The integrase from the *Streptomyces* phage φC31 carries out efficient recombination between the attP site in the phage genome and the attB site in the host bacterial chromosome. In this paper, we show that the enzyme also functions in human cells. A plasmid assay system was constructed that measured intramolecular integration of attP into attB. This assay was used to demonstrate that in the presence of the φC31 integrase, precise unidirectional integration occurs with an efficiency of 100% in *Escherichia coli* and >50% in human cells. This assay system was also used to define the minimal sizes of attB and attP at 34 bp and 39 bp, respectively.

Furthermore, precise and efficient intramolecular integration of an incoming plasmid bearing attP into an established Epstein–Barr virus plasmid bearing attB was documented in human cells. This work is a demonstration of efficient, site-specific, unidirectional integration in mammalian cells. These observations form the basis for site-specific integration strategies potentially useful in a broad range of genetic engineering applications.

**Materials and Methods**

**Integrase-Expressing Plasmids.** Integrase-expressing plasmids were constructed as follows. The φC31 integrase gene was amplified by the PCR from the plasmid pIJ8600 containing the φC31 integrase and attP (a gift from Mervyn Bibb, John Innes Institute, Norwich, U.K.) with the following primers: 5'-GAACATAGTCGTAGGGTCGCCGACATGACAC-3 and 5'-GGGATCCGGGTGTCTCGCTACGGCCGTAC-3'. The PCR product was ligated into pCR2.1 (Invitrogen) to make the plasmid pTA-Int. The lacZ gene was removed from pCMVSPORTβGal (Life Technologies, Gaithersburg, MD) by digestion with the restriction enzymes BamHI and SpeI, and was replaced by the integrase gene from pTA-Int with BamHI and SpeI-compatible ends, creating the plasmid pCMVInt (Fig. 1B), which expresses φC31 integrase in mammalian cells under control of the cytomegalovirus (CMV) immediate-early promoter.

A BamHI/PstI fragment from pCMVInt containing the integrase gene was cloned into pACYC 177 (with resistance to ampicillin and kanamycin; a gift of Stanley N. Cohen, Stanford; ref. 10) treated with BamHI and PstI to remove part of the ampicillin-resistance gene. Finally, the lacZ promoter was removed from pBCSK+ (Stratagene) by digestion with SacI and SapI. The integrase-expressing pACYC plasmid was digested with PstI and SacI, and the lacZ promoter was inserted upstream of the integrase gene with a linker (5'-GCTCGGCCAAAAAG-GGCTGACGAG-3' and 5'-GGCCCTTTTGGCCG-3'), creating the plasmid pInt (Fig. 1A), expressing the φC31 integrase under control of the lacZ promoter.

**Intramolecular Integration Assay in *E. coli.*** The intramolecular integration assay plasmid was constructed as follows. A 285-bp fragment containing the bacterial attachment site for φC31 and attB sites are documented in this paper. If the φC31 integrase functioned, for example, in mammalian cells and an attB site were present in the genome, it could serve as an efficient target for gene addition. The two additional requirements for integration would simply be presence of an appropriate attP site on the incoming DNA and transient presence of the integrase enzyme, which can be arranged by coinsertion of an integrase expression cassette or the protein. We demonstrate here that the φC31 integrase does indeed function in mammalian cells by using compact recognition sites. As expected, a much higher integration frequency is produced by this unidirectional integrase than by recombinases such as Cre that carry out reversible reactions.
(attB) was amplified by PCR from Streptomyces lividans genomic DNA (a gift of Stanley N. Cohen) with the primers 5'-CAGGTACCGTCGAGGATCTACGTCGTCGC-3' and 5'-CGCGATCGCGCCGCTGACCG-3'. This fragment was ligated into pCR2.1 to create the plasmid pTA-attB. The phage attachment site (attP) was amplified by PCR from pJ8600 with the primers 5'-CGACTAGTCGACGATGTAGGTCACGGTC-3' and 5'-GTACTAGTCGCGCCGCTGACG-3' and was ligated into pCR2.1 to create the plasmid pTA-attP, containing a 221-bp attP region. The lacZa region was removed from pBCSK+ by digestion with PvuI and KpnI, treatment with T4 polymerase, and religation. The full-length lacZ gene from pCMVSPORTβgal was removed by digestion with SpeI and HindIII and cloned into the SpeI and HindIII sites of the lacZa-deficient pBCa to make pββgal. The attP was then removed from pTA-attP and cloned into the SpeI site of pββgal to create pββgal-attP. The attB was removed from pTA-attB by SalI digestion and cloned into the SalI site of the attP-containing pββgal-attP to create the assay plasmid pBCBP+ (Fig. 1C), in which the TTG cores of the two att sites are in the same orientation. In addition, a control plasmid, pBCBP−, in which the att sites are in opposite orientations, was also constructed.

The pInt plasmid was then transformed into DH10B bacteria. Cells were grown under kanamycin selection and made electrocompetent by a standard protocol. The resulting electrocompetent DHInt cells were used in the bacterial intramolecular integration assay, which was conducted as follows. A 0.2-ng sample of the assay plasmid of choice was electroporated into DHInt cells, which were allowed to recover for 1 h, spread on LB plates containing 25 μg/ml chloramphenicol, 60 μg/ml kanamycin, and 50 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), and grown at 37°C. The lac inducer isopropyl β-D-thiogalactoside (IPTG) was not present or necessary. If an intramolecular integration event occurs, the lacZ gene located between the attB and attP sites will be excised, and a resulting colony will be white. The frequency of intramolecular integration was calculated as the number of white colonies divided by the total number of colonies × 100.

**Intramolecular Integration Assay in Human Cells.** Subconfluent (60–80%) 60-mm plates of human 293 cells (American Type Culture Collection; ref. 11) grown in DMEM supplemented with 9% FBS and 1% penicillin/streptomycin were transfected with Lipo-fectamine (Life Technologies) at a ratio of 6 μg of Lipofectamine per μg of DNA. Experiments were performed with 100 ng of the assay plasmid of interest and 2 μg of pCMVInt. Negative controls performed in each experiment included no DNA, pCMVInt only, pββgal (assay plasmid with no att sites), pββgal + pCMVInt, and pBCBP+ alone.

At 24 h after transfection, the medium was supplemented with 50 units/ml DNase I to reduce the background of untransfected DNA. Low molecular weight DNA was recovered 72 h after transfection as described by Hirt (12). A portion of this DNA was electroporated into competent DH10B E. coli cells and spread on plates containing chloramphenicol and X-Gal to select for the assay plasmid only. The intramolecular integration frequency was determined to be the number of white colonies divided by the total number of colonies × 100.

PCRs on Hirt extracts contained 100 nM forward (5'-GGCGAGAAGGAAGGAAAGAAGA-3') and T3 (5'-ATTAAC-CCTCACTAAAGGAAA-3') primers, 1.5 units of Taq DNA polymerase, 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTPs, and either 250 pg of plasmid DNA as a control or 1/40 fraction of a Hirt extract from a 60-mm plate. Reactions were overlaid with mineral oil, and PCR was conducted as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 45 sec. A final extension period of 72°C for 7 min then was performed. PCR products were analyzed on 2% agarose (NuSieve 3:1; FMC) in TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3).

Unreacted pBCBP+ plasmid DNA was used as a negative control in the PCR. The positive recombination control was generated from pBCBP+ in vitro by incubating plasmid DNA with a crude protein extract from integrase-expressing E. coli. In vitro recombination reactions contained 100 ng of pBCBP+, 1 μg of integrase extract, 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, and 1% glycerol in a final volume of 20 μl. Reactions were incubated on ice, incubated at 37°C for 1 h, and

![Diagram of five plasmids](image-url)
Plasmids for Intermolecular Integration Assay. The attB and attP plasmids needed for this study were constructed as follows. The target hygromycin-resistant Epstein–Barr virus (EBV)-based plasmids were based on p220.2 (13). The negative control plasmid p220Kan was made by inserting the kanamycin-resistance gene from the Kan-resistant Genblock (Amersham Pharmacia) into the XmnI site of the ampicillin-resistance gene of p220.2. To make attB-containing p220 plasmids, the ampicillin-resistance gene of p220.2 was removed by digestion with BspHI. The kanamycin-resistance gene described above was isolated by digestion with PstI and cloned into amp-deleted p220.2 with BspHI/PstI linkers (5’-CATGAGGCCCAAAAAAG- GCCCTGCA-3’ and 5’-GGCTTTTGGCCCTTGC-3’ to create the plasmid p220K. The full-length attB was removed from the plasmid pTA-attB by SalI digestion and cloned into the SalI site of p220K, creating the plasmid p220KattB (Fig. 1D). The 35-bp attB was cloned into the SalI and BamHI sites of p220K by using the oligonucleotides 5’-GATCCGATATCCGGCC-CCGGGGAGCCCAAGGGCACGCCCTGGCACCG-3’ and 5’-TCGCAGGGTCAGGCGGTCCCTGGCCTGCCC-GGGCGCATATCG-3’, creating the plasmid p220KattB35.

These EBV plasmids, p220Kan, p220KattBfull, and p220KattB35, were established in human 293 cells as follows. Human 293 cells were grown in DMEM containing 9% FBS and 1% penicillin/streptomycin to ~70% confluence in 100-mm plates. Samples (~8 μl) of p220KattBfull, p220KattB35, or the control p220Kan were introduced by transfection with Lipofectamine according to the manufacturer’s protocol. At 24 h posttransfection, the cells were split 1:4, and at 48 h posttransfection, hygromycin selection (350 μg/ml) was begun. At 11 to 14 days after starting selection, the cells were expanded and frozen.

The attP-containing plasmid pTSA3 (Fig. 1E) was constructed as follows. A multiple cloning site (oligos 5’-ATTATT CCAGGCGCGCCGCGCTTATTACCGATATCCGGCC-3’ and 5’-ATTATCCAGGCGCGCCGCGCTTATTACCGATATCCGGCC-3’) was cloned into the EcoRI site of the plasmid pWTLox2 (18) upstream of lacZ, regenerating one EcoRI site. The attP site was removed from the plasmid pTA-attP by digestion with EcoRI and cloned into the regenerated EcoRI site of pWTLox2 to create the plasmid pES1. The lacZ promoter was removed from pBCPB1 by digestion with PvuII and SacI and cloned into pES1 that had been digested with Pmel and SacII. The region containing attP, the lacZ promoter, and the lacZ gene was removed by digestion with BamHI and BglII and cloned into the BamHI site of pTSA3 (a gift of Gregory Phillips, Iowa State University, Ames; ref. 14) to create the donor plasmid pTSDS. pTSA30 and its pTSDS derivative are temperature-sensitive for plasmid replication in E. coli.

Intermolecular Integration Assay in Human Cells. To perform the integration assay, EBV plasmid-containing cells were grown to confluence in DMEM containing 9% FBS, 1% penicillin/streptomycin, and 200 μg/ml hygromycin in 100-mm plates. These plates were split into eight 60-mm plates and grown in the above medium without hygromycin for 24–48 h until they were ~60–80% confluent. pCMVInt (Fig. 1B) and pTSDS were transfected in equimolar amounts (~10 μg of total DNA) by using 50 μl of Superfect (Qiagen, Valencia, CA), according to the manufacturer’s protocol. As negative controls, no DNA, 4 μg of pCMVInt, or 6 μg of pTSDS was cotransfected with salmon sperm DNA to 10 μg. In addition, an equimolar amount of a plasmid encoding the green fluorescent protein gene under control of the CMV promoter (a derivative of pEGFP-C1; CLONTECH) with salmon sperm DNA to 10 μg was transfected in parallel into the EBV plasmid-containing cells to monitor transfection efficiency. Transfected cells were trypsinized, suspended in PBS, and placed on slides, and several fields were counted by eye under a fluorescence microscope.

Cells were grown in medium containing serum and 50 units/ml DNase I at 24 h after transfection, and they were harvested 72 h after transfection. Low molecular weight DNA was purified by Hirt extraction and transformed into DH10B E. coli by electroporation. Also, 24 h after transfection, transfection efficiency was measured by counting the green fluorescent protein-expressing cells relative to the total number of cells. The transfection efficiencies typically ranged from 6% to 18%. Because untransfected cells would have no opportunity to undergo integration but would still contribute EBV plasmids to the bacterial assay in the form of white colonies, the transfection efficiency was used to obtain the correct integration frequency.

In a typical experiment, 15 μl of a transformation was spread on each of three plates containing 60 μg/ml kanamycin, 50 μg/ml X-Gal, and 10 μg/ml isopropyl-β-d-thiogalactoside (IPTG), and 150 μl of the same transformation was spread on each of three plates containing 100 μg/ml ampicillin, 100 μg/ml mecthinil, 60 μg/ml kanamycin, 50 μg/ml X-Gal, and 10 μg/ml IPTG. The bacteria were grown overnight at 42°C for ~16 h. The elevated temperature prevented replication of pTSAD, which has a temperature-sensitive plasmid origin of replication. Integrants were scored as blue colonies on the plates containing both kanamycin and ampicillin. Integration frequency was calculated as the number of blue colonies on kanamycin and ampicillin plates divided by (the total number of colonies on kanamycin plates × 10) for each set of transfections. The colony number on kanamycin plates was multiplied by 10 because only 10% of the transformation was spread on these plates, relative to the amount spread on kanamycin and ampicillin plates. Raw numbers for integration frequency were divided by transfection efficiency to obtain accurate values for integration frequency.

Results

Intramolecular Integration Assay in E. coli. To create a simple and rapid assay for integration, we developed a plasmid that would provide a quantitative measure of intramolecular integration frequency. The pBCPB+ plasmid carries the 5′C31 attP and attB sites in direct orientation flanking a lacZ gene on a chloramphenicol-resistant ColE1 derivative (Fig. 1C). The plasmid carries a 221-bp region derived from 5′C31 DNA and known to contain the attP site (9). Because an 84-bp subregion from within this fragment gives good integration function (7), it was likely that the 221-bp fragment contained the complete attP site. Plasmid pBCPB+ also contains a region of ~265 bp known to contain the attB site, obtained by PCR from the S. lindvianus genome. The 5′C31 integrase gene, under the control of the lacZ promoter, was placed on a kanamycin-resistant derivative of pACYC177 to form pInt (Fig. 1A). These two plasmids, pBCPB+ and pInt, belong to different compatibility groups and can therefore coexist in E. coli.

When the pBCPB+ assay plasmid is transformed into an E. coli strain, DHInt, in which the pInt plasmid is resident, growth will catalyze integration of attP into the attB site, resulting in excision of the lacZ gene. This integration event subsequently produces a color change from blue to white when the bacteria form colonies on agar plates containing X-Gal.

When this assay was carried out in DHInt bacteria carrying pInt by using pBCPB+, all colonies were white, indicating efficient integration (Table 1). The same plasmid produced only blue colonies in DH10B bacteria, in the absence of the integrase gene. These results verify that our assay plasmid carries functional attB and attP sites and that the 5′C31 integrase functions.
efficiently in *E. coli* with no added cofactors. In contrast, the plasmid pBCPB−, which carries the *att* sites in inverted orientation, resulted in blue colonies, because the *lacZ* gene was merely inverted, not excised, by the integration reaction. The assay plasmid with no *att* sites, pBCGgal, also yielded only blue colonies in DHInt cells. Restriction-enzyme digestion of plasmid DNA purified from a representative number of white colonies verified that the intramolecular integration reaction occurred as expected and resulted in the deletion of *lacZ* between the *attB* and *attP* sites. DNA sequencing of an integrant demonstrated the expected exact recombination reaction mediated by the integrase.

### Intramolecular Integration Assay in Human Cells

To test whether the φC31 integrase can function in a mammalian cell environment, we transfected pBCPB+ and an integrase expression plasmid, pCMVInt (Fig. 1B), that carries the CMV promoter active in mammalian cells, into human 293 cells. After 72 h, plasmid DNA was purified from the cells by Hirt extraction (12) and transformed into bacteria for scoring. Using this assay system in mammalian cells, we determined that the φC31 integrase catalyzed recombination between the full-length *attB* and *attP* sites of pBCPB+ at a frequency of 52.4% (Table 1; mean of 16 experiments, SE = 2.32%). This frequency is likely to be an underestimate, because plasmid DNA that never came in contact with the φC31 integrase was probably present, despite our efforts to remove untransfected DNA with DNase I.

To assure that this recombination was happening in the human cells and not later in the bacteria, pCMVInt and pBCPB+ were coelectroporated into DH10B *E. coli*. In this control, 1.08% white colonies were observed, verifying that the vast majority of the recombinants occurred in the human cells. To further verify that the site-specific recombination was occurring in the mammalian cells, low molecular weight DNA isolated by Hirt extraction from the human cells was subjected to PCR with specific primers that would amplify a 401-bp product only from the recombinant. These experiments revealed the expected fragment only when both pBCPB+ and pCMVInt were cotransfected into the human cells (Fig. 2). We determined the DNA sequence of this PCR fragment, revealing that the recombination occurred as expected in integrase-mediated site-specific recombination between *attP* and *attB*.

To verify site-specific recombination, 96 of the white colonies were picked, and plasmid DNA was prepared and examined by restriction digestion. Of these, 97% contained a plasmid that represented the expected site-specific recombinant. The remaining colonies contained plasmids that carried large rearrangements that disrupted *lacZ*. The low-frequency rearrangement of transfected plasmids has been observed with all plasmids, with and without integrase and *att* sites, and can be attributed to transfection-associated mutation of newly introduced DNA (15). This result indicates that the φC31 integrase is active in mammalian cells and efficiently carries out the integration reaction when transiently introduced with its *att* sites.

### Determination of Minimal Sizes of *attB* and *attP*

Before this study, the minimal sizes for the φC31 attachment sites *attB* and *attP* had not been reported. The *attB* site had been localized to ~280 bp, and the *attP* region had been localized to 84 bp (7). We used the intramolecular integration assay described above to determine the minimal functional sizes for these *att* sites. Short double-stranded adaptor molecules containing *att* sites of various lengths were created by annealing single-stranded oligonucleotides. These shorter sites were used to replace the full-length *att* sites in the pBCPB+ assay plasmid, and recombination efficiencies were determined by electroporation into *E. coli*.

To determine the minimal functional size of *attB*, the BamHI and HindIII fragment of pBCBgal-attP was replaced by a series of synthetic shorter sites having ends permitting their orientation-appropriate cloning. The resulting plasmids were electroporated into DHInt *E. coli* cells, and recombinants were scored as white colonies, as described above. Fig. 3 (Upper) shows the results of these experiments. *attB* sites of 50, 40, 35, and 34 bp all provided full recombination function in this assay, i.e., they functioned at 100% of the efficiency of the full-length *attB*. Reduction of the site to 33 bp produced a marked decrease in recombination activity, so we concluded that 34 bp is the minimal functional size of *attB*. This determination is somewhat arbitrary, because under more stringent reaction conditions, such as less time, sites of 34 bp in length might be revealed to function less well than does the full-length site. However, 34 bp clearly provides a substantial level of function and probably retains most of the critical sequences. In addition, a site of this length encompasses the major inverted repeats present in the *attB* region (6).

Once *attB* was determined to be 34 bp long, *attP* was subjected to a similar set of reductions. The reduced *attP* sites were assayed in a plasmid carrying *attB34* rather than full-length *attB*. To perform these experiments, the full-length *attP* surrounded by SacII and SpeI sites was replaced with a series of synthetic annealed oligonucleotides bearing ends that permitted their correct orientation-specific cloning into pBCBPattB34+. Fig. 3 (Lower) depicts the results of these experiments. The function of *attP* dropped off as its size was reduced from 40 to 36 bp. The

### Table 1. Intramolecular integration frequencies

<table>
<thead>
<tr>
<th>Cells</th>
<th>Plasmid</th>
<th>n</th>
<th>White</th>
<th>Total</th>
<th>Intramolecular integration frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHInt <em>E. coli</em></td>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>pBCBgal</td>
<td>3</td>
<td>0</td>
<td>2,369</td>
<td>&lt;0.04</td>
<td></td>
</tr>
<tr>
<td>pBCPB−</td>
<td>1</td>
<td>0</td>
<td>1,398</td>
<td>&lt;0.07</td>
<td></td>
</tr>
<tr>
<td>pBCPB+</td>
<td>3</td>
<td>2,284</td>
<td>2,284</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>293 Human</td>
<td>None</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>plint</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>pBCBgal</td>
<td>8</td>
<td>5</td>
<td>6,090</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>pBCBgal and plint</td>
<td>8</td>
<td>30</td>
<td>7,643</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>pBCPB−</td>
<td>8</td>
<td>7</td>
<td>4,403</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>pBCPB+ and plint</td>
<td>16</td>
<td>6,971</td>
<td>13,293</td>
<td>52.44</td>
<td></td>
</tr>
</tbody>
</table>

Numbers of blue and white colonies and integration frequencies for plasmids assayed in DHInt *E. coli* and for plasmids recombined in human 293 cells and assayed in DH10B. *n*, Number of independent experiments for each plasmid; NA, not applicable.
DNA sequence reveals that the 38-bp site encompasses the major inverted repeat evident in attP, but it is apparent from this data that one of the next outermost base pairs conveys some function (P39B). From this analysis, we conclude that the minimal size of attP is 39 bp, subject to the same caveats discussed previously for attB.

To determine the frequency at which the reduced att sites function in mammalian cells, the same panel of plasmids was transfected into human 293 cells along with pCMVInt. After 72 h, colonies that grew on plates containing kanamycin, ampicillin, and having a temperature-sensitive origin of replication (pTSAD; Fig. 1D) were determined not to be integrants. Some appeared to replicate and transformed into DH10B E. coli cells for scoring of recombinants. The results of these experiments showed that minimal sizes for attB and attP similar to those determined in E. coli also apply in mammalian cells (data not shown). We were able to achieve ~60–90% of the efficiency of the full-length att sites with the same reduced att sequences that worked at 100% efficiency in E. coli, probably because the overall reaction is somewhat less efficient in the mammalian cell environment. The lesser frequencies perhaps reveal with more sensitivity that the attB site is somewhat less efficient in the mammalian cell environment. The results of the integration assays shown in Table 2 are in agreement with these observations.

Bimolecular Integration Assay into a Model Chromosome in Mammalian Cells. We showed that the φC31 integrase efficiently catalyzes site-specific intramolecular integration in mammalian cells. We wanted to test whether the integrase could catalyze efficient site-specific integration of exogenous DNA into mammalian chromosomes in cell culture. EBV-based plasmids are good models for chromosomes and are much easier to work with. EBV vectors are retained in the nucleus, replicate in synchrony with the chromosomes, and bear chromatin indistinguishable from that of the chromosomes (16). They can easily be purified from cells and transformed into E. coli for rapid scoring of integration events, thus they have great utility in characterization of the integration reaction in human cells.

In these experiments, one kanamycin-resistant EBV plasmid was equipped with the full attB site (Fig. 1D) and another with a minimal attB site (attB35), and each was established in human 293 cells to create a stable attB-containing human cell line. An ampicillin-resistant plasmid carrying attP and lacZ and having a temperature-sensitive origin of replication (pTSAD; Fig. 1E) was then cotransfected into the attB cell line, along with a plasmid expressing the φC31 integrase. To assay for integration products, after 3 days, plasmid DNA was extracted, transformed into bacteria, and grown at the nonpermissive temperature. Blue colonies that grew on plates containing kanamycin, ampicillin, and X-Gal were scored as integrants, whereas total colony number could be obtained by plating on kanamycin alone.

Table 2 lists the integration frequencies obtained with each EBV test plasmid and the negative controls. Each line of the table represents a minimum of three separate transfections. For p220Kan, which lacks the attB site, a negligible frequency of blue colonies was detected. On restriction analysis, these plasmids were determined not to be integrants. Some appeared to represent homologous recombination events that occurred through common amp sequences on the two plasmids. For p220KattB35, carrying a smaller attB, significant numbers of blue colonies were detected. When corrected for the transfection efficiency in these experiments, the integration frequency was 1.7%. For p220KattBfull, the integration frequency was even higher at 7.5%.

Results are shown for human cells carrying one of three EBV plasmids, p220Kan, a negative control lacking attB; p220KattB35, which carries a similar attB; and p220KattBfull, carrying the full-sized attB. Integration frequencies are shown for experiments when no DNA was transfected, when either the integrase expression plasmid pCMVInt or the attB-bearing plasmid pTSAD were transfected alone, or when both pCMVInt and pTSAD were transfected together. Only the latter condition, in the presence of a plasmid bearing attB, led to integration events. Integration frequencies were corrected for transfection efficiency to give the accurate corrected integration frequencies in the last column. p220KattBfull produced the highest integration frequency at 7.5%.

Table 2. Bimolecular integration assay in human cells

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Blues/total</th>
<th>% integration</th>
<th>Corrected % integration</th>
</tr>
</thead>
<tbody>
<tr>
<td>p220Kan</td>
<td>0/7,970</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>pCMVInt only</td>
<td>0/13,600</td>
<td>0.007%</td>
<td>0.005%</td>
</tr>
<tr>
<td>pTSAD only</td>
<td>1/11,830</td>
<td>0.008%</td>
<td>0.005%</td>
</tr>
<tr>
<td>pCMVInt + pTSAD</td>
<td>3/32,680</td>
<td>0.009%</td>
<td>0.006%</td>
</tr>
<tr>
<td>p220KattB35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No DNA</td>
<td>0/64,530</td>
<td>0.002%</td>
<td>0.007%</td>
</tr>
<tr>
<td>pCMVInt only</td>
<td>0/42,720</td>
<td>0.002%</td>
<td>0.002%</td>
</tr>
<tr>
<td>pTSAD only</td>
<td>0/39,930</td>
<td>0.003%</td>
<td>0.002%</td>
</tr>
<tr>
<td>pCMVInt + pTSAD</td>
<td>382/157,710</td>
<td>0.242%</td>
<td>1.69%</td>
</tr>
<tr>
<td>p220KattBfull</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No DNA</td>
<td>0/70,350</td>
<td>0.001%</td>
<td>0.001%</td>
</tr>
<tr>
<td>pCMVInt only</td>
<td>0/41,960</td>
<td>0.002%</td>
<td>0.002%</td>
</tr>
<tr>
<td>pTSAD only</td>
<td>0/39,740</td>
<td>0.003%</td>
<td>0.002%</td>
</tr>
<tr>
<td>pCMVInt + pTSAD</td>
<td>1,799/166,890</td>
<td>1.08%</td>
<td>7.50%</td>
</tr>
</tbody>
</table>
the integrase gene (pCMVInt) and the attP site (pTSAD). Because EBV vectors are nuclear, chromatinized minichromosomes, the high integration frequency obtained in this system may be predictive of integration frequencies into att sites located on the chromosomes.

**Discussion**

This study demonstrates that the φC31 integrase, derived from a Streptomyces phage, can function efficiently in mammalian cells. The demonstration that the enzyme could function in *E. coli* and that purified φC31 integrase was active in an *in vitro* system implied a lack of requirement for Streptomyces-specific cofactors (7). On this basis, we hypothesized that the enzyme might have the ability to function in mammalian cells. It was necessary to express the enzyme in a mammalian cell environment and demonstrate activity in integration assays to provide evidence that the enzyme could function in higher eukaryotic cells. In this way, pairing and reacting attP and attB sites in the context of chromatin, large genome size, and other features distinct from the prokaryotic environment where φC31 integrase had previously been shown to work, were tested.

By placing the attB sequence on a stable chromatinized EBV plasmid established in human cells, then demonstrating the specific and efficient integration of a plasmid carrying an attP site into attB, we have provided proof that the enzyme can locate and recombine its recognition sites in mammalian cells. This finding suggests that the enzyme may mediate chromosomal integration in a wide range of host environments, including plants, insects, other mammals, and diverse microorganisms.

Our demonstration of 34-bp and 39-bp minimal sizes for the attB and attP sites, respectively, makes it unlikely that they will be present even in the large genomes of mammals and most plants. However, the enzyme may be able to mediate integration at naturally occurring pseudo-att sites in eukaryotic genomes having significant similarity to native att sequences (18). The expected rarity of good matches with att sites may limit recombination to a small number of chromosomal pseudo-att sites, which may produce usable integration frequencies at endogenous locations in the chromosomes.

This integration system is significantly more efficient and specific than currently available alternatives. The integration frequency into an attB site located on an EBV plasmid is several orders of magnitude higher than the frequency of random integration (1). The site-specificity of the enzyme also distinguishes it from the random integration mediated by retroviral integrases and most transposases. With regard to other recombinases, because there is no accompanying excision reaction with the φC31 integrase, the integration frequency is more than two orders of magnitude higher than that of the Cre recombinase (6). Recently, integration mediated by the integrase protein of lambdoid phage HK022 has been reported in mammalian cells (17). This integrase requires host cofactors in *E. coli* and seems to produce a net integration frequency in mammalian cells at least an order of magnitude lower than that of the φC31 integrase. The high integration frequency mediated by an autonomous unidirectional integrase such as that of φC31 may enable a variety of genomic modifications desirable for research, commercial, or therapeutic purposes.

We thank Drs. Mervyn Bibb, Stanley N. Cohen, and Gregory Phillips for plasmids and DNA. This work was supported by grants from the National Institutes of Health (DK51834) and the Cystic Fibrosis Foundation (to M.P.C.). E.C.O. was supported by a Ford Foundation predoctoral fellowship. A.C.G. was supported by a predoctoral fellowship from Public Health Service National Research Service Award 5 T32 CA09302 from the National Cancer Institute.