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Integration and Stable Germ Line Transmission of Genes Injected into Mouse Pronuclei

Abstract. *Genetic material has been successfully transferred into the genomes of newborn mice by injection of that material into pronuclei of fertilized eggs. Initial results indicated two patterns of processing the injected DNA: one in which the material was not integrated into the host genome, and another in which the injected genes became associated with high molecular weight DNA. These patterns are maintained through further development to adulthood. The evidence presented indicates the covalent association of injected DNA with host sequences, and transmission of such linked sequences in a Mendelian distribution to two succeeding generations of progeny.*

The successful introduction of exogenous DNA into cultured mammalian cells (*1–4*) has led to the development of a novel gene transfer system that has yielded new information about gene regulation in higher eukaryotes. One difficulty with this system is that cultured cells are not capable of organismal development and differentiation. DNA sequences cloned by recombinant DNA technology can be microinjected into the pronuclei of fertilized mouse oocytes and can be subsequently located in the DNA of newborn mice (*5*). This system allows the study of transferred gene sequences in the context of normal embryonic development. Since development is a process that includes maturation to adulthood, reproduction, and senescence, it is important to examine the fate of transferred genes beyond the point of birth. We have now followed this injected material through further stages of mouse development.

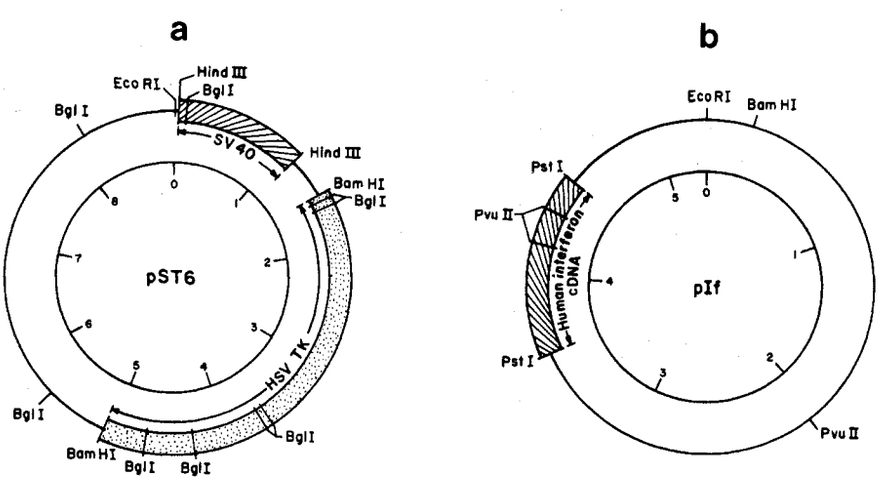

Fig. 1. Simplified diagrams of recombinant plasmids used for microinjection of mouse embryos. The Eco RI site of pBR322 is marked at 0 kb for reference.

Two recombinant plasmids were used for microinjection. The first, designated pST6 (*5*), was composed of the Hind III C restriction endonuclease fragment of SV40 virus and the herpes virus thymidine kinase (TK) genes cloned in plasmid pBR322; the second, pIf (*6*), contained human leukocyte interferon complementary DNA (cDNA) also cloned in pBR322 (*7*). A simplified diagram of each plasmid with its relevant restriction sites is shown in Fig. 1. Between 1000 and 35,000 copies of each plasmid were injected into each zygote. All microinjections were carried out as described (*5*).

The feasibility of producing such genetically transformed mice, which we call "transgenic" mice, depends upon several factors. Our experience has been that higher copy number gives a higher rate of transformation, but that the viscosity of concentrated preparations increases embryo mortality at the time of injection. Injection of 1000 copies of pST6 gave a survival rate of 50 to 70 percent with a third of the survivors eventually giving rise to live young. About 1 in 30 of such young retained transferred genes (*5*). When 30,000 copies of this plasmid were injected, embryo survival was reduced to 30 to 50 percent, but 1 in 15 mice retained the sequences. The pIf plasmid is smaller than pST6 and was therefore more easily injected. Survival of microinjection of 10,000 copies of this plasmid varied between 50 and 75 percent. Ten mice were born from 33 embryos thus far implanted, a rate which compares well with survival rates of embryos injected with pST6 (*5*). Of these ten mice, one was transgenic. This rate appears higher than that obtained from pST6 injections, but statistically significant numbers allowing a rigorous comparison of these experiments are not yet available.

Southern blot hybridization has been used to evaluate plasmid sequences in newborn and adult mice (*5, 8–10*). In the case of adults, DNA was extracted from spleens. Whether or not the donor material was integrated into the host genome was assessed by three criteria: (i) the acquisition of resriction sites in the host genome but not in the recombinant plasmids, (ii) the mobility of plasmid sequences in agarose gels when the DNA applied to the gels was undigested, and (iii) the ability of the plasmid sequences to be transmitted through the germ line to succeeding generations.

Two mice (73 and 9.02) injected with either pST6 or pST9 (pST9 is identical to pST6 except that the orientation of the SV40 insert is reversed) and one mouse

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(If-4) injected with pIf retained plasmid sequences whose restriction patterns were consistent with integration. When undigested, the DNA of all three mice gave single bands of high molecular weight upon filter hybridization. This result suggests an association of the plasmid sequences with high molecular weight DNA. When cut with the restriction enzymes Xba I and Xho I, which do not recognize sites in either recombinant plasmid, DNA from mice 9.02 and 73 again gave single high molecular weight bands. The mobility of these bands could not be distinguished from each other or from that of the band produced by undigested DNA. Digests with enzymes that excise internal fragments of pST6 and pST9 showed no evidence of concatamerization of the plasmid in DNA from mice 73 and 9.02. These high molecular weight bands were thus suggestive of integration. When digested with Xba I, DNA from mouse If-4 yielded a single band of 13.5 kilobases (kb), a much larger size than the original 5.2-kb plasmid. These patterns are again consistent with integration into the host genome at a single site. However, these results do not conclusively demonstrate covalent association of the plasmid with the host DNA.

Double digests of the DNA from mice 9.02 and 73 provided additional evidence for integration. The first digest, with Bam HI, was followed by digests with Xba I or Xho I (Fig. 2). The 7.8-kb Bam HI band in mouse 73 was converted to 5.6 kb by Xba I. Similarly, the 18-kb band in mouse 9.02 was reduced to 15 kb by Xba I. These alterations in mobility indicate the acquisition of Xba I sites, a result consistent with integration.

Similar results permitted the same conclusion regarding the state of pIf sequences in the spleen DNA of animal If-4. A partial digest with Xba I yielded several high molecular weight bands after hybridization with the pIf probe (Fig. 3c). The smallest of these bands, 13.5 kb, was the only band produced by a complete digest with Xba I. The latter pattern obtained by partial digestion indicates linkage of the plasmid to DNA sequences containing multiple Xba I sites. The 13.5-kb band was generated by cutting at the Xba I sites closest to the point of attachment of the plasmid, whereas the larger fragments were produced when one or both of these closest sites was not digested, but more distant sites were cleaved. These results thus demonstrate for all three mice that plasmid DNA had become ligated to host genomic sequences, but they do not conclusively demonstrate integration into a host chromosome. A satisfactory test of this possibility is breeding of the transformed adult. Integration into a single chromosomal homolog should result in Mendelian transmission of the plasmid sequence as a heterozygous marker. Since mice 73 and 9.02 were killed at birth, only mouse If-4 could be subjected to progeny testing.

We applied this test to the adult mouse If-4, and the results obtained were consistent with chromosomal integration of the injected plasmid. This mouse was

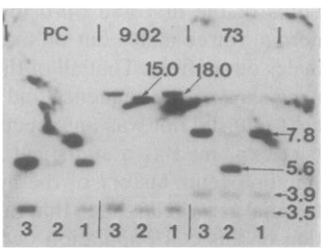

Fig. 2. Digestion of DNA from mice 73 and 9.02 with Bam HI (lane 1), Bam HI plus Xba I (lane 2), and Bam HI plus Xho I (lane 3). This experiment was performed because the single bands generated by Xba I and Xho I alone were so large that their mobilities could not be distinguished from each other or from the band produced by undigested DNA. Thus, although these large bands were consistent with integration, they did not rule out the possibility that the plasmid DNA existed as a large independent piece without sites for Xba I or Xho I. Cutting first with Bam HI produced smaller bands whose mobilities were altered to a greater degree by a given change in size. The association of the plasmid with DNA containing Xba I sites was therefore more readily demonstrated when Bam HI digestion was performed prior to Xba I. Clearly Xba I alters the Bam HI pattern in both mice. Although the Xba I digest did not proceed to completion in mouse 73, almost all of the 7.8-kb Bam HI band was converted to 5.6 kb by Xba I. The 18-kb Bam HI band in mouse 9.02 was altered to 15 kb by Xba I. Thus, at least 2.2 kb of genomic DNA was ligated to plasmid sequences in mouse 73, and 3 kb of genomic material was ligated to plasmid DNA of mouse 9.02. As expected, dougle digests of the positive control (*PC*) did not alter the Bam HI pattern. Failure of Xho I to alter the mobilities of the Bam HI fragments indicates that the genomic portions of these fragments did not contain Xho I sites. Positive controls in these and all other experiments were formulated by adding purified plasmid to DNA from uninjected mice at a ratio of 1 to 10^6, by weight.

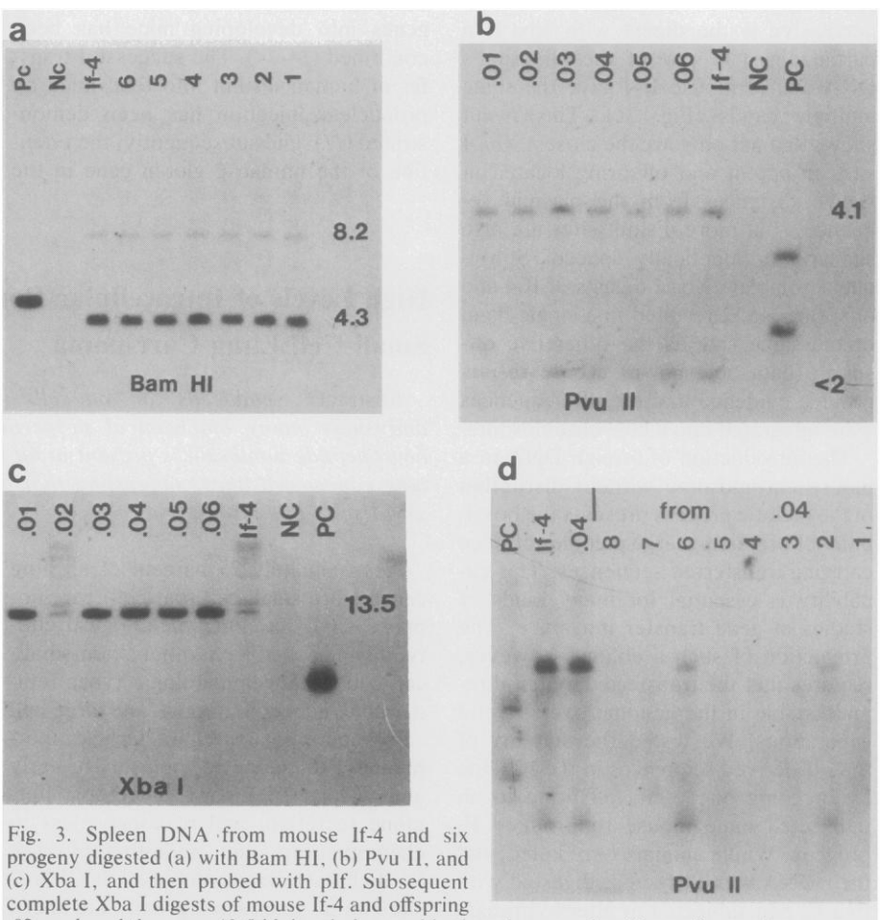

Fig. 3. Spleen DNA from mouse If-4 and six progeny digested (a) with Bam HI, (b) Pvu II, and (c) Xba I, and then probed with pIf. Subsequent complete Xba I digests of mouse If-4 and offspring .02 produced the same 13.5-kb band observed in the other samples. These digests demonstrate germ line transmission of pIf sequences. (d) Pvu II digest of spleen DNA from mouse If-4, its offspring mouse .04 and eight progeny of .04. Of these eight second-generation progeny mice, 2 and 6 show inheritance of pIf sequences; *PC* indicates the positive control; *NC* a negative control.

crossed to an uninjected male. Nine of 15 progeny from three litters thus far tested have inherited the pIf-derived sequences. The numbers of offspring with these sequences in each litter were six of six, zero of four, and three of five. The second litter was killed at birth and therefore the sex of the litter members was not examined; however, the sex ratios of the first and third litters were normal (three males out of six, and two males out of five). That all of the first six mice showed the sequence and all of the next four did not was unexpected, but is best explained as a statistical anomaly. The ontogenic history of the mouse primordial germ cell is such that randomization of this cell population occurs prior to entry into genital ridge. At present, there is no evidence that suggests a special relation between oocytes ovulated during any particular estrous cycle.

Consistent with the notion that the sequences were inherited is the observation that the restriction patterns of the DNA from the offspring were indistinguishable from those of the parent. Digestion with Bam HI, Pvu II, or Xba I gave identical patterns in parent and offspring (Fig. 3, a to c). Particularly persuasive is the digest with Xba I; a partial digest of one of the offspring's DNA and of mouse If-4 gave the same multiple bands (Fig. 3c). This result shows that not only are the closest Xba I sites in parent and offspring located at similar distances from the plasmid sequences, but more distant sites are also similarly or identically spaced. Subsequent complete Xba I digests of If-4 and offspring No. 2 resulted in a single band of the same size as the other five offspring (data not shown). These results provide evidence that the pIf sequences were integrated into a host chromosome.

The introduction of foreign DNA in a mouse chromosome without disruption of the meiotic process presents the possibility of producing large colonies of mice carrying transferred sequences. This capability is essential for many kinds of studies of gene transfer into mice. The production of such a colony, however, requires that the transferred material remain stable in the genome over several generations. We tested the stability of the pIf-derived sequences in the If-4 line by breeding one of its offspring to an uninjected male mouse to produce F_2 progeny. Whole animals were killed; and their DNA was extracted, digested with Pvu II, and subjected to filter hybridization with pIf as the probe. Two of the first eight offspring produced by one of the F_1 mice showed clear homology to the probe, with a restriction pattern indistinguishable from the F_1 parent or from the original transformed mouse, If-4 (Fig. 3d). This second generation of germ line transmission constitutes evidence for the stability of the transferred material.

The integration of plasmid sequences and their transmission to offspring means, for example, that mice can be backcrossed to produce homozygotes for the transferred sequences, making possible the study of crossover events within a DNA segment whose sequence is well defined, and facilitating mapping studies by both Mendelian and somatic cell genetic approaches. Sequences present in small organs can be studied by pooling tissue from many animals. Breeding tests can also be used to determine whether genes transferred into mice are integrated randomly or reproducibly into a specific site. This issue is of importance if attempts at gene replacement are to be made.

Our data, as well as those from several other laboratories, indicate promise for the technique of pronuclear injection for studying gene action during mammalian development. Our initial report that such injections could succeed in transferring genes into developing mice has been confirmed (*11–14*). The successful transfer of human insulin into fetal mice by pronuclear injection has been demonstrated (*11*); and subsequently, the retention of the human β-globin gene in the DNA of fetal mice was described (*12*). Supporting evidence for germ line transmission of transferred genes has also been gathered (*13, 14*), and expression of genes injected into the pronucleus has been observed at late fetal stages and in adult mice (*12, 14*).

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High Levels of Intracellular Bombesin Characterize Human Small-Cell Lung Carcinoma

Abstract. *"Small cells" or "oat cells" characterize a virulent form of lung cancer and share many biochemical properties with peptide-secreting neurones. The neuropeptide bombesin is present in all small-cell lines examined, but not in other lung cancer cell lines, suggesting that bombesinergic precursor cells in lung may give rise to this disease.*

Approximately 25 percent of all lung cancers are small-cell (oat cell) carcinomas (SCCL), a clinicopathological entity, distinguished from other "non-small-cell" lung cancer histologic types (epidermoid, adenocarcinoma, and large-cell carcinoma) by its characteristic morphology, tendency to metastasize early and widely, frequency of ectopic hormone secretion, and responsiveness to chemotherapy and radiotherapy (*1*). Well-characterized, clonable SCCL tissue culture lines have greatly advanced our knowledge of the biology of SCCL (*2*). These SCCL lines are distinguished from those of the other lung cancer types by the presence of neurosecretory granules, frequent polypeptide hormone secretion, high levels of L-dopa decarboxylase, high levels of the isoenzyme of creatine kinase found in brain, and neuron-specific enolase, as well as a lack of substrate adhesion and characteristic growth factor requirements (*2–4*). Amine precursor uptake and decarboxylating (APUD) cells consist of a widely distributed network of neuroendocrine cells programmed to secrete certain amines and polypeptide hormones (*5*); SCCL and the more benign pulmonary carcinoids are presumed to arise from normal APUD cells in the respiratory tract (*6*). We now report that the 17 SCCL culture lines tested have high quantities of intra-

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