Site-specific genomic integration produces therapeutic Factor IX levels in mice

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Published online 15 October 2002; doi:10.1038/nbt753

We used the integrase from phage ϕC31 to integrate the human Factor IX (hFIX) gene permanently into specific sites in the mouse genome. A plasmid containing *attB* and an expression cassette for hFIX was delivered to the livers of mice by using high-pressure tail vein injection. When an integrase expression plasmid was co-injected, hFIX serum levels increased more than tenfold to ~4 μg/ml, similar to normal FIX levels, and remained stable throughout the more than eight months of the experiment. hFIX levels persisted after partial hepatectomy, suggesting genomic integration of the vector. Site-specific integration was proven by characterizing and quantifying genomic integration in the liver at the DNA level. Integration was documented at two pseudo-*attP* sites, native sequences with partial identity to *attP*, with one site highly predominant. This study demonstrates *in vivo* gene transfer in an animal by site-specific genomic integration.

Lasting gene therapy may be facilitated by the permanent introduction of exogenous DNA into the chromosomes of patients. Here we describe the use of bacteriophage ϕC31 integrase to achieve site-specific genomic integration of therapeutic genes into the genomes of adult mice. In nature, the integrase mediates unidirectional recombination of the phage genome into the bacterial chromosome through a site-specific reaction between the phage *attP* site and the host *attB* site[1,2]. We have shown that this integrase is functional in mammalian cells[3], and that it can efficiently integrate plasmids containing an *attB* site into pseudo-*attP* sites in mammalian genomes[4]. These pseudo sites have enough identity with wild-type *attP* to support integrase-mediated recombination, and persistent gene expression occurs from vectors integrated at such sites[4]. In this study, we have applied these principles *in vivo* to integrate *attB* vectors bearing the gene for human blood clotting Factor IX (hFIX) into the chromosomes of mice at native pseudo-*attP* sites, resulting in the generation of persistent normal levels of the protein.

Results

Integrase-mediated expression of hAAT in mouse liver. We constructed plasmids that contained a ϕC31 *attB* site and either a cDNA expression construct of the human α_1-antitrypsin gene (hAAT)[5] or a minigene version of hFIX optimized for gene expression[6]. To evaluate the activity of the ϕC31 integrase *in vivo*, we injected 25 μg of the hAAT-*attB* plasmid with either 25 μg of carrier plasmid pCS or the integrase expression vector pCMV-Int (ref. 3) in ~1.8 ml saline into the tail vein of mice over 5–8 s. This hydrodynamic-based method delivers naked DNA to mouse hepatocytes at efficiencies of up to 40% without delivering substantial amounts of DNA to nonliver tissues[5,7,8]. We deliberately used a cDNA AAT construct known to express at levels lower than what has been seen when the full human genomic AAT gene is introduced by plasmid[9] or by high doses of gutless adenovirus[10] vectors, so as to maximize our ability to see increased expression in the presence of integrase. Serum levels of hAAT were highest one day after injection and then dropped steadily in the absence of integrase (Fig. 1). When pCMV-Int was included, hAAT levels stabilized within one to two weeks and remained at levels ~12-fold higher than when the carrier plasmid was used, indicating that the integrase substantially increased and stabilized expression levels. More pCMV-Int did not appreciably increase the stable levels of hAAT beyond that obtained using 25 μg of integrase (Fig. 1). Neither an hAAT plasmid lacking *attB* plus 100 μg pCMV-Int, nor the hAAT-*attB* plasmid plus 100 μg of pCMV-S12F-Int, a mutant substituting the catalytic serine and lacking DNA cleavage activity[11], raised expression levels. These experiments proved that the increased hAAT expression levels were dependent on the presence of the *attB* site on the hAAT plasmid and the catalytic serine of the integrase protein. We thus concluded that the introduced integrase gene was expressed and catalytically active in liver cells.

High-level hFIX expression persists after partial hepatectomy. We constructed an hFIX-*attB* plasmid by using a cassette optimized for liver-specific hFIX expression in mouse[6]. The hFIX protein produced by this minigene construct is known to be functional because it ameliorates the clotting disorder in Factor IX–deficient mice[12]. Mice that received the hFIX-*attB* and integrase plasmids by hydrodynamic tail vein injection produced 3,970 ± 1,040 ng/ml hFIX, ~12-fold more than mice that received the hFIX-*attB* plasmid alone (327 ± 82 ng/ml) at day 100 (Fig. 2). To investigate whether or not the DNA responsible for hFIX expression was integrated, we performed two-thirds partial hepatectomies (PH) on subsets of the mice. This procedure stimulates liver cell division and removes a large fraction of unintegrated DNA[12]. The high hFIX levels of the mice that received integrase remained stable after PH, while those of the mice that did not receive integrase dropped substantially (Fig. 2). This result suggested that, while unintegrated plasmids can express hFIX, essentially all of the high-level hFIX expression observed in the

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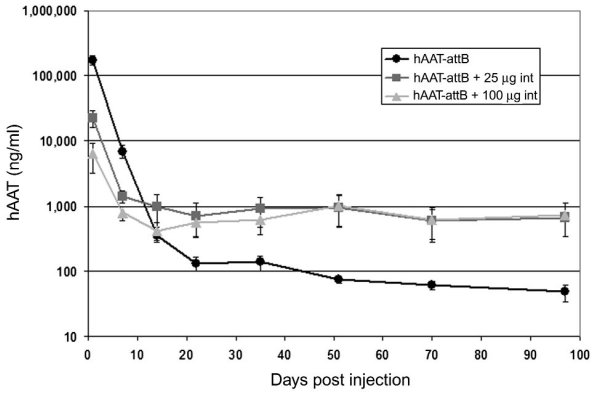

Figure 1. Integrase mediates increased and prolonged expression of hAAT in mouse liver. Groups of four mice received a large-volume tail vein injection of 25 µg of hAAT-*attB* vector alone (circles) or with 25 µg (squares) or 100 µg (triangles) integrase expression plasmid. Serum levels of hAAT were monitored by ELISA over the course of the experiment.

presence of integrase was due to integrated hFIX plasmid, rather than to residual extrachromosomal DNA. The low level of hFIX expression in post-PH animals that did not receive integrase may reflect residual extrachromosomal DNA or random integration. As demonstrated by the final time point in Figure 2 (open circles), this expression was barely above background, indicating that there was little or no detectable hFIX expression due to random integration.

Analysis of total and extrachromosomal vector DNA in liver cells. To quantitate extrachromosomal hFIX-*attB* plasmids in various mouse liver samples, we transformed electrocompetent *Escherichia coli* with DNA prepared from livers before and after PH. Resulting colonies on selective medium were counted. As shown in Figure 3A, the PH caused loss of ~80–90% of the extrachromosomal hFIX-*attB* plasmids from the livers, independent of integrase. Furthermore, mice that received integrase had substantially fewer extrachromosomal vectors than those that did not receive integrase, consistent with integrase-mediated integration of a large portion of delivered vectors.

A primer–probe set for *Taq*Man quantitative PCR[13] that detects the chloramphenicol resistance gene in the hFIX-*attB* vector was used to quantify the amount of vector DNA persisting in the liver after PH. This method measures the total number of vector molecules present, whether they are integrated or extrachromosomal. As shown in Figure 3B, the pre-PH amounts of total vector are approximately equivalent for all mice. Post-PH levels reveal that inclusion of integrase causes greater persistence of the hFIX-*attB* vector, suggesting its genomic integration.

Identification of genomic pseudo-*attP* integration sites *in vivo*. To prove that ϕC31 integrase-mediated genomic integration had occurred, we sought to demonstrate covalent linkage of the hFIX plasmid with sequences in the genomes of liver cells. The persistence of transfected extrachromosomal plasmid DNA in the liver[9,14] made standard plasmid rescue methods unfeasible, so a nested inverse PCR approach was used to amplify specifically integration junctions (*attL*) that would indicate genomic integration. Using this approach, we identified two different genomic integration sites that we named mpsL1 and mpsL2, for mouse pseudo-site from liver (Fig. 4A). We were unable to rescue any additional sites, suggesting a low number of integration sites. By using primers in the *attB* site in combination with primers flanking the mpsL1 and mpsL2 genomic regions, we amplified by PCR the *attL* and *attR* vector–genome junctions from the liver genomic DNA of mice that received integrase and the *attB* donor. The resultant bands were sequenced and aligned with the genomic sites. The switch from *attB* to genomic sequence at or near the TTG core and the detectable sequence identity between the genomic sequence and *attP* confirmed ϕC31-mediated integration at genomic pseudo-*attP* sites. These results further demonstrated the expression and catalytic activity of integrase in liver cells.

The PCR primers that detect mpsL1 *attL* were used to screen livers harvested from animals that received the hAAT-*attB* or hFIX-*attB* plasmid, with and without integrase. All (13/13) of the animals that received integrase were positive for the PCR band indicating integration at mpsL1 (Fig. 4C), whereas 0% (0/7) of the animals that did not receive integrase showed an integration event at mpsL1. This result was statistically significant ($P < 10^{-5}$) and suggested that integration at the mpsL1 pseudo-*attP* site in liver cells was a frequent event specifically mediated by the ϕC31 integrase.

Quantitation of *in vivo* site-specific integration events. We employed *Taq*Man quantitative PCR to determine the fraction of genomes in a given liver that contained integration events at mpsL1 and mpsL2. A primer–probe set that specifically amplified the *attL* junction resulting from integration of *attB* at mpsL1 (Fig. 4B) was used in combination with another probe set that allowed quantitation of haploid genomes using the single-copy target gene *RAD52*. The percentage of haploid liver genomes that were positive for an integration at mpsL1 ranged from 0.62 to 3.41, with an average of 1.8% ± 0.89% (Fig. 4D). No signal was detected for livers that did not receive integrase, indicating that there was no detectable integration at mpsL1 in the absence of integrase. To estimate the integration efficiency per transfected cell, the percentage positive cells must be multiplied by the inverse of the transfection efficiency. Considering that a maximal liver transfection efficiency of 40% has been reported for the hydrodynamic injection method[7], our integration frequency at mpsL1 per transfected cell appears to be well into the percentage range. A quantitative PCR analysis at the mpsL2 site revealed that integrations at this position were close to the lower limit of detection and >100-fold lower than the integration frequency at mpsL1. Because

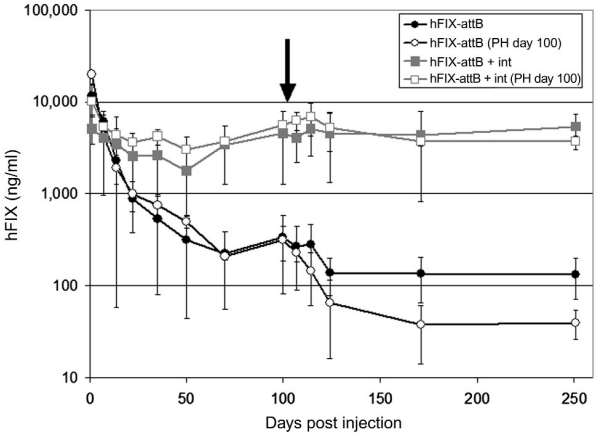

Figure 2. Therapeutic levels of hFIX persist after partial hepatectomy. Mice received a large-volume tail vein injection of 25 µg of hFIX-*attB* plasmid alone (circles) or with 25 µg of integrase expression plasmid (squares). Two-thirds partial hepatectomies were performed on the indicated groups 100 days after injection (indicated by the vertical arrow).

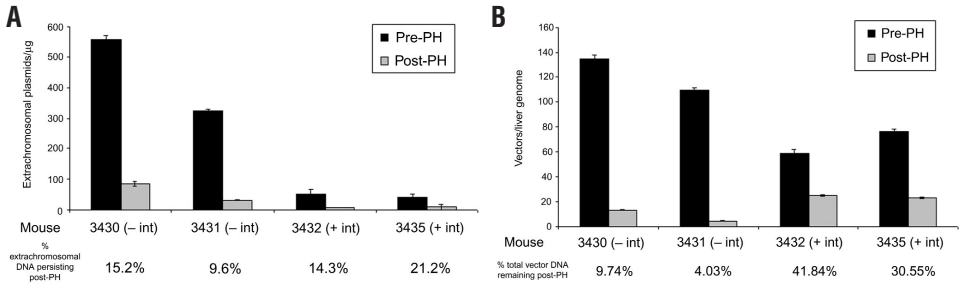

Figure 3. Quantitation of extrachromosomal vectors and total vectors persisting in four mouse livers after partial hepatectomy. (A) Total DNA prepared from four mouse liver samples before and after partial hepatectomy was transformed into electrocompetent *E. coli*. Resultant chloramphenicol-resistant colonies are represented here as a reflection of the quantity of extrachromosomal vectors present. (B) The same genomic DNAs were subjected to quantitative PCR for the chloramphenicol resistance gene vector backbone. These numbers reflect an estimate of the total amount of vector DNA remaining in the livers, independent of whether it is extrachromosomal or integrated.

we were able to rescue at least one integrant at mpsL2, we concluded that integration occurs at mpsL2 at a low frequency.

Integration appears to be largely restricted to the liver with this delivery method[7,8]. We analyzed DNA from brain, heart, kidney, spleen, lung, and liver of animals that received hFIX-attB and integrase plasmids. We conducted PCR to detect presence of the chloramphenicol resistance gene in the hFIX-*attB* plasmid backbone, and quantitative PCR to detect integration at the genomic mpsL1 site. Abundant plasmid DNA was detected by PCR only in liver; plasmid was present at 1,000-fold lower levels in kidney, and was near background or undetectable in the other tissues. Integration at mpsL1 was detected only in liver with the very sensitive quantitative PCR technique. Therefore, the overwhelming majority of expression of the introduced gene was due to DNA delivery to the liver, in agreement with expression studies by others[8]. Furthermore, the presence of a liver-specific promoter on our hFIX cassette[6] reinforced this specificity. Studies of high-pressure DNA injection into limb arteries in primates suggest that a similar procedure may be applicable to larger animals and humans[15].

Discussion

This study documents that phage ϕC31 integrase site-specifically integrates introduced plasmid DNA bearing an *attB* site into endogenous positions in the genome of mouse liver cells. The frequency of integration and the robust level of gene expression resulting from integrated DNA were sufficient to provide normal plasma levels of human Factor IX that remained stable long-term (Fig. 2). Therefore, we expect that introduction of the FIX gene using this integrase system will provide a permanent cure for hemophilia in mouse models, and, given comparably effective DNA delivery, in patients. The hFIX expression levels achieved by site-specific integration are, for example, 20-fold higher than those obtained using lentiviral delivery[16]. The integrase system has no apparent upper size limit and readily integrated the >9.7-kilobase hFIX plasmid. Furthermore, the prolonged survival (more than eight months) of Factor IX–expressing hepatocytes that we documented in this study suggests that there is no substantial immune response against integrase in this setting.

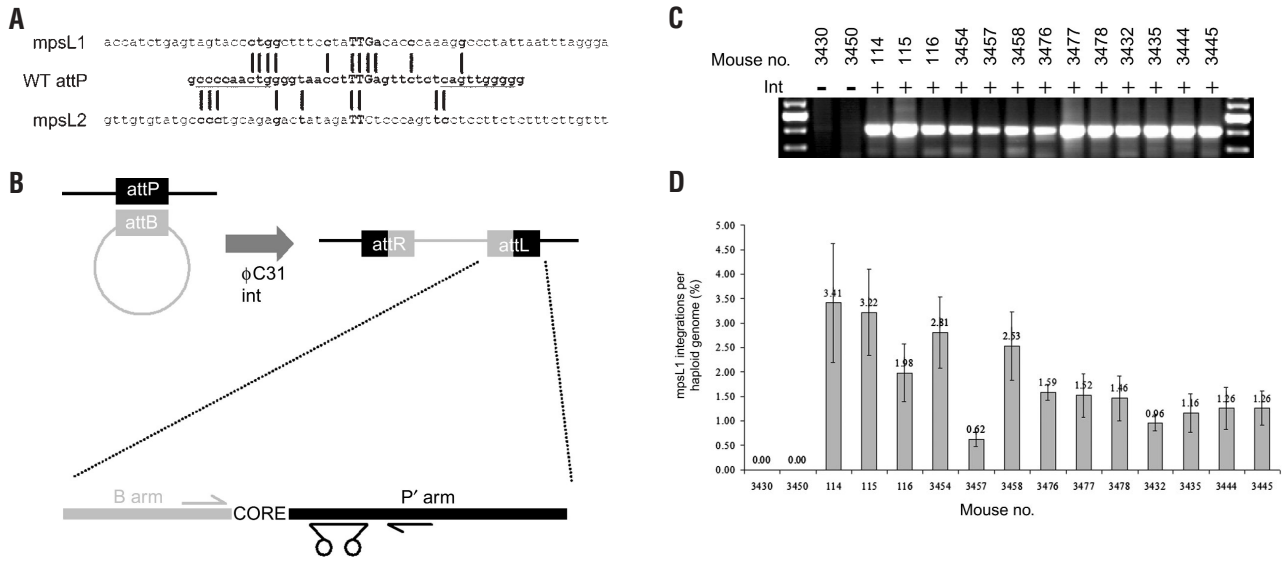

Figure 4. Detection of integrase-mediated site-specific recombination at the DNA level. (A) Comparison of wild-type *attP* sequence with the two pseudo-*attP* sites detected in liver DNA. Underlined bases in wild-type *attP* denote the inverted repeats. Bold bases indicate identity between *attP* and the pseudo-site. These identities over a 40 bp region are 30% and 23% for mpsL1 and 2. Higher percentage identity can be obtained by introducing small gaps in the assembly. The left arm of *attP* is more conserved, as we have seen before[4]. GenBank accession numbers for mpsL1 and mpsL2 are AC079573 and AL593846, respectively. (B) Schematic diagram of integrase-mediated recombination and its detection by real-time quantitative PCR. Specific *attL* junctions were detected using a primer that lies in the left half of the *attB* site (B arm), in combination with a dual-labeled probe and primer that lie in the right half of the pseudo-*attP* site (P′ arm) adjacent to the TTG identical region, which defines the CORE. (C) Ethidium bromide–stained gel depicts detection of integration at mpsL1 with standard PCR. Mice no. 3430 and no. 3450 did not receive integrase, while all other mice received 25 µg pCMV-Int. The expected band size is 513 bp. The brightest band in the size marker lane is 600 bp. (D) Quantitative *Taq*Man PCR detection of *attL* junction at mpsL1. Specific mpsL1/*attL* junction was quantified using real-time quantitative PCR and normalized to a single-copy genomic probe to determine the fraction of haploid genomes that contained a site-specific recombination event.

Integrase-mediated gene transfer required the presence of the phage *attB* site on the hFIX expression plasmid and of catalytically active ϕC31 integrase. Site-specific genomic integration was expected on the basis of tissue culture studies with the integrase[4,17] and was further suggested by the robust survival of hFIX expression after PH. Genomic integration was proven by recovery of integrated plasmid DNA from liver genomic DNA and demonstration of covalent junctions between introduced plasmid DNA and native mouse sequences.

The DNA sequences at the integration sites displayed substantial, though limited, identity with the wild-type phage *attP* sequence (Fig. 4A) and are termed pseudo-*attP* sites[4,18]. Our analysis revealed two such sites in the mouse genome, mpsL1 and mpsL2. Quantitative PCR demonstrated that mpsL1 was frequently targeted for integration events in the transfected liver cells. The number of integration events per cell is under investigation. The mpsL2 site was used far less often. The mpsL1 and mpsL2 integration sites are present in the mouse GenBank database, on chromosomes 2 and 11, respectively. Neither site lies within known coding sequences. A retrospective analysis of a collection of integration sites mediated by the ϕC31 integrase in mouse 3T3 tissue culture cells[4] revealed that integration at mpsL1 occurred in 3T3 cells, along with a minimum of 56 other integration sites. The integration specificity seems to be higher in differentiated tissues than in immortalized cell lines and may reflect a requirement for open chromatin, which is rarer in primary differentiated cells and may be needed for access by the large, 613–amino acid integrase protein. Judging from the robust, durable expression of hFIX at mpsL1, good integration sites may tend also to display good gene expression, because integration frequency could be influenced by some of the same variables that favor transcriptional activity, such as chromatin accessibility. The limited sequence identity between wild-type *attP* and pseudo-*attP* sites seen here and previously[4] is consistent with an influence of secondary factors such as chromatin accessibility in determining the locations where integration may occur in mammalian genomes. We hypothesize that the preference for integration at mpsL1 over mpsL2 is partly due to such contextual features. The high specificity of integration for one genomic site is beneficial for gene therapy because it reduces the risk of insertional mutagenesis.

We measured the amount of extrachromosomal hFIX-*attB* plasmid DNA remaining in liver cells transfected with and without integrase and before and after PH. The decrease in amount of extrachromosomal DNA remaining in the liver cells was not accompanied by a corresponding fall in hFIX expression (Figs 2, 3A). This finding is consistent with gene expression primarily coming from the integrated, rather than extrachromosomal, vectors, and further reinforces the suggestion that integration at preferred pseudo-*attP* sites supports strong, durable gene expression. This favorable circumstance predicts that integrase-mediated gene transfer is a route to obtaining long-term, efficient expression of introduced genes that is likely to be generally applicable to a wide variety of genes and tissues. Given that integrase frequency and specificity can be further improved and manipulated by directed evolution[17], the ϕC31 integrase seems to represent a useful tool for gene transfer. The existence of pseudo-*attP* sites in the human genome, including a hotspot on chromosome 8, has been demonstrated[4], opening the possibility of using the ϕC31 integrase for gene transfer in humans.

Experimental protocol

Animal studies. We obtained six- to eight-week-old C57Bl/6 mice from the Jackson Laboratory (Bar Harbor, ME) and housed them under specific-pathogen-free conditions. Experimental protocols were submitted and approved by the Administrative Panel on Laboratory Care (A-PLAC) at Stanford University. Animals were treated according to the NIH Guidelines for Animal Care and the guidelines set forth by the A-PLAC. Plasmid DNA in 0.9% saline (1.8 ml) was injected into the tail vein over 5–8 s (refs 7,8). Mice were periodically bled by the retro-orbital technique. In some cases, mice were subjected to a surgical two-thirds partial hepatectomy (PH) as described[12].

Blood analysis. We analyzed mouse serum for total hAAT or hFIX antigens by an ELISA assay[5]. We assessed liver injury in mice following plasmid injection by analyzing serum glutamate pyruvate transaminase (SGPT) levels for up to nine days as described (Sigma Procedure No. 505; Sigma, St. Louis, MO) and found no evidence of integrase-dependent liver toxicity.

Plasmid construction. The ϕC31 integrase expression plasmid pCMV-Int has been described[3]. Carrier plasmid pCS was generated by removing the integrase coding sequence from pCMV-Int using *Spe*I-*Nhe*I followed by re-ligation. A 2.0 kb *Xho*I fragment from pRSV.hAAT.bpA (ref. 12) was cloned into the *Xho*I site of pBCSK(+) (Stratagene, La Jolla, CA) to form pBC-hAAT. pTA-*attB* (ref. 3) was cut with *Eco*RI to release a 312 bp fragment encompassing the full-length ϕC31 *attB* site. The cohesive ends were blunted, and the *attB* fragment was ligated into the blunted *Not*I-*Sal*I sites of pBC-hAAT to create pBC-hAAT-B. The human Factor IX minigene was removed from vector pBS-ApoEHCR-hAATp-hFIXmg-bpA (ref. 6) with *Spe*I and ligated into the *Xba*I-*Spe*I sites in the polylinker of pBCSK(+) to create pBC-hFIX. The pBC-hFIX plasmid was digested and blunted at the *Bam*HI-*Xho*I sites, and the same blunted *att*B fragment from pTA-*attB* was ligated to make the 9.7 kb pBC-hFIX-B.

Pseudo site rescue by nested inverse PCR. Mice that received pBC-hAAT-B and integrase were killed. Liver genomic DNA was subsequently prepared, 10 μg of which was digested with pairs of restriction enzymes that have incompatible cohesive ends and that cut at least once in pBC-hAAT-B. The digests were extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 500 μl of 1× ligation buffer, to which was added 1,000 units of T4 DNA ligase (New England Biolabs, Beverly, MA). Low-concentration ligations were incubated at 17°C overnight, extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 20 μl TE. Primary amplifications with primers attB-F2 (5′-ATGTAGGTCACGGTCTCGAAGC-3′) and attL-iPCR-1 (5′-CCTACTCAGACAATGCGATGC-3′) were carried out on 0.5 μl of the ligation for 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s. Primers were removed from the primary reactions using the QiaQuick PCR purification kit (Qiagen, Valencia, CA), and a portion of the eluate was reamplified with nested primers attB-F3 (5′-CGAAGCCGCGGTGCG-3′) and attL-iPCR-2 (5′-GGAGGGGC AAACAACAGAT-3′) using an annealing-temperature gradient from 55°C to 72°C. Approximately 10 bands were excised from the gel, purified, and cloned into pCR2.1-TOPO using the TOPO Cloning Kit (Invitrogen, Carlsbad, CA). Insert-containing colonies were sequenced using standard primers. Two of the ten bands represented coherent *attL* fragments, and the genomic locations they represented were named mpsL1 and mpsL2. By using specific primers designed for mpsL1 (mpsL1-forward, 5′-GTGGCACATTCCTTAATCCC-3′, and mpsL1-reverse, 5′-TGAGGAGGAGCCTTAGCAAC-3′) and mpsL2 (mpsL2-forward, 5′-TGAGTCTGCCTTGAGCCTTA-3′, and mpsL2-reverse, 5′-CAAAGGGCCTGACCTAGAGT-3′) in combination with vector-specific primers attB-F3 and hAATB-R (5′-CAATACGCAAACCGCCTCT-3′), *attL* and *attR* recombination junctions were amplified from genomic liver DNA.

Quantitative PCR of *in vivo* recombination junctions. All quantitative PCR reactions were conducted in an ABI Sequence Detector 7700 with ABI *Taq*Man PCR Core Reagents. Reaction conditions were as recommended by ABI.

In combination with primers attB-F4 (5′-CGGTGCGGGTGCCA-3′) and mpsL1-qPCR2 (5′-AAGCCTTTAATAAAATGAAGCAAAAGTTC-3′), the probe (6FAM-5′-TGTTTTAGTTTTGCACCTTCCCATTATTCACAG-3′-TAMRA) allows measurement of all integrase-mediated events that took place at mpsL1. Similar quantitation of total mpsL2 *attL* recombination was conducted using primers attB-F4 and mpsL2-qPCR1 (5′-AGAGCACAGCAGAGGTGACCA-3′) and probe (6FAM-5′-TCTTGTTTTGCTTCCTGTCCATCAAGGG-3′-TAMRA).

Quantities of *attL* junction in each sample were normalized to genome copy number using a probe (VIC-5′-CAACAACCAGGACAGCGTCCCA-

CA-3′-TAMRA) and primer set (rad52-forward, 5′-CAAACTTCTGCCACTCGAACCT-3′, and rad52-reverse, 5′-TGGTTTCTGATGGCAATGGA-3′) that detect the single-copy mouse *RAD52* gene. Standard dilution curves for the *attL* of interest and the *RAD52* set were done on known quantities of standard plasmid, which contained the attL sequence of interest (mpsL1 or mpsL2) along with the *RAD52* region.

To quantitate total plasmids per genome, a probe (6FAM-5′-TGATGCCGCTGGCGATTCAGG-3′-TAMRA) and primer set (forward, 5′-CGCAAGGCGACAAGGTG-3′; reverse, 5′-CCATCACAAACGGCATGATG-3′) were designed to detect a portion of the chloramphenicol resistance gene included on all *attB* vectors. Genome copy number was again measured using the *RAD52* probe/primers described above.

Analysis of extrachromosomal plasmid DNA. Genomic DNA was prepared (Qiagen Blood and Cell Maxi Kit) from a portion of liver taken during the partial hepatectomy (pre-PH) and another portion of the liver taken at the terminus of the experiment (post-PH). Electrocompetent *E. coli* were transformed with 2 μg of DNA and spread on chloramphenicol plates to select for the *attB* vector. After 16–20 h at 37°C, colonies were counted to determine plasmids/μg genomic DNA.

Acknowledgments

This work was supported by NIH grants HL68112 to M.P.C. and DK49022 to M.A.K.

Competing interests statement

The authors declare that they have no competing financial interests.

Received 15 May 2002; accepted 17 September 2002

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