Drosophila P Element-Enhanced Transfection in Mammalian Cells

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We constructed a gene transfer vector containing the herpes simplex virus type 1 thymidine kinase (TK) gene flanked by *Drosophila* P element terminal repeats (W. R. Engels, *Annu. Rev. Genet.* 17:315-344). This vector was introduced into mouse LTK⁻ cells and enhanced the frequency of stable transformation to the TK⁺ phenotype by approximately 50-fold relative to a similar plasmid lacking the P element terminal repeats.

The study of *cis*-acting genetic elements has been greatly facilitated by the use of DNA-mediated gene transfer systems in mammalian cells, in mouse and *Drosophila* embryos, and in other systems (5a, 15, 17). In the *Drosophila* system, DNA sequences from the P element family of transposable elements have been used in gene transfer vectors (2, 5, 13, 15). In this system, the gene of interest must be flanked by the 31-base-pair (bp) inverted terminal repeats common to the P element family, and it must be cotransferred with an intact P element. The intact P element is thought to supply, in *trans*, a putative transposase function (15).

Figure 1 shows a diagram of the plasmid Carnegie 4 (12), a derivative of pUC8 (16), containing a fragment of *Drosophila* P element DNA and a small amount of "white" DNA. The P element insert contains the 31-bp inverted terminal repeats along with a small amount of internal P element DNA and a polylinker region (12). Plasmid pPTK2 was constructed by inserting the thymidine kinase (TK)-containing 3.4-kilobase (kb) *Bam*HI fragment of herpes simplex virus type 1 (HSV-1) DNA into the *Bam*HI site of the polylinker region of Carnegie 4. This was accomplished by digesting Carnegie 4 DNA with *Bam*HI, followed by dephosphorylation at the 5' terminus with calf intestinal alkaline phosphatase as previously described (8). The cohesive end HSV-TK fragment was inserted bidirectionally into Carnegie 4. The transcriptional orientation of the TK gene in pPTK2 is from left to right as seen in Fig. 1 and from right to left in plasmid pPTK4 (data not shown). Plasmid pΔPTK4 was constructed by digesting pPTK4 with *Nde*I and *Sal*I, which removed the 5' portion of the *Drosophila* DNA including the 5' P element terminal repeat and the 5' portion of white DNA from the 3' end of the TK gene in pPTK4. The digested plasmid was made blunt ended by treatment with the Klenow fragment of *Escherichia coli* DNA polymerase, and the plasmid was recircularized by blunt-end ligation. *E. coli* HB101 was then transformed with the recombinant plasmid and selected for ampicillin resistance as previously described (6). Plasmids were then used to transfect LTK⁻ cells by a modification of the method of Wigler et al. (18). LTK⁻ cells were seeded at a density of 5×10^5 cells in 100-mm plastic tissue culture dishes in 15 ml of Dulbecco modified Eagle medium plus 10% fetal calf serum (E medium) 24 h before transfection. The E medium was replaced with 9 ml of fresh E medium 4 h before transfection. For transfection, 1 ml of a calcium phosphate-DNA coprecipitate containing 0.5 μg of each of the appropriate plasmid(s) was added, and the cells were allowed to incubate for 20 to 24 h.

The medium and precipitate were then removed and replaced with 15 ml of HAT medium (E medium plus 0.1 mM hypoxanthine, 0.14 μM aminopterin, 10 μM thymidine) (7). HAT medium was changed every 4 days, and after 7 to 11 days colonies were fixed with methanol and stained with Giemsa. Colonies containing 50 or more cells were counted.

Table 1 shows the results of a number of such transfection experiments. It can be seen that plasmid pPTK2 gives, on the average, approximately a 50-fold enhancement in the frequency of stable transformation (relative to the frequency obtained with plasmid pHSV106 [1, 10], which also contains the 3.4-kb *Bam*HI fragment of HSV-1 DNA but no P element sequences). Although the absolute transformation frequency varied from experiment to experiment, significant enhancement with plasmid pPTK2 was always observed. The greatest degree of enhancement observed in any one experiment was over 100-fold. The highest absolute frequency of transformation observed was 0.5%.

To successfully transform *Drosophila* embryos with a P element vector, the vector must be cotransferred with an intact P element, which is thought to provide a transposase function (15). Thus, tests were performed to determine whether the plasmid pπ25.1, which contains an intact P element, further enhances the frequency of stable transformation with pPTK2. Cotransformation of 0.5 μg of pπ25.1 with 0.5 μg of pPTK2 did not increase the transformation frequency of pPTK2 (Table 1). Thus, unlike the *Drosophila* system, a *trans*-acting element is not required in mammalian cells for enhanced transfection of the TK gene in pPTK2. It was also seen that the cotransfer of 0.5 μg of pHSV106 with 0.5 μg of the unmodified Carnegie 4 vector did not increase the stable transformation frequency over that seen with pHSV106 alone, indicating that the inverted repeats found in Carnegie 4 do not provide *trans*-acting enhancement functions. Cotransfer of Carnegie 4 with pPTK2 also did not augment the stable transformation frequency, as the enhancement of transformation was somewhat lower in the presence of Carnegie 4 and pPTK2 (32-fold) than in the presence of pPTK2 alone. In another set of experiments, pPTK2 was cut with the restriction endonuclease *Xba*I, which linearizes the plasmid and severs one inverted repeat from the 3.4-kb *Bam*HI fragment of viral DNA (Fig. 1). When this linearized plasmid was transfected into LTK⁻ cells, the frequency of stable transformation was increased 51-fold over pHSV106, the same as seen with uncut pPTK2. In another set of experiments, the TK gene was inserted into Carnegie 4 in a transcriptional orientation opposite that in pPTK2. When this plasmid (pPTK4) was introduced into LTK⁻ cells, enhancement of the transformation was similar to that seen

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FIG. 1. Schematic diagram of plasmid Carnegie 4 which contains P element sequences (left) and plasmid pPTK2 transforming vector (right). The Carnegie 4 P element plasmid was obtained from G. Rubin and has been previously described (12). The 3.4-kbp *Bam*HI fragment of plasmid pHSV106 (1, 10) containing the entire HSV-TK gene was cloned into the *Bam*HI site of the polylinker region of Carnegie 4. The cross-hatched region represents the 31-bp P element inverted repeat DNA and a small amount of flanking white locus DNA. The shaded area represents other P element DNA. The stippled area of pPTK2 contains the HSV-TK gene, and the unshaded region represents pUC8 plasmid DNA. The direction of transcription of the TK gene in pPTK2 is from left to right.

with pPTK2. To test whether both P element repeats were required for enhancement of transfection, LTK⁻ cells were transfected with plasmid Δ pPTK4, in which the 5' P element DNA and the adjoining white DNA were deleted from pPTK4 by digestion with the restriction endonucleases *Sal*I and *Nde*I. This effectively removes the 5' *Drosophila* DNA from the 3' end of the TK gene in pPTK4, leaving 3' *Drosophila* DNA adjacent to the transcriptional start site of the TK gene. Transfection with this plasmid gave transformation frequencies similar to those seen with plasmids containing both P element terminal repeats, indicating that only one repeat is required for enhancement of transfection.

Experiments were carried out to test the stability of expression of the transfected TK gene in the absence of selective pressure. Two P element-containing transfectants selected in HAT medium were grown for approximately seven generations in nonselective E medium. The cells were then plated in E or HAT medium, and the plating efficiencies were the same in both media. Thus, at least for seven generations, the expression of the TK gene in the P element-containing transfectants appeared stable in the absence of selective pressure.

To analyze the arrangement of TK-containing sequences in the transfectants, several TK⁺ colonies growing in HAT medium were isolated and grown into mass culture. DNA from transformed and untransformed lines was then purified,

and 10 μ g of DNA was digested with 10 U of *Bam*HI restriction endonuclease for 16 h at 37°C and electrophoresed in a Tris-acetate 1% agarose gel for 16 h at 1.6 V/cm. The DNA was transferred to nitrocellulose filter paper (14) and hybridized with the appropriate probe labeled with ³²P by nick translation (9).

Figure 2 shows DNA from various transformed lines digested with *Bam*HI and blotted and probed with either the 3.4-kb *Bam*HI fragment of HSV-1 DNA (1, 10) (Fig. 2A) or with pUC8 (16) DNA (Fig. 2B). All of the cell lines transformed with pHSV106 alone (Fig. 2A, lane 4) or pPTK2 alone (lane 5), or cotransformed with Carnegie 4 (lane 6) or p π 25.1 (lanes 7 to 10), yielded a prominent band hybridizing with the ³²P-labeled 3.4-kb *Bam*HI probe and corresponding to the full-length 3.4-kb *Bam*HI fragment of HSV-1 DNA contained in pPTK2. Most cell lines also contained a few minor bands which hybridized with the 3.4-kb *Bam*HI fragment and probably represent small amounts of integrated rearranged HSV-1 DNA. Figure 2B shows the same cell lines as seen in Fig. 2A, but the blots were probed with ³²P-labeled pUC8. It can be seen that all of the cell lines transformed with pPTK2 (either alone or with Carnegie 4 or p π 25.1 [lanes 5 to 10]) yielded a prominent 3.6-kb fragment of DNA corresponding to the full-length pUC8 sequence contained in pPTK2. Again, the presence of varying numbers of other bands probably represents rearranged plasmid sequences.

The presence of unit-length molecules corresponding to entire pUC8 and HSV-1 TK-containing regions of pPTK2 suggests several possible interpretations. One is that free pPTK2 exists in all of the transformed cell lines. To test this, DNA from several of the transformants was digested with *Hpa*I, which does not cut within the plasmid sequences. If the plasmid was not integrated, then this digestion should release an intact plasmid which would comigrate with uncut plasmid on an agarose gel. However, it was found that the TK-hybridizing sequences did not comigrate with pPTK2 but rather remained associated with DNA of much higher molecular weight (data not shown). These data are consistent with integration of the TK-containing sequences into high-molecular-weight genomic DNA. Although other possibilities exist, the most likely explanation for the presence of both a full-length 3.4-kb fragment of HSV-1 DNA and a full-length 3.6-kb pUC8 fragment in all of the cell lines tested is that the transforming plasmids have been linked in tandem before integration into the host cell genome. This is consistent with the observations that the majority of molecules transferred into mammalian cells by various means become linked in head-to-tail concatamers (3, 11). Based on reconstruction experiments wherein approximately 1 genome equivalent of pPTK2 (lane 2) or 1 genome equivalent of p π 25.1 (lane 3) was added to LTK⁻ DNA before digestion

TABLE 1. P element-mediated transformation of mouse LTK⁻ cells

Plasmid(s)	No. of experiments	Total no. of TK ⁺ colonies	No. of TK ⁺ colonies ^a	Enhancement ^b
pHSV106	4	141	64 (7-132)	
pPTK2	4	6,114	1,696 (320-4,972)	52 (9-113)
pPTK2 + p π 25.1	4	3,092	1,372 (1,000-1,744)	53 (12-149)
pHSV106 + Carnegie 4	2	59	72 (43-100)	0
Δ pPTK4	4	2,273	758 (169-1,055)	40 (7-113)

^a Number of TK⁺ colonies is expressed as the number of colonies per microgram of TK-containing DNA per 10⁶ cells. At least two independent preparations of each plasmid were tested. The numbers represent the averages of all the experiments, and the ranges are presented in parentheses.

^b Enhancement is the average (ranges in parentheses) and represents the average of the enhancement values for all of the experiments and not the increase in the average colony number over all of the experiments.

FIG. 2. Genomic DNA from transformed and untransformed cells lines digested with *Bam*HI restriction endonuclease, transferred to nitrocellulose filters, and hybridized with a 32 P-labeled 3.4-kb *Bam*HI fragment of HSV-1 DNA (A) or with the plasmid pUC8 (B). Lanes: 1, LTK⁻ cell DNA; 2, LTK⁻ cell DNA and approximately 1 genomic equivalent of pPTK2 DNA added before *Bam*HI digestion; 3, LTK⁻ cell DNA and 1 genomic equivalent of p π 25.1 added before *Bam*HI digestion; 4, LpTK1-1, transformed with pHSV106; 5, LpPTK2-1 transformed with pPTK2 alone; 6, LpPTK2 Car 4, transformed with pPTK2 and Carnegie 4; 7 to 10, LpPTK2-1, -2, -3, and -5, respectively, independent lines cotransformed with pPTK2 and p π 25.1, a P element plasmid containing an entire P element. The prominent band in B which is larger than 3.6 kb represents sequences hybridizing to p π 25.1 (lane 3) and to pBR322 sequences in pHSV106 (lane 4).

and blotting, it can be estimated that each transformed cell line contains at least one but not more than a few copies of the transferred plasmid(s).

We have demonstrated that insertion of the HSV-TK gene into a plasmid containing *Drosophila* P element sequences including the 31-bp inverted terminal repeats significantly increased the frequency of stable transformation of the HSV-TK gene into mammalian cells. We have also shown that this increase does not require *trans*-acting functions coded for by an intact P element, in contrast to the transformation of *Drosophila* embryos. The P element sequences could increase the frequency of mammalian cell transfection by a variety of mechanisms. One possibility is that integration of the TK sequences may be facilitated by the P element termini. It is also possible that the P element termini contain an enhancer function which could permit more efficient transcription of the transferred TK genes. It should also be noted that the P element vectors used in these studies include a small amount of DNA corresponding to the white

locus in *Drosophila* which may contain regulatory sequences which could influence the TK transformation frequency (7). If white DNA contains regulatory sequences which could influence expression of the TK gene in our constructs, then these sequences would in all likelihood be contained in the 5' region of white DNA. However, deletion of these 5' white DNA sequences does not diminish the enhancement of transformation frequency (Table 1). Thus, it seems more likely that the terminally repeated elements are responsible for enhanced transformation, perhaps by facilitating integration or expression of the TK gene as has been previously shown for other terminally repeated sequences (4). Further analysis of this system should elucidate the mechanism by which the *Drosophila* P element sequences are functioning in mammalian gene transfer. Furthermore, this system may provide a general means of enhancing the transfer of a variety of genes into mammalian cells of different types and also possibly enhancing the transfer of genes into mammalian embryos.

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