was added (0.016U) and incubated (37^{o}C, 20 min). Subtilisin was inactivated (80^{o}C, 10 min), the DNAs were separated by PAGE and analysed as described in the legend to Figure S1 for the DNA binding assays. The amount of radioactivity in the products (blue lines) versus the cleaved substrates (orange lines) was quantified and plotted against time. The results are the average of two independent experiments ± standard error.

Supplementary Material and Methods

Construction of plasmids for Rowley *et al,*

pMSX18. The *attL* and *attR* sites were first created by performing an *in vitro* recombination reaction between pHS20 (*attP*) and pHS23 (*attB*) to form pHS2023 containing *attL* and *attR* (1). *attL* was then amplified with primers PRMS116 and PRMS117 (Table S1a) and pHS2023 as a template and then ligated into XbaI-HindIII digested pSP72 (Promega) to generate pMSX14. *attR* was amplified (primers PRMS118 and PRMS119; Table S1a) using pHS2023 as a template and inserted into KpnI-EcoRI cut pSP72 to make pMSX15. *attL* from pMSX14 was then excised with HindIII and XbaI and ligated to HindIII-XbaI cut pMSX15 to make pMSX16. pMSX18 was then constructed by inserting a blunt-ended SapI-PsiI fragment from pGEM7 into pMSX16 cut with BamHI and blunted ended.

pPAR1000. A 1654 bp ApaLI-BglII fragment from pMSX18 containing *attL* and *attR* flanking *lacZ*α was inserted into BamHI-SfcI cut pZMR100 (2).

pMSX22. An MluI(blunted)-XbaI fragment encoding *attP* was inserted into PvuII-XbaI cut pRT702 to generate pMSX21. pMSX22 was then constructed by inserting a blunt-ended SapI-PsiI fragment from pGEM7 encoding *lacZ*α into pMSX21 cut with XbaI and blunted.

pMSX24. An XbaI-HindIII fragment encoding *attL* was isolated from pMSX14 and inserted into pRT702 cut with XbaI and HindIII to form pMSX23. The blunted SapI-PsiI fragment encoding *lacZ*α was ligated with pMSX23 cut with XbaI and blunted-ended to form pMSX24.

pMSX26. A HpaI-HindIII fragment encoding *attL* from pMSX14 was inserted into pRT602 cut with PvuII and HindIII to make pMSX25. The blunted SapI-PsiI fragment from pGEM7 encoding *lacZ*α was ligated with pMSX25 cut with HindIII and blunt-ended to generate pMSX26.

pMSX28. A HindIII-EcoRV fragment encoding *attR* was inserted into pRT602 cut with PvuII and HindIII to form pMSX27. To make pMSX28 the blunted SapI-PsiI fragment from pGEM7 encoding *lacZ*α was ligated with pMSX27 cut with XbaI and blunt-ended.

pMSX30. Oligonucleotides PRMS120 and PRMS121 (Table S1a) were annealed and ligated into pMSX15 cut with XhoI and SphI to generate pMSX29. pMSX30 was made by inserting the blunted SapI-PsiI fragment from pGEM7 encoding *lacZ*α into pMSX29 cut with XbaI and blunt-ended.

pMSX32. Oligonucleotides PRMS122 and PRMS123 (Table S1a) were annealed and ligated to pHS23 cut with XbaI and SphI to create pMSX31. pMSX32 was then made by inserting the blunted SapI-PsiI fragment from pGEM7 encoding *lacZ*α into pMSX31 cut with XbaI and blunt-ended.

pMSX34. Oligonucleotides PRMS124 and PRMS125 (Table S1a) were annealed and ligated to pMSX14 cut with BamHI and ClaI to generate pMSX33. The blunted SapI-PsiI fragment from pGEM7 encoding *lacZ*α was ligated with pMSX33 cut with XbaI and blunt-ended to make pMSX34.

pMSX36. Oligonucleotides PRMS126 and PRMS127 (Table S1a) were annealed and ligated to XhoI-SphI cut pMSX15 to generate pMSX35. The blunted SapI-PsiI fragment from pGEM7 encoding *lacZ*α was ligated with pMSX35 cut with XbaI and blunt-ended to create pMSX36.

pPARX34 and pPARX36. Mutagenic primers pairs PRX25 / PRX26 and PRX27 / PRX28 were used to generate PCR products from pMSX34 and pMSX36, respectively. The PCR was carried out using Phusion^{TM} polymerase (New England Biolabs) using DMSO and GC buffer with the program: 95^{o}C for 30 s (denaturation), then 30 cycles of: 95^{o}C for 10 s (denaturation), 54^{o}C for 30 s (annealing) and 72^{o}C for 15 s (extension). All other PCR conditions were as recommended by the manufacturer. PCR products were digested with BglII and KpnI (PRX25 / PRX26) and NdeI and HindIII (PRX27 / PRX28) and ligated into pMSX34 and pMSX36 respectively that were digested with the same combinations of restriction enzymes.

pPARX18Laa and pPARX18Raa. Both mutant plasmids were created by the SDM of pMSX18 changing the central dinucleotide of *attL* (primers; attLaaFWD and attLaaREV) or *attR* (primers; attRaaFWD and attRaaREV from TT to AA.

pMS92, pMS93, pMS94, pMS95. pRT603701 (3) was digested with BamHI and the two fragments allowed to self-ligate to form pMS92 containing *attL*^{TA} and pMS93 *attR*^{TA}. The individual plasmids were isolate by transformation of DH5α. pMS94 containing *attL* and pMS95 containing *attR* were isolated in an analogous way from pRT602700 (3).

References

1. Thorpe, H.M. and Smith, M.C.M. (1998) In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. *0027-8424*, **95**, 5505-5510.

2. Rogers, M., Ekaterinaki, N., Nimmo, E. and Sherratt, D. (1986) Analysis of Tn7 transposition. *Mol Gen Genet*, **205**, 550-556.
3. Smith, M.C.A., Till, R. and Smith, M.C.M. (2004) Switching the polarity of a bacteriophage integration system. *Mol Microbiol*, **51**, 1719-1728.

Table S1a. Oligonucleotides used in plasmid construction and sequencing

Plasmid	Description of oligo	Sequence
PRMS116	PCR primer to amplify *attL* from pHS2023	GAACCAAGCTTACCGCGGGGCCATCATCTTG
PRMS117	PCR primer to amplify *attL* from pHS2023	GCTCCTCTAGACTCGAGGAATTCTCG
PRMS118	PCR primer to amplify *attR* from pHS2023	GGGAGGGTACCGGATCTTCGATGAAGTCATG
PRMS119	PCR primer to amplify *attR* from pHS2023	GACCACGATATCCAGCGGTTTCGAGGGCGAGGG
PRMS120	Encodes upper strand *attP*	TCGAGGAATTAGTAGTGCCCCAACTGGGGTAACCTTTGAGT TCTCTCAGTTGGGGGCGTACGAAATCGGCATG
PRMS121	Encodes bottom strand *attP*	CCGATTTCGTACGCCCCCAACTGAGAGAACTCAAAGGTTAC CCCAGTTGGGGCACTACTAATTCC
PRMS122	Encodes upper strand *attB*	CTAGAGAATTCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGG GCTCCCCGGGCGCGTACTCCCGAAATCGATAGCATG
PRMS123	Encodes bottom strand *attB*	CTATCGATTTCGGGAGTACGCGCCCGGGGAGCCCAAGGGCA CGCCCTGGCACCCGCACCGCGGAATTCT
PRMS124	Encodes upper strand *attL*	GATCCTTCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGAGTT CTCTCAGTTGGGGGCGTACGAAAT
PRMS125	Encodes bottom strand *attL*	CGATTTCGTACGCCCCCAACTGAGAGAACTCAAGGGCACGC CCTGGCACCCGCACCGCGGAAG
PRMS126	Encodes upper strand *attR*	TCGAGATTAGTAGTGCCCCAACTGGGGTAACCTTTGGGCTC CCCGGGCGCGTACTCCCGAAGCATG
PRMS127	Encodes bottom strand *attR*	CTTCGGGAGTACGCGCCCGGGGAGCCCAAAGGTTACCCCAG TTGGGGCACTACTAATC
PRX9	Forward primer pZMR100	CTG TTT TAT CAG ACC GCT TCT GC
PRX10	Reverse primer pZMR100	GCT GTT GAC AAT TAA TCA TCG GCT CG
HS43	Sequencing primer #2, *int* gene	GAT TGT TTC CAC TCA GGA AG
HS44	Sequencing primer #3, *int* gene	CTT CCC TTC AAG CCG GGC AG
HS45	Sequencing primer #4, *int* gene	TGG ACG GCA GGG GGC GCG GC
HS46	Sequencing primer #5, *int* gene	CGA ACG GGC GAA CCT TGT TG
T7	Sequencing primer T7-7	TAA TAC GAC TCA CTA TAG GG
PRX9	Forward primer pZMR100	CTG TTT TAT CAG ACC GCT TCT GC

Table S1b, Primers for site-directed mutagenesis[1]

Mutation	Sequence
E449A	CGG CAA GCT CAC TGC GGC GCC TGA GAA GAG C
E449H	CGG CAA GCT CAC TCA CGC GCC TGA GAA GAG C
E449R	CGG CAA GCT CAC TCG GGC GCC TGA GAA GAG C
E449F	CGG CAA GCT CAC TTT CGC GCC TGA GAA GAG C
E449Y	CGG CAA GCT CAC TTA CGC GCC TGA GAA GAG C
E449D	CGG CAA GCT CAC TGA CGC GCC TGA GAA GAG C
E449N	CGG CAA GCT CAC TAA CGC GCC TGA GAA GAG C
E449K	CGG CAA GCT CAC TAA GGC GCC TGA GAA GAG C
E449V	CGG CAA GCT CAC TGT GGC GCC TGA GAA GAG C
E449L	CGG CAA GCT CAC TCT GGC GCC TGA GAA GAG C
E449Q	CGG CAA GCT CAC TCA GGC GCC TGA GAA GAG C
S12A	GCT TAC GAC CGT CAG GCG CGC GAG CGC
E452K	CAC TGA GGC GCC TAA GAA GAG CGG CG
E456K	CTG AGA AGA GCG GCA AAC GGG CGA AC
D466K	GTT GCG GAG CGC GCC AAG GCC CTG AAC GC
A470K	CGA CGC CCT GAA CAA GCT TGA AGA GCT GTA CG
E472K	CCT GAA CGC CCT TAA AGA GCT GTA CGA AG
E473K	CCT GAA CGC CCT TGA AAA GCT GTA CGA AG
E476K	GAA GAG CTG TAC AAA GAC CGC GCG GCA GG C
D477K	GAA GAG CTG TAC GAA AAG CGC GCG GCA GG C
attLaa	GCC AGG GCG TGC CCA AGA GTT CTC TCA GTT GG
attRaa	CAA CTG GGG TAA CCT AAG GGC TCC CCG GGC GCG TAC

[1] These are the top strand only. Second strand primers with sequences complementary to the top strand primers were used in addition for each mutant but are not shown here.

Table S1c. Oligonucleotides used for DNA binding assays

***att* site**	**Name**	**Sequence**
attP	PRXO1	GGG GCT GCC CCA ACT GGG GTA ACC TTT GAG TTC TCT CAG TTG GGG GCG TG
	PRXO2 Complement	CAC GCC CCC AAC TGA GAG AAC TCA AAG GTT ACC CCA GTT GGG GCA G -3'
attB	PRXO3	5'- GGG GCG CGG GTG CCA GGG CGT GCC CTT GGG CTC CCC GGG CGC GTA CTC CG3
	PRXO4 Complement	-CGG AGT ACG CGC CCG GGG AGC CCA AGG GCA CGC CCT GGC ACC CGC G
attL	PRXO5	GGG GAC CGG GTG CCA GGG CGT GCC CTT GAG TTC TCT CAG TTG GGG GCG TG -3'
	PRXO6 Complement	5'-CAC GCC CCC AAC TGA GAG AAC TCA AGG GCA CGC CCT GGC ACC CGG
attR	PRXO7	GGG GCT GCC CCA ACT GGG GTA ACC TTT GGG CTC CCC GGG CGC GTA CTC CG
	PRXO8 Complement	CGG AGT ACG CGC CCG GGG AGC CCA AAG GTT ACC CCA GTT GGG GCA G