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Transformation by Iontophoretic Microinjection of DNA: Multiple Integrations Without Tandem Insertions

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DNA transformations of mouse tissue culture cells and mouse embryos were carried out by iontophoretic microinjection of DNA. Iontophoresis involves the use of an externally applied electric current to expel DNA molecules from the injection micropipette into the impaled cell (microelectrophoresis). Restriction analysis of transformants obtained by using this procedure demonstrated that this method gave strikingly different results from those previously obtained by others with transformants generated by pressure injection. First, tandem insertions were never observed in any of our transformants. Second, despite the lack of tandem insertions, we have obtained transformants which have integrated many copies of the injected DNA sequences, probably via a comparably large number of independent integration events. And third, in one transformant, an injected *Bam*HI restriction fragment was found to have been integrated in its entirety; this included the preservation of the terminal *Bam*HI recognition sequence. Based on these observations, we discuss the potential usefulness of iontophoretic DNA microinjections in DNA transformation studies that are focused either on the analysis of the regulation of gene expression or on the targeting of DNA sequences into the eucaryotic cell genome.

Transformation of eucaryotic cells by direct microinjection of DNA has been successfully implemented with a number of different cell types, including mouse tissue culture cells (2, 4), mouse embryos (3, 5, 11, 30, 31), frog embryos (23), and *Drosophila* embryos (22). One of the ultimate goals in such experiments has been the use of this approach for studying the developmental regulation of gene expression. However, this has been hampered by the fact that the insertion of developmentally regulated genes into embryonic cells frequently does not result in the expression of those genes in the differentiated tissues. This may be the result of the apparent lack of site specificity of integration in DNA transformation. Thus, in these experiments, it may be advantageous to generate transformants with insertions at a large number of independent loci to increase the probability of obtaining some transformants with expressible insertions. In all of the microinjection studies reported thus far, pressure injections were utilized to introduce the DNA molecules, and in all cases, the number of separate integration events found in any one transformant was always very small. Here we investigated the feasibility of a different microinjection method for transforming cells, that of iontophoresis. The results we have obtained suggest that this approach may be useful for obtaining transformants with a large number of independent insertions and also may be useful for attempting to target DNA sequences in DNA transformation.

Iontophoretic microinjection involves the use of a microelectrode to electrophorese charged molecules into an impaled cell. The main difference between this method and pressure injection is that with iontophoresis, there is no net fluid displacement. This difference may have several important consequences. First, with iontophoresis, the amount of mechanical shearing which occurs during microinjection is minimized (9). This may be particularly important for the injection of very large DNA molecules. Second, the injected molecules will not accumulate locally in high concentration, but instead will be freely disseminated into the injected cell as a result of the migration induced by the electric current. This may have a bearing on the ultimate fate of the injected DNA molecules (see below). Third, a very large amount of DNA can be injected—probably much more than with pressure injection, since one would not be limited by the problems of forcing a highly viscous solution out of a very small orifice or injecting too much fluid into the cell or cell nucleus (see below). Using iontophoretic injection, we have transformed mouse tissue culture cells and mouse embryos. Restriction analysis of transformants revealed that some transformants have a large number of

independent insertions, but that despite this fact, no tandem insertions were ever observed.

MATERIALS AND METHODS

Iontophoretic injection. Iontophoretic injections were carried out under conditions essentially identical to those in previous studies (15). Glass microelectrodes measuring 20 to 30 mΩ in resistance (filled with 3 M KCl) were used. They were pulled by using a horizontal Micropipet Puller M1 (Industrial Science Associates, Inc.) and were backfilled by using a 1-ml syringe with a Hamilton syringe needle. A high-impedance amplifier M 707, and a 302 T anapulse stimulator, both from W. P. Instruments, Inc., were used in conjunction with a Tektronix oscilloscope. The progress of cell penetration was monitored on the oscilloscope, and successful impalements were indicated by the measurement of a membrane potential of −10 to −40 mV. Injections were conducted by using DNA solutions of 25 to 150 ng/μl in 50 mM KCl or in distilled water. The DNA was injected either as linear fragments or as circular supercoiled molecules. For each injection, current pulses of 0.2 to 10 nA with a duration of 0.5 s at a frequency of once per second were maintained for 2 to 5 min. We have estimated that with current pulses of 0.1 to 10 nA, a maximum of 5×10^4 to 5×10^6 molecules, respectively, have been injected per cell. This is an upper estimate, since a portion of the current may be carried by other electrolytes in the aqueous solutions. However, note that even if only 10% of the injection current is being carried by the DNA molecules, 10^5 molecules are still being introduced with a 10-nA current pulse. This compares with a maximum of approximately 10^4 molecules being injected with pressure injection (5, 11, 27). Since pulses of even higher current can be tolerated by mouse embryos (unpublished data), the amount of DNA which can be iontophoretically injected should be even greater.

Cell culture. Thymidine kinase (TK)-deficient L cells (Ltk^-) were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum and 50 U of penicillin-streptomycin (50 μg/ml). They were maintained in a 5% CO_2 incubator at 37°C. At 24 h after injection, the culture medium was replaced with medium containing HAT medium (14 μg of hypoxanthine, 0.15 μg of aminopterin, and 3 μg of thymidine per ml) to select for cells transformed to a TK^+ phenotype. Such cell colonies were isolated by using glass cloning rings and were subsequently maintained as separate cell lines for further analysis.

Mouse embryos. C57BL/6J female mice were superovulated with 2 IU of pregnant mare serum gonadotropin (PMSG) 48 h before receiving 4 IU of human chorionic gonadotropin (HCG) and then were immediately paired with SJL/J males. The next morning, mated females were sacrificed, and the embryos were flushed from the oviducts. The embryos were maintained in modified Whitten medium (13, 34) in 5% CO_2 at 37°C. The injections were carried out in PB1 medium (35) at room temperature. After all of the embryos had been injected, they were surgically transferred to pseudopregnant CD1 females for further development. At day 14 to 16 of gestation, these females were sacrificed, and the fetuses and their placentas were harvested for DNA extractions.

DNA restriction analysis. DNA was isolated from the tissue culture cells by published procedures (6). DNA from the mice was isolated by first freezing the tissue in liquid nitrogen and grinding the tissue into a fine powder, using a mortar and pestle. The powder was then solubilized in lysis buffer and prepared as described above. Restriction enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, and Amersham Corp., and restriction digestions were carried out according to manufacturers' specifications. All digestions were carried out overnight and in at least a 5- to 10-fold enzyme excess to assure complete digestion. Restriction digests were phenol extracted, ethanol precipitated, separated on 0.8% agarose gels, and blotted according to Southern (26), with some modifications (32). DNA fragments between 1 and 30 kilobases can be resolved with this gel-blotting procedure. Fragments which are much smaller than 1 kb do not resolve well (they tend to be very diffuse) and generally do not bind efficiently, if at all, to nitrocellulose. Fragments which are much greater than 30 kb do not resolve in 0.8% gels and also do not bind to nitrocellulose, probably as a result of interference by Ficoll (which was used in the loading buffer and which migrates in this size range; unpublished data). The blots were hybridized in the presence of dextran sulfate, with nick translated probe (16) generated from purified DNA fragments and plasmid DNA (3.6-kb ptk *Bam* fragment, 1.9-kb *Bam-Hpa*I pMBJ fragment, or pBR322). The DNA fragments were isolated from 1% low melt agarose gels and were purified on a Franklin column (Whatman, CF11 cellulose [8]). Hybridizations were conducted at 65°C for 12 to 24 h, and subsequent washes were carried out in 0.1× SET at 50°C for *tk*, 55°C for pBR, and 65°C for globin (two times for 20 min each). Autoradiography was carried out by using Kodak XAR-5 film with a DuPont intensifying screen at −80°C for 1 to 5 days.

RESULTS

Tissue culture cell injections. Ltk^- cells were injected with the plasmid ptk. ptk consists of pBR322 with a 3.6-kb *Bam*HI insert containing the herpesvirus TK gene (*tk*) (Fig. 1). Injections were carried out by using either *Hin*dIII-linearized ptk or *Bam*HI-digested ptk. The DNA was dissolved in 50 mM KCl at a concentration of 25 ng/μl. Iontophoresis was conducted with a current pulse of 0.1 nA at a frequency of once per minute for a total of 2 to 5 min. Of approximately 150 cells injected, two survived selection in HAT medium (designated as LC1 and LC2).

Clone LC1, which was obtained after injection with *Bam*HI-digested ptk, had two insertions. One insert contained only a small portion of the injected *Bam* fragment and was detectable only with some restriction enzymes. The other insert consisted of a single intact copy of the entire 3.6-kb *Bam*HI fragment, including the terminal *Bam*HI recognition sequence. The *tk* insert in LC1 was characterized and mapped as follows. When LC1 DNA was digested with *Bam*HI, a 3.6-kb *tk*-hybridizing band was observed (Fig. 2,

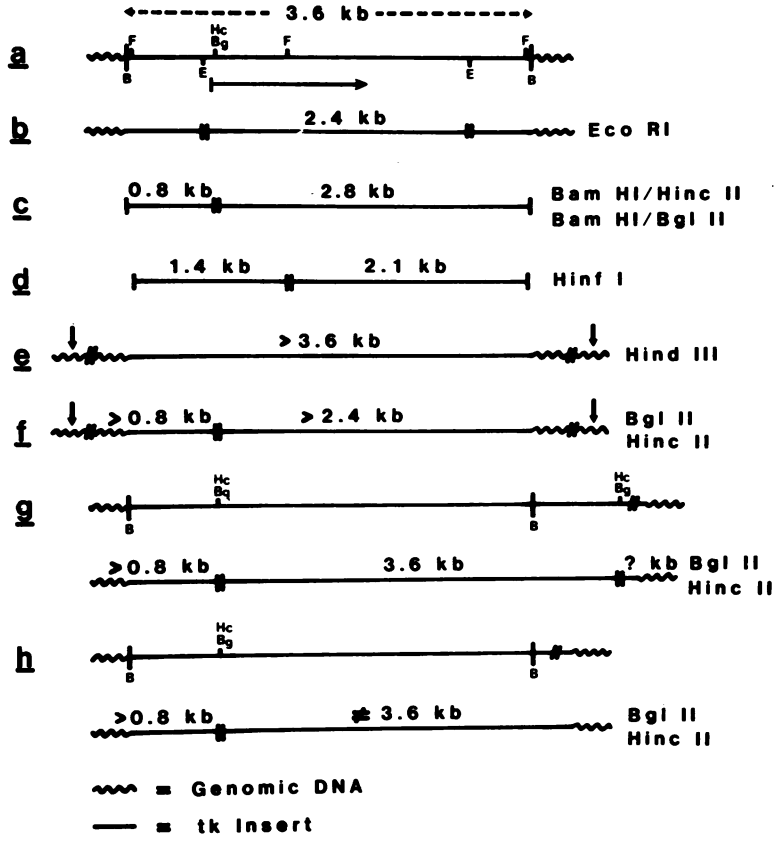

FIG. 1. (a) Restriction map of the 3.6-kb herpesvirus *Bam*HI fragment of ptk. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Hc, *Hinc*II; F, *Hin*fI. The arrow indicates the direction of transcription. (b to f) *tk*-Hybridizing fragments expected from a single 3.6-kb *Bam* insert. (g) Tandem insertion of an intact copy of the 3.6-kb *Bam*HI fragment with a second partially deleted copy in which the *Hinc*II-*Bgl*II site has been preserved. The bottom part of this section represents *tk*-hybridizing fragments which would be expected upon *Bgl*II or *Hinc*II digestion. (h) Tanden insertion of an intact copy of the 3.6-kb *Bam* fragment with a second partially deleted copy in which the *Hinc*II-*Bgl*II site has been deleted. The bottom part of this section represents the *tk*-hybridizing fragments which would be observed upon *Bgl*II of *Hinc*II digestion.

lane 7; the additional faint band is due to partial digestion). That this hybridizing band was indeed identical to the injected 3.6-kb *Bam* fragment was confirmed by the results obtained with *Eco*RI digestion and double digestion with *Bam*HI-*Hinc*II and *Bam*HI-*Bgl*II. Thus, *Eco*RI digestion released the expected 2.4-kb internal fragment and some faint bands possibly corresponding to junction fragments (Fig. 1b; Fig. 2, lane 8), and double digestion of LC1 DNA with either *Bam*HI-*Hinc*II or *Bam*HI-*Bgl*II released the expected 2.4- and 0.8-kb hybridizing bands (Fig. 1c; Fig. 2, lanes 3 and 4). To determine whether this *Bam* fragment was integrated into L cell genomic DNA, LC1 DNA was digested with *Hin*dIII, a noncutter enzyme. If integration had occurred, *Hin*dIII digestion should have released a hybridizing band greater than 3.6 kb in size, corresponding to the *tk* insert straddled on either side by genomic DNA sequences (Fig. 1e). In fact, *Hin*dIII digestion did release a single hybridizing band that was 4.1 kb (Fig. 2, lane 2). In summary, these results suggest that only a single insert is present in LC1 and that it consists of an intact copy of the entire 3.6-kb *Bam* fragment. However, results with other restriction enzymes indicated that there must be an additional insert in LC1. Thus, digestion with *Hin*fI revealed not only the expected 1.4- and 2.1-kb bands from the intact *Bam* insert (Fig. 1d) but also an additional band at 13.8 kb (Fig. 2, lane 1). Similarly, digestion with *Bgl*II, a single cutter, released three bands instead of just two, as would have been expected if only a single insert were present. The fact that this second insert was not detected at all in digestions with *Bam*HI, *Eco*RI, *Hinc*II, and *Hin*dIII digests may be a reflection of the inherent limitation of our analysis for detecting very small or very large fragments (<1 kb or >30 kb; see above). Consis-

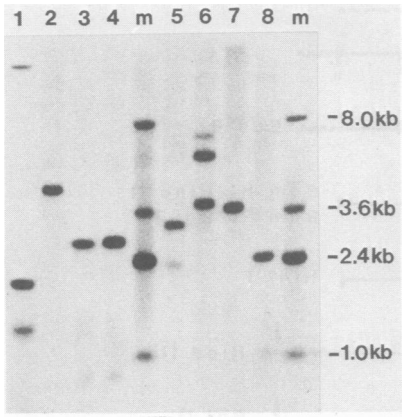

FIG. 2. Genomic Southern blot of clone LC1. The probe used was the 3.6-kb *Bam*HI fragment of ptk. Restriction enzymes used in each lane were as follows: (1) *Hin*fI, (2) *Hin*dIII, (3) *Bam*HI-*Hin*cII, (4) *Bam*HI-*Bgl*II, (5) *Hin*cII, (6) *Bgl*II, (7) *Bam*HI, (8) *Eco*RI, and (m) molecular weight standards. Note that the DNA fragments which are less than 1 kb do not resolve well and bind only very poorly to nitrocellulose.

tent with this latter suggestion was the observation that digestion with *Hin*cII released only two bands, instead of three as would be expected for a single cutter and as was observed with *Bgl*II. Furthermore, the results obtained with these single cutters (*Bgl*II and *Hin*cII) also suggest that this second insert is not tandemly linked to the 3.6-kb *Bam* insert. Thus, if this second insert were tandemly linked and had preserved its *Hin*cII-*Bgl*II site, then *Bgl*II or *Hin*cII digestion should have released a 3.6-kb fragment (Fig. 1g), but this was not observed. If, however, the *Hin*cII and *Bgl*II sites were deleted in such a tandem arrangement (Fig. 1h), a 3.6-kb band would not be expected, but in such a case, only two bands should be released in a *Bgl*II digest, not three as was observed. Nevertheless, these results cannot rule out the possibility that these two fragments are covalently linked, since only a single band was observed in *Hin*dIII noncutter digests. However, it is important to note that, as was mentioned above, this latter observation may be only a mere reflection of the limitation of the techniques used. That these insertions are stably integrated in LC1 was indicated by the fact that the restriction pattern described above has remained unchanged during continuous growth of these cells in culture for a period of 3 years.

The second clone, LC2, was obtained after injection with *Hin*dIII-linearized ptk. Similar restriction analysis of LC2 indicated that originally, a single DNA fragment containing the herpesvirus *tk* gene was integrated in LC2. *Bam*HI and *Eco*RI digestion of LC2 revealed a single *tk*-hybridizing band of 2.0 kb (data not shown). This indicates that a substantial portion of the injected 7.96-kb ptk DNA was lost before integration, presumably by exonucleolytic attack. This clone was generated in October 1979 and has been grown almost continuously in culture since that time. In contrast to clone LC1, we found that during this time span, the original insert in LC2 has amplified or increased in copy number (data not shown) and in the process has also undergone a number of rearrangements such that *tk* inserts with new flanking DNA sequences are now detected (Fig. 3 and 4). Thus, for example, the presence of rearrangements in LC2 was suggested by the hybridization pattern observed in the blot in Fig. 3, in which LC2 DNAs extracted from cells at timed intervals during a period of several months were analyzed by restriction digestion with *Eco*RI, *Bam*HI, and *Bgl*II. Lanes 4, 5, and 6 demonstrate the restriction pattern of the earliest LC2 DNA sample analyzed in this series. It reveals, respectively, six *Bgl*II bands, three *Bam*HI bands, and two *Eco*RI bands hybridizing with a *tk*-specific probe. This is consistent with at least three copies of the *tk* insert being present in LC2. DNA made from LC2 cells 5 months later showed the same *Bam* and *Bgl*II patterns but an extra, faint *Eco*RI band at 3.1 kb (Fig. 3, lanes 1, 2, and 3). With DNA made from LC2 another 10 months later, this *Eco*RI band became very prominent, and in addition, a new 4.1-kb *Eco*RI

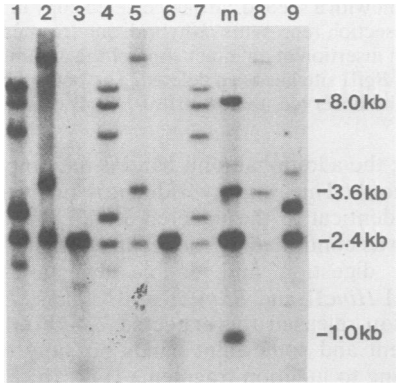

FIG. 3. Genomic Southern blot of clone LC2 DNA, using DNA samples isolated from cells grown continuously in culture over a period of 15 months. The probe used was the 3.6-kb *Bam*HI fragment of ptk. DNA used in lanes 4, 5, and 6 was isolated in January of 1980; DNA used in lanes 1, 2, and 3 was isolated 5 months later; and DNA used in lanes 7, 8, and 9 was isolated yet another 10 months later. Restriction enzymes used were as follows: lane (1) *Bgl*II, (2) *Bam*HI, (3) *Eco*RI, (4) *Bgl*II, (5) *Bam*HI, (6) *Eco*RI, (7) *Bgl*II, (8) *Bam*HI, (9) *Eco*RI, (m) molecular weight standards.

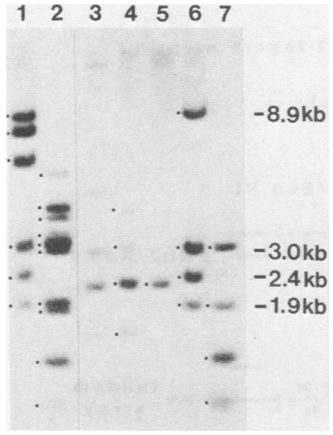

FIG. 4. Genomic blot of subclones LC2-3 and LC2-6. Lanes 1, 2, and 4 are digests of DNA isolated from LC2-6, and lanes 5, 6, and 7 are digests of DNA isolated from LC2-3. Lane 3 is of DNA isolated from LC2 mass culture. The restriction enzymes used were as follows: lane (1) *Bgl*II, (2) *Hin*fI, (3) *Eco*RI, (4) *Eco*RI, (5) *Eco*RI, (6) *Bgl*II, and (7) *Hin*fI.

band was observed (Fig. 3, lane 9). Throughout all of these experiments, we took a number of precautions to ensure that our observations were not the result of incomplete digestion. This included using large enzyme excesses, adding known plasmid or lambda DNA to follow the course of digestion, reprobing the blots with globin, α-fetoprotein, or α-actin sequences to monitor the completeness of digestion of the corresponding endogenous sequences, and monitoring for the release of repetitive sequence bands observed with some restriction enzymes such as *Bam*HI, *Bgl*II, and *Eco*RI (10, 17). To determine whether this changing restriction pattern was due to artifacts resulting from heterogeneity in our original LC2 culture, we isolated 24 subclones of LC2 and analyzed their DNA by restriction analysis as described above. We found that all of the subclones shared the same characteristic 2.0-kb *Bam*HI and *Eco*RI fragments and had in common many of the same *Hin*fI and *Bgl*II bands (Fig. 4). However, as expected, a subset of restriction fragments were unique to some of the subclones. This is illustrated in Fig. 4, in which the subclone, LC2-6, was found to have 4 *Eco*RI bands, 10 *Hin*fI bands, and 6 *Bgl*II bands (Fig. 4, lanes 4, 2, 1), whereas LC2-3 has a subset of these bands, as shown by the 1 *Eco*RI, 4 *Hin*fI, and 4 *Bgl*II bands (Fig. 4, lanes 5, 6, and 7). These results indicate that although the subclones have a heterogenous restriction pattern reflecting the changing patterns in the LC2 mass cultures, they nevertheless must all have been derived from one parent cell initially. Furthermore, these results also clearly demonstrate that the amount of *tk*-hybridizing sequences can vary considerably between subclones. This amplification and rearrangement process is still ongoing, since blots of DNA isolated more recently revealed the presence of additional *tk*-hybridizing fragments (Fig. 4, lane 3); moreover, the intensity of hybridization of some bands has greatly increased. Interestingly, our subclone analyses have demonstrated that these changes actually reflect a general instability at the site of insertion, since in addition to amplifications and rearrangements, deletions of the *tk* inserts also occur at a comparably high frequency (data not shown). We are currently constructing a genomic library of LC2 DNA so that we might further characterize the nature of this phenomenon by isolating and identifying the DNA sequences which mediate these instabilities.

Mouse embryo injections. Mouse embryos (C57BL/6J × SJL/J) at the one-cell stage were injected with the plasmid pMBJ. pMBJ consists of pBR322 with a 7.0-kb insert at the *Eco*RI site which contains the mouse β major globin gene (Fig. 5a). This genomic fragment was isolated from a mouse λ phage library by Tilghman et al. (29) and was kindly provided to us by P. Leder. The recipient mice were homozygous for the β single globin locus and therefore had a restriction pattern distinguishable from that of the injected DNA (14, 33). The plasmid was injected as circular supercoiled DNA, *Kpn*I-linearized DNA, or *Eco*RI-digested DNA. The DNA was dissolved in distilled water at a concentration of 150 ng/μl, and current pulses of 1 to 5 nA were used. Positive transformants were identified by restriction digestion and Southern blotting. Due to the presence of repetitive sequences in the 7-kb globin-containing insert, only the 1.9-kb *Bam*HI-HpaI fragment of pMBJ was used as the globin-specific probe (Fig. 5a).

From a total of 64 fetuses obtained, 4 were positive transformants (no. 24, 35, 60, 65). The fact that the pMBJ sequences were integrated into high-molecular-weight DNA was demonstrated by the observation that uncut DNA did not yield any hybridizing bands (see Fig. 7, lane 1; other data not shown). Furthermore, digestion with *Sau*3A and *Mbo*I gave identical restriction patterns, thereby definitively demonstrating that the hybridizing sequences were eucaryotic in origin and were not derived from contaminating plasmid DNA (data not shown). In three of four transformants (no. 24, 35, and 60), integration must have occurred very soon after injection, since the restriction patterns obtained by using eight or more enzymes with the fetal DNA were identical to that obtained with the placental DNA (see, for example, Fig. 9, lanes 2 and 3 of placental DNA and lanes 7 and 8 of fetal DNA of

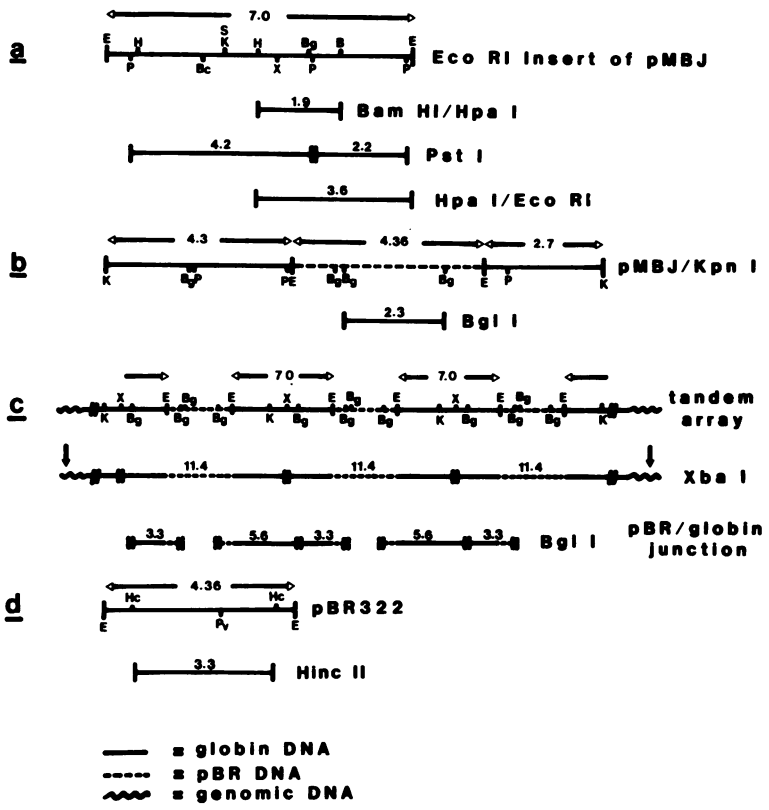

FIG. 5. (a) Restriction map of 7.0-kb *Eco*RI mouse genomic fragment in pMBJ. The 1.9-kb *Bam*HI-*Hpa*I fragment was used to make the globin-specific probe. The presence of the 3.3-kb *Hpa*I-*Eco*RI fragment and of the 4.2- and 2.5-kb *Pst* fragments is diagnostic of the presence of the 7.0-kb *Eco*RI fragment in transformants. Abbreviations: B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*I; E, *Eco*RI; H, *Hpa*I; K, *Kpn*I; P, *Pst*I; S, *Stu*I; X, *Xba*I, (b) Restriction map of *Kpn*I-linearized pMBJ. The 2.3-kb *Bgl*I fragment is diagnostic of the presence of intact pBR322. Restriction enzyme abbreviations are the same as in (a). (c) Insertion of a tandem array of the injected *Kpn*I-linearized pMBJ. *Xba*I digestion should generate a major hybridizing band at 11.4 kb, corresponding to the size of the linear plasmid and two junction fragments of indeterminate size. *Bgl*I digestion should release a 3.3- and a 5.6-kb pBR-globin junction fragment—corresponding to the region of pMBJ—which contain the point of ligation of pBR with globin. The restriction enzyme abbreviations are the same as in (a). (d) Restriction map of pBR322 (linearized with *Eco*RI). *Hin*cII digestion should release a 3.3-kb fragment if the pBR sequences inserted are intact copies of the *Eco*RI-linearized plasmid. Abbreviations: Hc, *Hin*cII; Pv, *Pvu*II.

transformant 35). However, in transformant 65, integration occurred much later since only the fetal DNA contained the injected pMBJ sequences. For each transformant, a detailed restriction analysis of the DNA isolated from the placentas and fetuses was carried out to determine the number of copies integrated, the minimum number of integration events in each transformant, and the approximate segments of the injected sequences which were integrated. The endogenous globin restriction fragments released by a number of restriction enzymes are illustrated in Fig. 6. In all subsequent figures, the endogenous globin bands are marked with a black dot to distinguish them from the exogenous sequences. The results obtained with each transformant are presented in detail below.

Fetus-placenta 24 was obtained after injection of mouse eggs with *Kpn*I-linearized pMBJ (Fig. 5b). Restriction digestion of DNA from this transformant with a number of enzymes which cut pMBJ only once (*Xba*I, *Stu*I, *Bcl*I; Fig. 5a) revealed the presence of a large number of extra or nonendogenous globin- and pBR-hybridizing bands (Fig. 7, lanes 3, 5, 8 for globin-hybridizing fragments). Some of these bands hybridized exclusively with either the pBR or the globin probe (Fig. 8; other data not shown). Thus, for example, *Stu*I digestion released eight bands, only four of which hybridized to both pBR and globin. Since the injected DNA consisted of a linear fragment which contained both pBR and globin sequences (*Kpn*I-linearized pMBJ), these results suggest that many of the injected DNA

molecules must have undergone exonucleolytic degradation before integration. These results also demonstrate that the integrated sequences are not arranged as tandem arrays. Thus, if tandem arrays were present, digestion with a single cutter should have released a major hybridizing band of 11.4 kb, corresponding to the size of the linear plasmid and junction fragments (see Fig. 5c, for example). In fact, although digestion with *Xba*I revealed a number of bands in this size range (11.2, 11.5, and 11.8 kb) (Fig. 7, lane 3), digestion with *Bcl*I did not release any bands above 11.2 kb (Fig. 7, lane 5), and *Stu*I digestion did not release any fragments between 11.0 and 12.0 kb (Fig. 7, lane 8). Also consistent with the absence of tandem arrays is the mere fact that a rather large number of hybridizing fragments were released by each of the single cutter enzymes: 10 by *Bcl*I, 10 by *Stu*I, and 11 by *Xba*I. Furthermore, if tandem arrays were present, digestion with *Bgl*I, which has five recognition sites in pMBJ, would be expected to release 3.3- and 5.6-kb pBR- and globin-hybridizing fragments, corresponding to the globin-pBR junction fragments (Fig. 5c). However, such fragments were not observed (Fig. 7, lane 6). All of these data conclusively show that no tandem arrays are present in this transformant. To assess the number of independent insertions present in this transformant, we attempted to restrict our DNA with *Bst*EII, the only known noncutter enzyme for pMBJ (11) (of which we are aware). Unfortunately, none of our DNA samples would cut with this enzyme. Nevertheless, a minimal estimate of the number of independent insertions can be arrived at by determining the number of exclusively pBR-hybridizing bands released by any one of the three single cutter enzymes. Thus, given that none of these enzymes cut pBR, any band which hybridizes only with pBR must be derived from pBR sequences which are flanked by *Stu*I, *Xba*I, or *Bcl*I cutting sites which are derived from neighboring mouse genomic DNA. Hence, each of these bands must represent a unique insertion into the mouse genomic DNA. When a fragment hybridizes to either globin alone or to both pBR and globin, it is not possible to determine whether the fragment is flanked by genomic DNA or more plasmid DNA. Thus, such DNA fragments cannot be distinguished as either independent inserts or pBR and globin DNA which are all covalently linked and integrated at one site. For our analyses, we regarded all such fragments as a single insert so as not to overestimate our integration efficiencies. Using this approach, we determined that there were at least five independent insertions in this transformant, since *Bcl*I digestion released four bands which were exclusively pBR hybridizing (all of the other hybridizing bands were counted as one insert [Fig. 8]). Further analysis of this transformant with *Eco*RI digestion revealed the presence of a 7.0-kb globin-

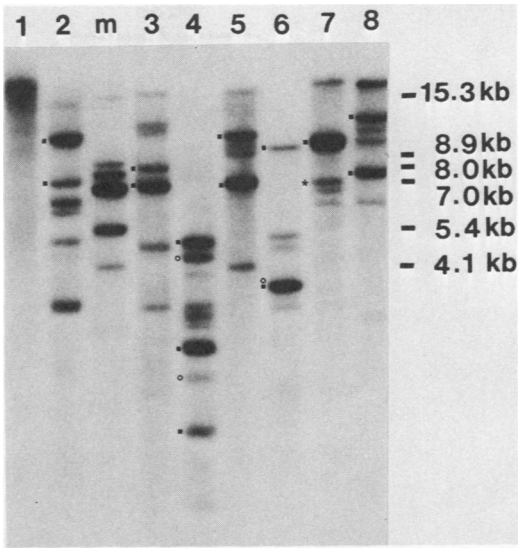

FIG. 7. Genomic Southern blot of mouse embryo transformant 24. The blot was hybridized with a globin-specific probe. The restriction enzymes used were as follows: lane (1) *Xma*I, (2) *Bgl*I, (3) *Xba*I, (4) *Pst*I, (5) *Bcl*I, (6) *Hpa*I-*Eco*RI, (7) *Eco*RI, (8) *Stu*I, (m) molecular weight standards. The black dots mark the positions of endogenous globin bands. The open circles in lanes 4 and 6 mark the positions of internal globin bands expected for the globin insert of pMBJ. The asterisk in lane 7 marks the position of a 7.0-kb globin-hybridizing band.

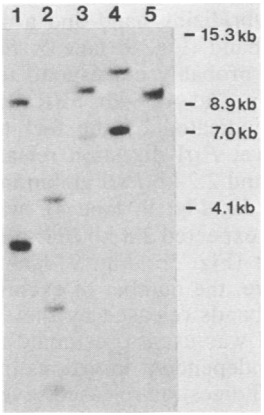

FIG. 6. Genomic Southern blot of normal mouse DNA (C57 × SJL). The blot was hybridized with a globin-specific probe to reveal the endogenous globin sequences. The restriction enzymes used were as follows: lane (1) *Eco*RI-*Hpa*I, (2) *Pst*I, (3) *Bgl*I, (4) *Stu*I, (5) *Eco*RI.

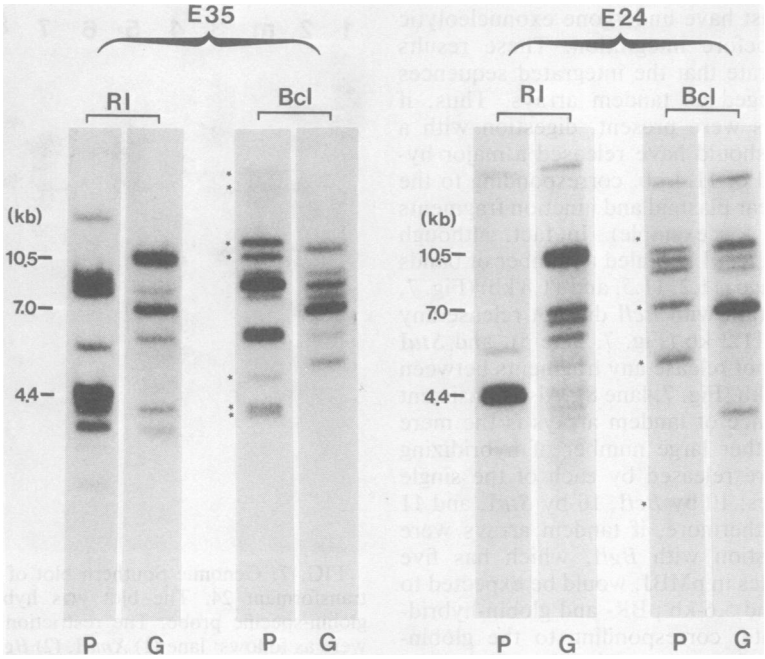

FIG. 8. Genomic Southern blot of mouse embryo transformants 24 and 35 for demonstrating the number of exclusively pBR-hybridizing fragments in *Bcl* digests and the presence of a 4.4-kb pBR-hybridizing and a 7.0-kb globin-hybridizing *Eco*RI fragment. *Eco*RI and *Bcl*I digests of E24 and E35 were hybridized with either a globin probe (G) or a pBR probe (P). In *Bcl*I digests, an asterisk was placed next to each band which was exclusively pBR hybridizing.

hybridizing band (Fig. 7, lane 7; Fig. 8). This was very surprising, since the injected DNA consisted of pMBJ that was cut with *Kpn*I, an enzyme which cuts once in the 7.0-kb globin insert. To determine whether the observed 7.0-kb fragment was indeed the authentic *Eco*RI globin insert, digestions with *Pst*I and double digestion with *Hpa*I-*Eco*RI were carried out; both digests gave the expected internal globin fragments (see Fig. 5A), 3.6 kb for *Hpa*I-*Eco*RI (Fig. 7, lane 6) and 4.2 and 2.2 kb for *Pst*I (Fig. 7, lane 4). These results suggest that the observed 7.0-kb fragment is, in fact, the intact 7.0-kb *Eco*RI insert of pMBJ. It is not clear how this insert was generated, but perhaps the injected linear plasmid was recircularized after injection and before integration. Note that also present in this transformant is the intact 4.36-kb pBR DNA. Thus, *Eco*RI digestion released a 4.4-kb pBR-hybridizing band (Fig. 8), and *Bgl*I digestion released the expected internal 2.3-kb pBR fragment (see Fig. 5b; other data not shown).

A similar analysis was carried out with embryo 35, which was obtained after microinjection with circular supercoiled pMBJ. Digestion of the DNA with the same set of single cutting enzymes revealed a large number of hybridizing bands (Fig. 9, lanes 2, 3, 7, 8), some of which, as described above, were only globin hybridizing or only pBR hybridizing. This indicated that partial nucleolytic degradation of the injected DNAs must have occurred before their integration. However, as with the previous transformant, the degradation was not so extensive as to preclude the insertion of either the intact 7.0-kb globin or the 4.36-kb pBR fragments. Thus, *Eco*RI digestion revealed the presence of a 7.0-kb globin-hybridizing band and a 4.4-kb pBR-hybridizing band (Fig. 9, lane 5; Fig. 8). That these bands probably correspond to the intact 7.0-kb globin and 4.36-kb pBR fragments of pMBJ was indicated by the fact that, as described above, *Pst*I digestion released the expected 4.2- and 2.2-kb *Pst*I globin internal fragments (Fig. 5a; Fig. 9, lane 4), whereas *Bgl*I released the expected 2.3-kb *Bgl*I pBR322 internal fragment (Fig. 5b; Fig. 9, lane 6). As described above, the number of exclusively pBR-hybridizing bands released by the single cutter enzyme *Bcl*I was used to estimate the minimal number of independent insertions in this transformant. *Bcl*I digestion released seven *Bcl*I fragments which hybridized exclusively with pBR; this indicates that at least eight independent integrants must have been present in this transformant (Fig. 8). As in transformant 24, restriction fragments released by the single cutters again revealed that no tandem arrays were pres-

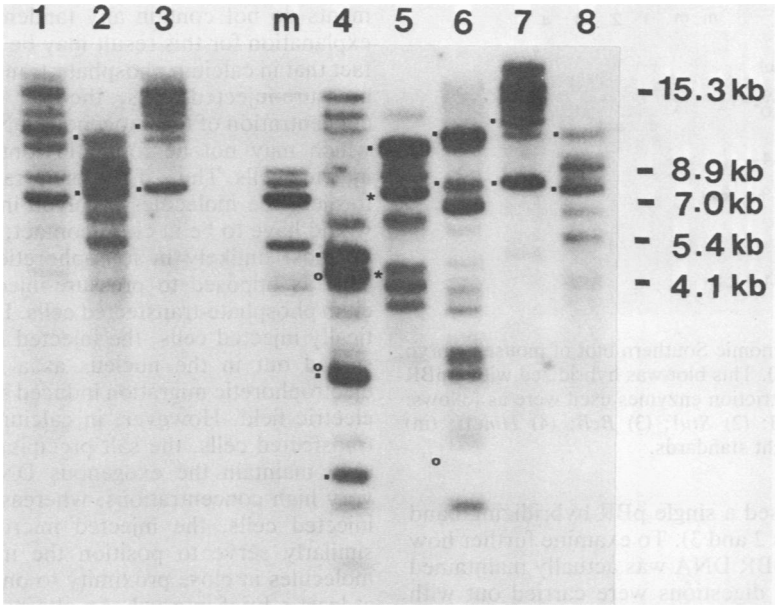

FIG. 9. Genomic Southern blot of mouse embryo transformant 35. This blot was hybridized with globin and pBR probes. Note that the pBr hybridization was weak due to the stringency of the wash conditions. Lanes 1 to 3 are digests of placental DNA, and lanes 4 to 8 are fetal DNA. Note that a high-molecular-weight band which is present in lane 7 but which is missing in lane 3 is actually normally observed but is absent here due to poor transfer in this gel. The restriction enzymes used were as follows: lane (1) *Bam*HI; (2) *Bcl*I; (3) *Stu*I; (4) *Pst*I; (5) *Eco*RI; (6) *Bgl*I; (7) *Stu*I, (8) *Bcl*I, (m) molecular weight standards. Endogenous globin fragments are marked on their left with a black dot. The open circles in lanes 4 and 6 mark the positions of bands corresponding to internal globin and pBR fragments expected from digests of pMBJ sequences (see the text). The asterisks in lane 5 denote the positions of a 7.0-kb globin-hybridizing band (top) and a 4.4-kb pBR-hybridizing band (bottom).

ent in this transformant. Thus, digestion with each of the single cutters did not result in the release of an 11.4-kb band (corresponding to the size of the linear plasmid). *Xba*I released an 11.7-kb fragment (data not shown); *Bcl*I did not release any fragment above 10.8 kb (Fig. 9, lane 8); and *Stu*I did not release any fragment between 11.0 and 12.0 kb (Fig. 9, lane 7; this is more clearly observed in lighter autoradiographic exposures of this blot and in gels which have been run even further). As described above, *Bgl*I digestion was carried out to examine this question further, and although *Bgl*I did release a 3.3-kb and a 5.6-kb band, corresponding to the sizes of the diagnostic pBR-globin junction fragments (Fig. 5c; Fig. 9 lane 6), in fact, the 5.6-kb band was found to hybridize only to pBR sequences (data not shown). These results all suggest that there were no tandem insertions in this transformant. Consistent with this conclusion are the observations that, as in transformant 24, a large number of fragments were released by the single cutter enzymes: 8 by *Stu*I, 7 by *Xba*I, and 12 by *Bcl*I. Furthermore, note that the release of 17 fragments by *Bgl*I and of 14 fragments by *Eco*RI would also be difficult to explain if tandem arrays accounted for any significant numbers of the inserts—with either enzyme, only two major hybridizing bands would have been expected (Fig. 5c).

Transformant 60 was obtained from the injection of *Eco*RI-digested pMBJ. This effectively consisted of injecting linear pBR322 and the linear 7.0-kb globin-containing fragment of pMBJ. Surprisingly, this embryo was found to contain only pBR DNA. Thus, *Eco*RI digestion revealed the presence of a single 13.5-kb pBR-hybridizing band (Fig. 10, lane 1). Since the pBR DNA that was injected was the *Eco*RI-linearized 4.36-kb pBR DNA, the observation of a single *Eco*RI fragment of 13.5 kb indicates that this transformant contains only a single insertion and that this insert is an exonucleolytically degraded fragment which is flanked by novel genomic DNA sequences. To further analyze the number of independent insertions in this transformant, digestions with pBR noncutter enzymes, *Bcl*I and *Stu*I, were carried out. Thus, the number of bands released by either enzyme should give a minimal estimate of the number of independent insertions in this transformant, and, consistent with the presence of only a single insert, each

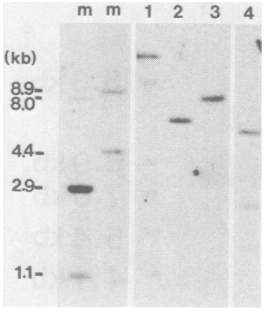

FIG. 10. Genomic Southern blot of mouse embryo transformant 60. This blot was hybridized with a pBR probe. The restriction enzymes used were as follows: lane (1) *Eco*RI; (2) *Stu*I; (3) *Bcl*I; (4) *Hin*cII; (m) molecular weight standards.

enzyme released a single pBR-hybridizing band (Fig. 10, lanes 2 and 3). To examine further how much of the pBR DNA was actually maintained in this insert, digestions were carried out with *Hin*cII, which has two recognition sites in pBR. *Hin*cII digests revealed that one of the two *Hin*cII recognition sites in pBR must have been deleted, since the expected 3.3-kb internal *Hin*cII fragment was not observed (Fig. 5d); instead, two bands at 5.1 and 2.5 kb were released (Fig. 10, lane 4). Note that this latter observation is also consistent with the presence of only a single insert. Thus, in this transformant, unlike the other two described above, only a single integration event took place, and this resulted in the insertion of a single copy of a partially deleted form of pBR322.

With transformant 65, *Eco*RI-digested pMBJ was also injected. In this transformant, pMBJ sequences were observed only in the fetal DNA. A 7.0- and a 5.0-kb fragment were detected after *Eco*RI digestion (data not shown). Because this embryo was very small and appeared to have atrophied at the time of harvesting, only a very small amount of DNA was obtained. Therefore, it was not possible to carry out more detailed restriction analysis with this transformant. However, it is interesting to note that this transformant contained a globin-hybridizing fragment that was the same size as the injected 7.0-kb *Eco*RI globin fragment.

DISCUSSION

Using iontophoresis, we have introduced the herpesvirus *tk* gene into mouse tissue culture cells and the mouse β major globin gene into mouse embryos. We have obtained stable transformants with one or more integrants, and, unlike transformants generated by either pressure injection (5, 7, 11, 19, 30) or calcium phosphate precipitates (20, 21, 24), our transformants do not contain any tandem arrays. The explanation for this result may be related to the fact that in calcium phosphate-transfected and in pressure-injected cells, there is a high local concentration of the exogenous DNA sequences which may not be found in iontophoretically injected cells. Thus, if tandem arrays were to be formed, the molecules involved in the reaction would have to be in close contact, and this may be most unlikely in iontophoretically injected cells as opposed to pressure-injected and calcium phosphate-transfected cells. In iontophoretically injected cells, the injected DNA may be spread out in the nucleus as a result of the electrophoretic migration induced by the applied electric field. However, in calcium phosphate-transfected cells, the salt precipitate may naturally maintain the exogenous DNA locally in very high concentrations, whereas in pressure-injected cells, the injected microdroplet may similarly serve to position the injected DNA molecules in close proximity to one another for at least a brief interval. An alternative explanation for the lack of tandem arrays in iontophoretically injected transformants may be related to the observed higher degree of nucleolytic degradation of the transfected DNA before integration. It is possible that this nucleolysis interferes with or somehow reduces the efficiency with which tandem arrays are formed. However, with no further data to support such a notion, it is equally possible that the higher degree of exonucleolysis is made possible because the injected sequences do not have their ends protected via the generation of tandem arrays; this again may be a consequence of the sequences being physically spread apart in the nucleus. At the present time, we cannot distinguish among these various possibilities, but we hope that future analysis of additional transformants will lead to further insights on this problem.

We have obtained transformants with either a single insert or multiple inserts, including one transformant with at least eight separate insertions. In contrast, very few inserts were observed in transformants generated by pressure injection or calcium phosphate precipitates—in fact, usually only one was observed per cell (3, 5, 7, 11, 19, 21, 25). Given that studies with yeasts have shown that free ends are required for integration of exogenous DNA (18), it is possible that the higher efficiency of integration in iontophoretically injected cells results from the presence (or maintenance) of more free ends in the injected DNA. This notion is consistent with the observation that many of the inserts in our transformants are only partial fragments of the injected DNA. Thus, if many of the iontophoretically injected molecules have exposed 5′ and 3′ ends, they may be more accessible to

exonucleolytic attack and may therefore be more prone to suffer partial degradation before integration. That this nucleolysis of the injected DNA must have occurred after its entry into the cell was demonstrated by the routine analysis of some of the DNA in the microelectrode before and after iontophoresis by agarose gel electophoresis. The DNA in all cases was observed to be intact.

The observation that one of our transformants contained an insertion which has preserved the terminal restriction enzyme cohesive ends of the injected DNA was rather surprising, since this has not been previously reported. Our injection procedure may favor such an event, since with iontophoresis, the injected DNA molecules do not become linked in tandem arrays; hence, more of the terminal restriction enzyme sites of the injected DNA fragments would be available for direct integration. If this integration proceeded in a completely random manner, it would have a 1 in 16 chance of occurring. However, it is also possible that this insertion proceeded via limited homologous recombination. Homologous insertions involving short stretches of DNA sequence homologies have been reported in a previous study of a simian virus 40-transformed cell line in which it was shown that a simian virus 40 insertion was mediated via a 5-base pair sequence homology in the genomic DNA of the recipient cell (28). In addition, limited homologous recombination between transfected DNA molecules themselves has been observed (1, 7, 24). At the present time, we do not know whether our integration is mediated by sequence homology.

In summary, our results demonstrate that DNA transformation can be achieved by iontophoretic microinjection. We are currently investigating the potential use of this injection method for targeting DNA sequences. This has been accomplished in yeasts (12), and it was found that the only requirement for homologous recombination was that the transfected DNA be linear and the linear molecules have homologous 5′ and 3′ ends (18). Given that free ends of injected linear molecules may be more abundant in iontophoretically injected cells as compared with other transformation protocols, iontophoretic injection may provide a means for obtaining the kinds of results found in yeasts. Furthermore, since iontophoretic injections may permit the generation of transformants with many more independent insertions, this approach may also be more efficient for obtaining expressing transformants for the purpose of studying gene expression. The particular inserts of interest can be subsequently isolated and identified simply by breeding the transformants to segregate out the various inserts into different animals.

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