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Genetic transformation of mouse embryos by microinjection of purified DNA

(gene transfer/mice)

JON W. GORDON*, GEORGE A. SCANGOS†, DIANE J. PLOTKIN*, JAMES A. BARBOSA‡, AND FRANK H. RUDDLE*‡

*Department of Biology and ‡Department of Human Genetics, Yale University, New Haven, Connecticut 06511

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ABSTRACT **A recombinant plasmid composed of segments of herpes simplex virus and simian virus 40 viral DNA inserted into the bacterial plasmid pBR322 was microinjected into pronuclei of fertilized mouse oocytes. The embryos were implanted in the oviducts of pseudopregnant females and allowed to develop to term. DNA from newborn mice was evaluated by the Southern blotting technique for the presence of DNA homologous to the injected plasmid. Two of 78 mice in one series of injections showed clear homology, though the injected sequences had been rearranged. Band intensities from the two positive mice were consistent with the presence of donor DNA in most or all of the cells of the newborns. These results demonstrate that genes can be introduced into the mouse genome by direct insertion into the nuclei of early embryos. This technique affords the opportunity to study problems of gene regulation and cell differentiation in a mammalian system by application of recombinant DNA technology.**

Introduction of specified gene sequences into mammalian embryos can be a powerful tool for the study of developmental genetic problems. The fate of such genes can be monitored throughout development by using sensitive probing techniques offered by recombinant DNA technology. In addition, the functioning of foreign genes in a normal host environment can be used to study the processes of gene regulation and to study the physiologic roles of products of such genes more precisely. Introduction of foreign DNA into all cells of an intact animal also provides an opportunity to pass sequences to offspring and to generate large numbers of transformed animals. In order to realize these benefits, it is necessary to transform embryos early in development and allow integration of foreign DNA into the cellular progenitors of the entire animal.

Such experiments with mammals are difficult. Zygotes must be maintained in culture conditions that at least grossly approximate the oviductal environment. Moreover, they can be maintained *in vitro* for only a few days, after which they must be returned to a female for implantation and further development. Insertion of material into early mammalian embryos is also difficult because of their small size.

Investigators have recently succeeded in constructing mosaic mice composed in part of descendants from cultured teratocarcinoma cells (1–3). This advance makes possible the introduction of genes into cultured cells, which might then be induced to cooperate in the formation of an intact adult mouse (4, 5). These cultured cells are often aneuploid, however, and some difficulty has been encountered in obtaining functional germ cells derived from them (6). Another problem with teratoma mosaics is that they are, indeed, mosaics. Thus, teratoma cells of XX chromosomal constitution cannot make sperm in mice that develop as males; the possibility of germ-line transmission in this system is accordingly reduced. Jaenisch and Mintz (7) have provided evidence that whole DNA of simian virus 40 (SV40), when placed in cavities of mouse blastocysts, may be found in the resultant offspring. Ideally, however, one would like to introduce a small amount of well-defined genetic material directly into normal embryos and allow this material to integrate and function within the host genome.

We have approached this problem by injecting DNA directly into the pronuclei of fertilized mouse oocytes. The one-cell stage was chosen in order to limit as much as possible the development of mosaicism during cleavage. To avoid the hazards of culture, injected embryos were immediately implanted into the oviducts of pseudopregnant recipients. The DNA chosen for injection was the bacterial plasmid pBR322 into which had been inserted fragments of herpes simplex and SV40 viral DNA. This plasmid was constructed because the SV40 fragment is known to contain an origin of DNA replication, whereas the herpes fragment codes for a gene product, thymidine kinase (TK), distinguishable from the endogenous mouse enzyme. DNA was extracted from newborn mice and screened by the Southern blotting technique for the presence of sequences homologous to the injected plasmid. Two of 78 mice evaluated in one experimental series were found to contain such sequences. In both instances the injected DNA had been modified, but it could be demonstrated to be derived from donor material. The intensity of the positive bands indicated that an amount of DNA roughly equivalent to one copy in every cell of the newborns was retained. We thus provide evidence that mice can be genetically transformed by direct insertion of DNA into early embryos.

MATERIALS AND METHODS

Mice. CD-1 mice were obtained from the Charles River Breeding Laboratories. B6D2F$_1$ mice were obtained from the Jackson Laboratory. All mice were maintained on a 14:10 light–dark schedule (lights off at 10 p.m., on at 8 a.m.). Six-week-old females were induced to superovulate with 5 international units of pregnant mares' serum (Gestyl, Organon) at 4 p.m. followed 48 hr later by 2.5 international units of human chorionic gonadotropin (Pregnyl, Organon) and placed immediately with males for mating. B6D2F$_1$ females were mated with CD-1 males; CD-1 females were mated with B6D2F$_1$ males. On the same evening other mature CD-1 females were placed with vasectomized CD-1 males. On the morning after mating (day 0) all female mice were examined for vaginal plugs. Six-week-old females were killed at 2 p.m. on day 0 and

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Abbreviations: SV40, simian virus 40; TK, thymidine kinase; kb, kilobase(s).

† Present address: Department of Biology, The Johns Hopkins University, Baltimore, MD 21218.

their oviducts were removed into Krebs–Ringer bicarbonate-buffered medium supplemented with bovine serum albumin (8) and hyaluronidase at 1 mg/ml. Oviducts were opened with forceps and the fertilized eggs with remaining follicle cells were expressed into the dish. After 1–2 min, eggs were removed and washed three times in 2 ml of culture medium equilibrated with 5% CO_2 in air at 37°C. Eggs containing pronuclei were identified under the dissecting microscope and placed in lots of 20 in a microdrop of equilibrated medium, which was placed in a 100-mm tissue culture dish and covered with mineral oil (Mallinckrodt 6358). Eggs were stored in this manner in the incubator until microinjected.

Microinjection. Microneedles were pulled from thin-walled no. 1211L Omega Dot tubing (Glass Co. of America) on a DK1 model 700C pipette puller. Holding pipettes were pulled by hand on a microburner from G-12 capillary tubing (Thomas), and fire polished on a Sensaur microforge. The tips of the microneedles were allowed to fill with plasmid suspension by capillary action and the barrels were then filled with Fluorinert (3M FC77). They were then secured in PE-190 intramedic tubing on a Leitz micromanipulator. Holding pipettes were also filled with Fluorinert and similarly secured in PE-90 tubing. The tubing was likewise filled with Fluorinert and attached to 1-cm^3 Hamilton syringes. All manipulations were carried out on a Leitz microscope.

Tissue culture dishes containing the fertilized eggs were placed on the microscope and eggs were positioned by holding the pipette such that a pronucleus near the plasma membrane was close to the microneedle. The microneedle was inserted into the pronucleus and enough plasmid suspension was injected to cause an approximate doubling of the pronuclear volume (approximately 1 pl). Eggs that survived microinjection were removed and stored in a 30-mm tissue culture dish containing 2 ml of equilibrated medium until all microinjections were completed. Injection of 40–60 embryos required 1–2 hr.

Implantation. Plugged pseudopregnant CD-1 females were anesthetized with Nembutal at 6 mg/100 g of body weight. Ovaries were located through a dorsal incision. The ovarian bursa was torn away with no. 5 Dumont watchmaker's forceps, taking care not to rupture large blood vessels. The ostium of the oviduct was visualized under the dissecting microscope and a pipette containing 10–20 microinjected embryos was inserted into it. The eggs were expelled into the oviduct and the wound was closed with wound clips. Mice were examined on days 18–21 for the delivery of live offspring. Newborn mice were stored at −80°C for later analysis. Sixty percent of the embryos survived microinjection; 30–50% of the survivors developed into live young. All newborns were normal in appearance. All microinjection work was carried out under P1 containment in accordance with National Institutes of Health guidelines.

DNA Isolation. DNA was isolated from whole newborn mice by the method of Blin and Stafford (9) with the following modifications. Powdered tissue was incubated for 4 hr at 50°C in 22 ml of 0.28 M EDTA/0.5% Sarkosyl, pH 7.0. The homogenate was subsequently extracted twice in phenol/chloroform/isoamyl alcohol (15 ml:5 ml:0.2 ml), and once in chloroform/isoamyl alcohol (15 ml:0.6 ml). The extract was dialyzed for 24 hr against 10 mM Tris·HCl, pH 8.0/10 mM NaCl/1 mM EDTA and precipitated with a 2-vol excess of 100% ethyl alcohol. Precipitated DNA was stored at −20°C until use.

Filter Hybridization. DNA was redissolved in 1× TEN (10 mM Tris·HCl, pH 7.75/10 mM NaCl/0.1 mM EDTA) to yield a final concentration of approximately 1 mg/ml. Twenty micrograms of DNA was digested at a 10- to 20-fold excess with appropriate restriction enzymes (Bethesda Research Laboratories, Rockville, MD). After overnight digestion at 37°C, samples were electrophoresed in 1% agarose in 160 mM Tris·HCl/80 mM NaOAc/80 mM NaCl/5 mM EDTA, pH 8, at 350 A for 22 hr. Samples were then blotted onto nitrocellulose filters according to the method of Southern (10).

Nick translations were performed by using the New England Nuclear nick translation kit with ^{32}P-labeled dCTP obtained from New England Nuclear. Filter hybridizations were performed as described by Wahl *et al.* (11). Filters were then used to expose Kodak X-Omat x-ray film, using intensifying screens, until band intensities were appropriate for analysis.

Construction of the Plasmid. The recombinant plasmids, called pST6, pST9 and pST12, carrying the SV40 origin of replication and promoters, and the herpes simplex virus TK gene were constructed by inserting the SV40 *Hin*dIII-C fragment (12, 13) into the available *Hin*dIII site in the plasmid pTKX-1 (14). DNA from the SV40 mutant 1265, kindly provided by C. Cole of Yale University, was digested to completion with restriction enzymes *Hin*dIII and *Hin*fI (New England BioLabs) simultaneously. The double digestion generated two fragments larger than 550 base pairs; the *Hin*dIII-C fragment (1099 base pairs; map position 0.649–0.859) and the *Hin*fI-B fragment (1085 base pairs; 0.992–0.199), which comigrated on a 1% Seaplaque agarose gel. The 1.1-kilobase (kb) doublet band was extracted from the gel and ligated with pTKX-1 that had been digested with *Hin*dIII and alkaline phosphatase [as described by Ullrich *et al.* (15) except that bovine alkaline phosphatase (Sigma) was used]. The molar ratio of the vector to target in the ligation mixture was 3:1. The ligation mixture was incubated at 4°C for 17 hr with one addition of phage T4 ligase at 11 hr. The mixture was used to transform *Escherichia coli* strain HB 101, and ampicillin-resistant colonies were selected. Colonies carrying the putative pST plasmids were identified by colony hybridization (16), using SV40 DNA as the probe. Approximately 20% of the ampicillin-resistant colonies contained SV40 sequences. Confirmation of the *Hin*dIII-C fragment insertion and determination of its orientation in the plasmid was done by restriction analysis of mini-DNA isolations (17). A restriction endonuclease map of the plasmid pST6 is shown in Fig. 1. This work was carried out under P2 containment in accordance with National Institutes of Health guidelines.

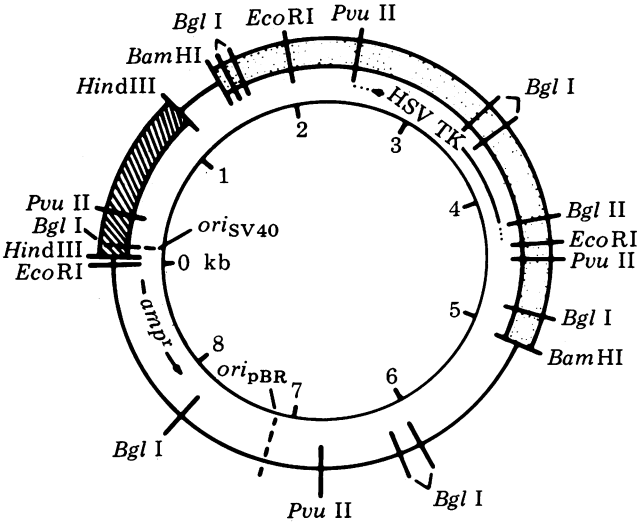

FIG. 1. The circular plasmid pST6, a derivative of pBR322. Hatched area shows the SV40 insert; stippled area denotes the herpes simplex virus TK insert. amp^r, ampicillin resistance gene; *ori*, origin of DNA replication.

Table 1. Summary of microinjection data

Exp.	Plasmid	Copies injected per cell	Offspring	Plasmid DNA positives
1	pST6	1,000	78	2
2	pST6	12,000	10	0
3	pST6 (linearized)	1,000	40	0
4	pST9	1,000	16	0
5	pRH 1.3Mm 1	1,000	27	0
6	pST12	500	2	0
7	Uninjected control	—	54	0

The pRH 1.3Mm 1 plasmid consists of a cloned fragment of a member of the highly repeated and interspersed *Eco*RI–*Bgl* II sequence family cloned in pBR322, provided by N. Arnheim (18). pST9 is identical to pST6, except that the orientation of the SV40 fragment is reversed. pST12 is a dimer of pST6. pST was linearized by *Sal* I digestion. A total of 187 mice were born from microinjected embryos.

RESULTS

Results of the plasmid microinjections are summarized in Table 1. In the first experimental series, injection of several hundred embryos yielded 78 live young. DNA was extracted from whole newborn mice for rapid and efficient determination of transformation frequency. The screening method gives a low estimate of the number of transformants; embryos with transforming DNA in a small percentage of their cells could have escaped detection. DNA from 2 of these 78 newborn mice contained sequences that hybridized strongly with the probe, pST6. The restriction endonuclease patterns of the incorporated sequences were significantly different between the two offspring, and are described below.

DNA from the first positive animal, no. 48, gave two intense bands with estimated sizes of 12.9 kb and 9.8 kb and a third band of very large size (>24 kb) when digested with *Bam*HI (Fig. 2). The positions of the two smaller bands were unaffected by digestion with *Hin*dIII, *Eco*RI, *Bam*HI, or *Xba* I (Fig. 2). This result suggested that the TK sequences, which had been inserted into the *Bam*HI sites, and the SV40 sequences inserted into the *Hin*dIII sites were not present in their native state in the incorporated material. The *Hin*dIII digestion, however, was incomplete as judged from the control track. We therefore probed with SV40 DNA alone. No sequences homologous to this

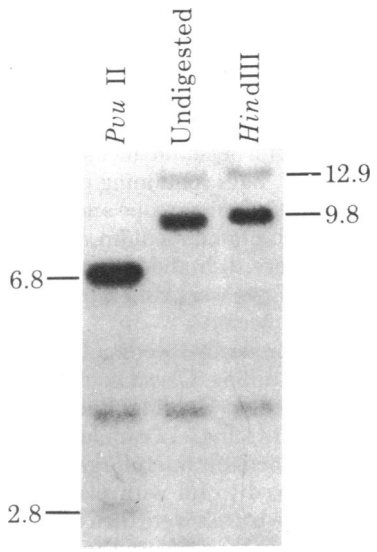

FIG. 3. DNA from mouse no. 48 digested with *Hin*dIII or *Pvu* II, or undigested; probed with pST6. Fragment sizes are indicated in kb.

probe were detected. The 12.9- and 9.8-kb fragments appeared in the undigested sample, consistent with their presence as free molecules. Digestion of the DNA with *Pvu* II generated two bands of altered mobility, 2.8 kb and 6.8 kb in size (Fig. 3). This result indicated that the sequences represented by the 12.9- and 9.8-kb bands contained at least one *Pvu* II site. We believe these results, taken together, are consistent with the existence of free circular molecules in the DNA of mouse no. 48.

The second positive, no. 73, showed a markedly different blotting pattern. In the undigested DNA, hybridizable material was not separable from the high molecular weight mouse DNA. Moreover, digestion with *Xba* I, which does not cut pST6, gave a single band of greater size than the highest molecular weight standard of 23.7 kb. Finally, several bands showed homology with probes synthesized from either purified SV40 DNA or TK fragment (Fig. 4). Thus, this animal had retained all or part of these portions of the plasmid.

Digestion with *Bam*HI yielded three major bands, 7.8 kb, 3.9 kb, and 3.4 kb. The largest band showed homology with

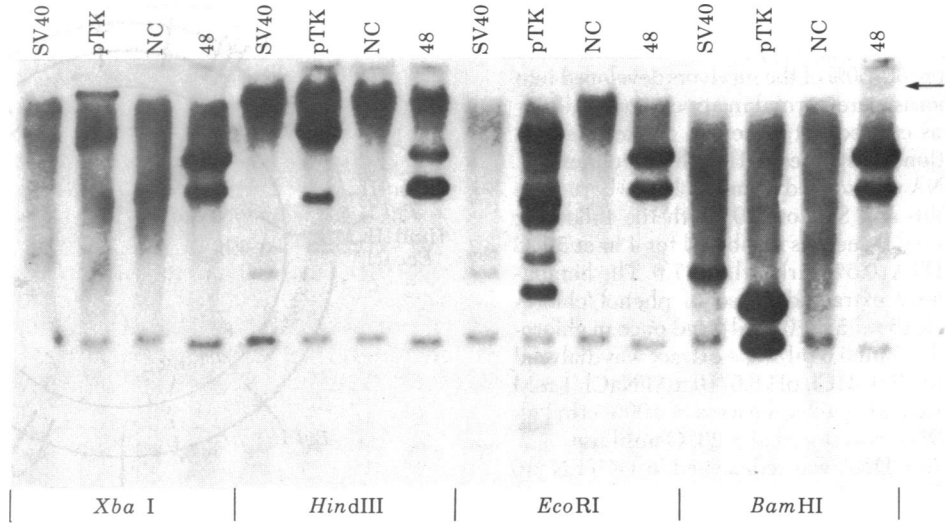

FIG. 2. DNA from mouse no. 48 digested with *Bam*HI, *Eco*RI, *Hin*dIII, and *Xba* I. The labeled probe was pST6 DNA. NC indicates the negative control (DNA isolated from uninjected mice). Positive controls include (*i*) NC DNA with SV40 DNA added (SV40) and (*ii*) NC DNA with the plasmid pTTX-1 added (pTK). Arrow indicates the high molecular weight band that appears reproducibly in *Bam*HI digests.

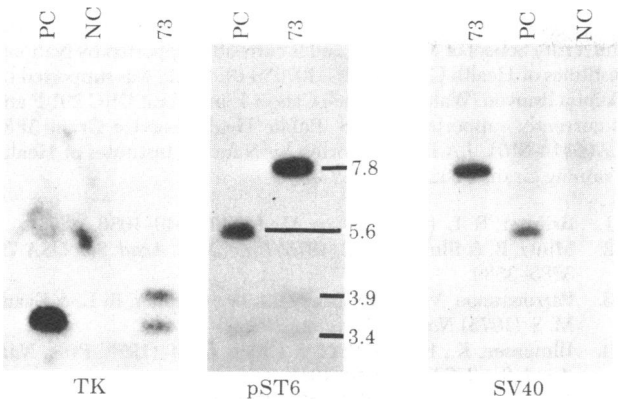

FIG. 4. DNA from mouse no. 73 digested with *Bam*HI and probed with pST6 (*Center*), SV40 DNA (*Right*), or TK fragment (*Left*). Positive control (PC) consists of pST6 added to mouse DNA to a concentration of 10^{-6} by weight. NC denotes the negative control (DNA isolated from uninjected mice).

SV40, SV40 + pBR322, and with the whole plasmid, pST6 (Fig. 4). The two smaller bands showed homology with TK fragment, but not with SV40 or pBR322 (Fig. 4). The probeable portions of these smaller pieces were thus composed entirely of TK-derived material. The smallest band, 3.4 kb, closely approximates the size of the TK fragment that was inserted into the *Bam*HI sites, suggesting that the entire TK gene had been retained in no. 73. Digestion with *Bgl* I, however, proved this supposition incorrect. An internal fragment of TK approximately 1.8 kb in size, defined by *Bgl* I sites, did not appear in the DNA (data not shown). This showed that the 3.4- and 3.9-kb *Bam*HI fragments were composed of portions of the TK fragment that had either been concatamerized or complexed with mouse DNA to yield molecular weights equal to or greater than the molecular weight of the original TK insert.

Digests with *Pvu* II and *Hin*dIII provided strong evidence that the entire SV40 sequence was retained. Digestion with *Hin*dIII produced a fragment very close in size to the SV40 insert of pST6. In addition, digestion with *Pvu* II gave two fragments that migrated indistinguishably from the *Pvu* II-defined SV40 fragments of pST6. Thus, two independent experiments support the contention that the entire SV40 fragment was present.

DISCUSSION

These data demonstrate that it is possible to use a recombinant plasmid as a vector for transfer of foreign genes directly into mouse embryos, and that these embryos can maintain the foreign genes throughout development. Moreover, the intensity of the bands on Southern blot analysis suggests that most or all of the cells of the newborns contained derivatives of the injected plasmid. Blotting experiments with hybrid cell populations have shown that sequences cannot be detected if present in fewer than 10% of the cells (19). We are thus confident that the two transformed mice contained enough plasmid DNA for distribution of one copy to at least this percentage of their cells. Our positive controls were adjusted to correspond to one copy of pST6 per diploid genome. The band intensities of no. 48 and no. 73 are comparable to the control. Thus, the transforming sequences are probably present in far greater amounts than the 10% threshold of detectability; the band intensities are more consistent with the presence of the plasmid derivative in most or all of the cells of the newborns. Our method of analysis cannot rule out the possibility that only a few of the cells contained all of the sequences while most of the cells were negative, but we consider unlikely the chances that cells carrying a large amount of additional genetic material would survive and compete successfully through development. If the transforming sequences were in fact distributed throughout the tissues of the mice, then integration must have occurred at an early stage, shortly after determination of the inner cell mass. Injection of one-celled embryos may be important for obtaining early integration. In addition, the high mortality caused by microinjection suggests that injection of only a fraction of the cells of a later cleavage stage might result in preferential survival of uninjected blastomeres and consequently give a lower rate of success.

The transformation rate reported here compares very favorably with other gene transfer systems involving mammalian cells. Calcium phosphate-mediated gene transfer into cultured cells results in transformation rates of 10^{-8} to 10^{-5} (20, 21), while microinjection of cultured cells gives approximately 5% success (22). Our transformation rate agrees well with these latter results. The reasons for higher rates in microinjection experiments are unknown but may include the facts that DNA is inserted directly into the nucleus and that gene expression is not required in the mouse system.

Significant differences were found between the two transformed mice. In mouse no. 48, SV40 and herpes viral TK DNA could not be detected. The remaining sequences, derived from pBR322, were complexed into three bands, all of higher molecular weight than the entire pBR322 plasmid. In addition, two of these bands represented DNA that probably existed free of the host genome. The presence of unintegrated sequences in no. 48 is intriguing. Two plausible models can be invoked to explain this observation: (*i*) these sequences may have replicated autonomously and persisted as plasmid-like units; (*ii*) alternatively, they may have been generated from an integrated segment. The former model requires that the free sequences have the capacity to replicate. The plasmid from which they descended did contain the pBR322 and SV40 origins. But, interestingly, SV40 DNA is undetectable in the retained material. It is also possible that a mouse origin was acquired as a result of interaction with the host genome.

It is more likely that the free sequences were generated from integrated material. Generation of free circular DNA from transformed cultured cells has been observed previously (23). Cells infected with viruses can also generate free DNA from the integrated viral genome (24). In addition, cells transformed in calcium phosphate-mediated gene transfer experiments can pass through an unstable phase during which the donated material is maintained independent of the host genome as high molecular weight "transgenomes" (25). An important characteristic of these independent transgenomes is their rapid loss from recipient cells; as many as 10% of the cells may lose the transforming sequences per day (25). The rearrangement of the donor material in no. 48 appears analogous to transgenome formation in cultured cells. If the unintegrated sequences were similar to independent transgenomes, we would expect them to be rapidly lost from the mouse cells during development and not detectable in the newborn. The marked intensity of the two bands in no. 48 rather suggests that they were continuously being produced from an integrated sequence. The presence of a high molecular weight band after digestion with *Bam*HI is also consistent with the integration model. This band may represent material from which the two smaller bands were generated.

In mouse no. 73, no free sequences were present. Both the undigested and *Xba* I-digested samples gave single bands of greater size than the highest molecular weight standard. Moreover, SV40 and TK sequences were retained in this animal. The patterns of bands present in mouse no. 73 is explained best

by plasmid integration into the mouse genome at a site within the TK region. In this model, digestion of the mouse DNA with *Bam*HI would generate three plasmid-derived fragments, two of which would consist of the TK fragment (now at both ends of the integrated molecule) linked to mouse DNA. The third fragment would be cleaved from within the integrated plasmid and would contain the SV40 and pBR322 moieties. The predicted size of this internal fragment is 5.5 kb. This model also predicts that the TK fragment would be disrupted and that the SV40 and pBR322 sequences would be intact. The DNA of mouse no. 73 contained two bands of 3.4 and 3.9 kb that hybridized only with the purified TK fragment and contained no sequences homologous to SV40 or pBR322, and a band of 7.8 kb that hybridized to SV40 and not to TK. The large size of this fragment relative to the expected 5.5-kb fragment might be due to partial internal duplication, which is consistent with independent observations of SV40 integration (26, 27). Digestion of the DNA of mouse no. 73 with *Bgl* I or with *Pvu* II failed to generate expected fragments from within the TK insert but indicated that most or all of pBR322 and SV40 were present. Additionally, *Hin*dIII digestion generated a band of the expected size of the SV40 insert, indicating that all of the SV40 sequences present on pST6 were also present in the DNA of mouse no. 73 (data not shown). Thus, our observations are consistent with a single integration event.

An important similarity between the two positive mice was the extensive rearrangement of the sequences. In the first instance, SV40 and herpes virus TK sequences were largely if not entirely removed from the injected DNA. In the second case, SV40 sequences and herpes virus TK sequences were demonstrable, but the TK gene was significantly rearranged. These observations raise the possibility that selection occurred against embryos that retained the TK gene intact and in an active state. The possibility that herpes virus TK is teratogenic to mouse embryos is consistent with our data. We consider this notion unlikely, however, because cells transformed in culture and under selection for TK demonstrate similar patterns of rearrangement (25, 28).

These initial results show that genetic transformation can be extended to whole mammalian organisms at a very early stage in their development. Further refinement of these techniques should lead to a reliable system of embryo transformation with its attendant applications for investigation of problems in development and cell differentiation.

Note Added in Proof. We have produced a third transformant by injection of 30,000 copies per cell of the plasmid pST9. Restriction analysis indicates that, as in mouse no. 73, the transforming sequences are integrated. Initial studies also indicate that at least one complete copy each of both the herpes virus TK and SV40 regions has been retained in this animal.

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