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Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs

(transgenic mice/pronuclear injection/germ line transformation/recombination)

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ABSTRACT **Microinjection of foreign DNA into fertilized mammalian eggs is a convenient means of introducing genes into the germ line. Some of the more important parameters that influence successful integration of foreign DNA into mouse chromosomes are described. The effects of DNA concentration, size, and form (supercoiled vs. linear with a variety of different ends) are considered as well as the site of injection (male pronucleus, female pronucleus, or cytoplasm) and buffer composition. The optimal conditions for integration entail injection of a few hundred linear molecules into the male pronucleus of fertilized one-cell eggs. Under these conditions about 25% of the mice that develop inherit one or more copies of the microinjected DNA. The overall efficiency also depends on the choice of mouse strains; for example, generating transgenic mice that express foreign growth hormone genes is about eight times easier with C57/BL6 × SJL hybrid mice than with inbred C57/BL6 mice.**

A number of investigators have successfully introduced foreign DNA into somatic tissues and the germ line of mice by microinjecting DNA into fertilized eggs (1–6). However, the factors that determine success with this technique are poorly defined. Therefore, we explored some of the parameters that influence stable integration of foreign DNA into chromosomes of mouse eggs. For most of these studies we have been concerned only with the efficiency of DNA integration rather than expression of the foreign DNA; therefore, we often analyzed DNA from 13- to 19-day fetuses—an age at which each fetus has enough DNA to make DNA analyses easy. Considering that normally only a few hundred molecules of DNA are injected but that detection of DNA by standard techniques requires about a million copies, foreign DNA would not be detected unless it were amplified during embryogenesis. Though replication of injected DNA is a formal possibility and does occur in *Xenopus* and sea urchin eggs (7, 8), we have not observed extrachromosomal replication of plasmid DNA in mouse eggs. Therefore, detection of foreign DNA in fetuses is assumed to represent chromosomal integration and amplification by cell division.

If integration occurs at the one-cell stage, then every cell of the fetus and placenta should contain the foreign DNA. However, if integration occurs after cleavage begins, then a blastocyst would be mosaic and the foreign DNA might be found only in the fetus or only in the placenta or in both (unpublished observations). When more than one copy of the foreign DNA integrates, they are generally all at a single chromosomal site as a tandem, head-to-tail array (7–9); however, examples of multiple integration sites have also been observed (10). Our experience is that mosaic mice and mice with more than one integration site each represent 10–20% of nearly a hundred transgenic mice that we have analyzed by outbreeding. For most of the analyses described here, we do not distinguish mosaics and multiple integration sites because they are most easily detected by breeding experiments (unpublished data).

In the analysis described here, we consider the influence of the number of DNA molecules injected, the form and size of the DNA, injection buffer, and the site of injection on the frequency of integration. Overall efficiency entails more than achieving a high percentage of positive fetuses. It must also include factors such as ease of obtaining fertilized eggs, ease of microinjection, survival of the eggs after microinjection, ability of the eggs to continue development after microinjection, and ability of the eggs to continue development after transfer to pseudopregnant recipients. These factors become important when considering adapting these conditions to other strains of mice or species.

METHODS

Preparation of DNA for Microinjection. All of the genes were propagated as plasmids or cosmids in *Escherichia coli*. Supercoiled molecules were isolated from bacterial cultures by standard techniques involving either lysozyme/Triton X-100 lysis or lysozyme/alkaline lysis followed by banding the supercoiled plasmids on ethidium bromine/CsCl gradients (11). Linear DNA molecules were prepared by digestion with restriction enzymes; after digestion the DNA was extracted with $NaDodSO_4$/phenol/chloroform, precipitated with ethanol, washed thoroughly with ethanol/salt, and then dissolved in TE buffer (10 mM Tris·HCl/0.25 mM EDTA, pH 7.5). When more than one fragment was generated, the fragments were generally separated by agarose gel electrophoresis and visualized by UV after staining with ethidium bromide and recovered by binding them to glass fiber filters in the presence of $NaClO_4$ followed by elution in 1 mM Tris·HCl/0.1 mM EDTA, pH 7.5 (12). The DNA concentration was determined by measuring the fluorescence of bisbenzimide H33258 (13) with a Perkin Elmer model 520-10M fluorimeter; calf thymus DNA was used as a standard. DNA samples were stored at 4°C and diluted in TE buffer for most microinjection experiments.

Egg Manipulation and Microinjection. For a typical experiment, about 30 C57/BL6 × SJL hybrid females were hormonally superovulated (14) and mated with C57 × SJL hybrid males. For some experiments eggs and sperm were from C57/BL6 inbred mice. The next morning, fertilized one-cell eggs were flushed from the oviduct with modified Brinster's medium (14) containing 0.1 mM EDTA (15). Cumulus cells were removed from the eggs with hyaluroni-

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Abbreviation: kb, kilobase(s).

dase (300 units/ml); then the eggs were washed free of debris and enzyme. For injection, the eggs were transferred to a depression slide in Brinster's medium modified by substitution of 25 mM Hepes buffer (pH 7.4) for the bicarbonate and inclusion of cytochalasin B (5 μg/ml). The medium was overlaid with silicone oil [Dow Corning 200 Fluid, 50 centistokes (1 centistoke = 10^{-6} m^2/sec)]. Eggs were sequentially held in place by a blunt pipet (outside diameter, $\approx$30 μm) while the tip of the injector pipet was inserted through the zona pellucida and vitellus and into one of the pronuclei. The DNA solution in the injector pipet was slowly discharged ($\approx$2 pl) by using a 100-μl Hamilton syringe connected to a micrometer. The injector pipet was filled with silicone oil except for the DNA solution. After injection the eggs were washed free of cytochalasin B and transferred to the oviducts of pseudopregnant, random-bred Swiss mice (16). A convenient assay for successful pronuclear microinjection is to inject a plasmid containing a transcriptionally active gene and assay its expression after incubation for 16–24 hr. We have successfully used the thymidine kinase gene from herpes simplex virus (17) as well as metallothionein–thymidine kinase and metallothionein–β-galactosidase fusion genes for this purpose (18). Activity assays for these gene products are sensitive enough to detect expression from a few hundred gene copies injected into a single egg. Toxicity studies were conducted by incubating the eggs for 4 or 5 days in Brinster's medium and counting the number that develop to the morula and blastocyst stage.

DNA Analysis. For routine detection of mice that had integrated the injected DNA, fetal or tail tissue (50–100 mg) was homogenized with a Tissumizer (Tekmar, Cincinnati, OH) in 4 ml of SET buffer (1% $NaDodSO_4$/10 mM Tris·HCl/5 mM EDTA, pH 7.5) containing 100 μg of proteinase K per ml, incubated for 2 hr at 37°C, and then incubated at ambient temperature for another 24 hr. Then 0.1 ml of 5 M NaCl was added, the sample was centrifuged for 10 min at 4000 × g, and an aliquot (0.4 ml) was mixed with 0.8 ml of ethanol and incubated overnight at −20°C. The nucleic acids were collected by centrifugation for 1 min at 15,000 × g and dissolved in 15 μl of 2 M NaCl/0.1 M NaOH by heating in a boiling water bath for 1–2 min. The sample was centrifuged briefly and mixed gently in a Vortex, and 5 μl was spotted directly onto nitrocellulose (Sartorius). The pipet tip was rinsed with ethanol prior to picking up the sample to facilitate sample dispersion on the nitrocellulose. After air drying, the nitrocellulose was washed briefly in 0.30 M NaCl/0.03 M sodium citrate, pH 7, and baked for 2 hr at 80°C. The filters were hybridized with nick-translated probes, washed, and exposed to x-ray film as described (9). This procedure results in about 2–5 μg of DNA being spotted on the nitrocellulose, which is sufficient to detect single-copy inserts with an overnight exposure. For quantitation, nucleic acids were isolated (19); then known amounts of DNA were spotted along with plasmid standards and hybridized as above.

RESULTS

Effects of DNA Concentration and Injection Buffer on Integration Efficiency. Table 1, experiment A, shows the effect of varying the number of linear plasmid molecules microinjected into the male pronucleus (the larger of the two pronuclei) on egg survival and integration frequency. As might be expected, injection of large amounts of DNA was toxic; this is demonstrated by a decrease in the number of eggs that developed to the morula/blastocyst stage in culture and by a lower recovery of fetuses after transfer of injected eggs to foster mothers. However, the integration frequency improved as the DNA concentration increased. In this series of experiments, the overall efficiency of obtaining fetuses that had integrated the foreign DNA was best (5.9%) when the DNA concentration was 1 ng/μl (Table 1, experiment A). In another series of experiments, we compared the integration frequency with a variety of different DNA fragments after injection at two different DNA concentrations. The integration frequency almost doubled in the range between 0.5 and 1.0 ng/μl but was constant between 1.0 and 2.0 ng/μl (Table 1, experiments B and C). Thus, for most experiments, the DNA was diluted to 1–2 ng/μl, which results in a few hundred copies of the foreign DNA being injected per egg.

The usual injection buffer contained 10 mM Tris·HCl and 0.25 mM EDTA (pH 7.5). We varied the concentrations of EDTA and $MgCl_2$ in the injection buffer as shown in Table 2. The integration frequency was good, averaging 38%, with up to 1 mM EDTA or $MgCl_2$, but fell to zero with either 5 mM EDTA or 3 mM $MgCl_2$. The overall efficiency was optimal with 0.1–0.3 mM EDTA.

Table 1. Effect of DNA concentration on embryo survival and integration frequency

DNA		Survival (%)		Integration efficiency (%)‡	Overall efficiency (%)
ng/μl	Copies per eggs	m + b/eggs*	Fetuses/eggs†		
		Experiment A			
No injection		61/61 (100)	44/60 (73)		
Buffer only		55/76 (72)	28/100 (28)		
0.01	5	112/139 (81)	48/209 (23)	2/48 (4.2)	2/209 (1.0)
0.1	53	123/152 (81)	34/175 (19)	6/34 (17)	6/175 (3.4)
1	530	115/138 (83)	33/170 (19)	10/33 (30)	10/170 (5.9)
10	5,300	72/161 (45)	10/104 (10)	2/10 (20)	2/104 (1.9)
45	23,800	40/88 (45)	8/96 (8.3)	3/8 (37)	3/96 (3.1)
		Experiment B			
0.5	50–160		251/1146 (22)	43/251 (17)	43/1146 (3.7)
1.0	100–320		203/1058 (19)	63/203 (31)	63/1058 (6.0)
		Experiment C			
1.0	40–430		143/662 (22)	36/143 (25)	36/662 (5.4)
2.0	80–860		142/675 (21)	40/142 (28)	40/675 (5.9)

In experiment A, a 3.5-kilobase (kb) linear DNA fragment with dissimilar ends was used; in experiment B, 13 separate experiments with different linear DNA molecules are included; in experiment C, 9 separate experiments with different linear DNA molecules are included. In experiments B and C, each DNA preparation was injected at two different concentrations.

*Fraction of eggs that developed to morula/blastocyst (m + b) stage after 4 days in culture.

†Number of fetuses that developed from injected eggs.

‡Fraction of fetuses that retained injected DNA.

Table 2. Effect of buffer composition on integration frequency

Buffer addition*	Survival (%)†	Integration frequency (%)‡	Overall efficiency (%)
3.0 mM $MgCl_2$	0/60 (0)	0/0 (0)	0/60 (0)
1.0 mM $MgCl_2$	21/140 (15)	7/21 (33)	7/140 (5.0)
None	31/263 (12)	4/31 (13)	4/263 (1.5)
0.1 mM EDTA	61/262 (23)	25/61 (42)	25/262 (10)
0.3 mM EDTA	31/140 (22)	13/31 (42)	13/140 (9.3)
1.0 mM EDTA	24/256 (9.4)	14/24 (58)	14/256 (5.5)
5.0 mM EDTA	0/39 (0)	0/0 (0)	0/39 (0)

*10 mM Tris·HCl (pH 7.5).
†Fraction of injected and transferred eggs that developed into fetuses.
‡Fraction of fetuses that retained injected DNA. The DNA sample was the same as used in Table 1 experiment A; about 740 copies were injected per egg.

Comparison of Nuclear and Cytoplasmic Injection. The previous results indicate that injection of linear DNA molecules into the male pronucleus of one-cell eggs gives rise to an integration frequency of 25–30% under optimal conditions. Considering that cytoplasmic injection is easier and less detrimental to survival than nuclear injection, we injected either linear or supercoiled plasmids into the cytoplasm of one-cell eggs. Table 3 shows that neither approach was very successful. Of 224 fetuses examined after cytoplasmic injection, only 2 were positive. We also assessed the feasibility of injecting both nuclei of two-cell eggs. There does not appear to be any advantage of injecting DNA at this later stage (Table 3), as both the survival and integration frequency were lower than when one-cell eggs were injected.

Effect of Plasmid Form on Integration Frequency. The efficiency of integration of DNA molecules microinjected into either the male or female pronucleus is shown in Table 4. The DNA molecules were either supercoiled or cut with restriction enzymes to yield molecules with "sticky" ends (*Eco*RI), blunt ends (*Eco*RI ends filled in with deoxynucleotides using the large fragment of DNA polymerase I), or dissimilar ends (*Eco*RI and *Kpn* I). The plasmids in this series were identical except for their ends. The results indicate that linear molecules with similar or different ends gave an overall integration efficiency of 24–31% compared to about 8% for blunt-ended linear or supercoiled molecules. The data in Table 4 suggest that DNA integration following injection into the male pronucleus may be slightly more efficient than injection into the female pronucleus.

We also measured the integration frequency with a variety of different DNA constructs. Table 5 summarizes the results. In this larger set of data, it is also clear that linear molecules with either similar or dissimilar, staggered ends integrated with similar efficiency (≈25%), whereas integration of supercoiled DNA was less efficient by a factor of 5. There was no apparent relationship between DNA length (ranging from 0.7 to 50 kb) and integration frequency.

Table 4. Effect of DNA form and ends on integration frequency

DNA*	Integration frequency (%)†		
	Injection into male pronucleus	Injection into female pronucleus	Total
Supercoiled	0/30 (0)	4/22 (18)	4/52 (7.7)
Linear			
Blunt ends	5/38 (13)	0/21 (0)	5/59 (8.5)
Similar ends	3/15 (20)	2/6 (33)	5/21 (24)
Different ends	11/29 (38)	2/13 (15)	13/42 (31)
Total	19/112 (17)	8/62 (13)	

*In each case, about 375 copies of a 4.8-kb DNA were microinjected. The plasmid was either supercoiled, cut with *Eco*RI and the ends filled in using Klenow fragment of DNA polymerase I (blunt ends), cut with *Eco*RI (similar ends), or cut with *Eco*RI and *Kpn* I (different ends).
†Number of fetuses that retained the microinjected DNA.

We also tried injecting linear plasmids cut within the mouse metallothionein-I promoter region and subsequently treated with exonuclease III to generate 400- to 600-nucleotide single stranded ends. In some cases these molecules were coinjected with rec A and single-strand binding proteins in an attempt to achieve homologous integration (20). The overall integration efficiency was 17.6% (68/368) but Southern blot analysis revealed no evidence for homologous integration into the endogenous metallothionein-I gene (data not shown).

Comparison of Using Eggs from C57 Inbred Mice and C57 × SJL Hybrid Mice. For some experiments it may be important to introduce genes into a defined genetic background; hence, we compared the efficiency of transferring genes into eggs of inbred or hybrid mice. Table 6 shows that nearly every aspect of working with inbred C57 mice was less efficient than working with hybrids. First, the number of eggs obtained per donor was lower (2.8 eggs per donor compared to 11.4); second, the percentage of the eggs that survived injection and developed into pups was lower (7.5% compared to 11.8%); and finally, the percentage of mice that retained the microinjected DNA was lower with the inbred mice (19.5% vs. 35.1%). Of the mice that were positive for the metallothionein growth hormone fusion genes (21, 22), 80% of the inbred mice and 59% of the hybrid mice expressed these genes. The overall efficiency, calculated as percentage of expressor mice that developed per female donor, was 3.3% for the inbred C57 mice compared to 27.1% for the hybrid mice. This 8-fold difference approximates the difference in work involved in using this inbred line compared to hybrids.

DISCUSSION

This paper describes our attempts to identify parameters that are critical for successful gene transfer into the germ line of mice by microinjection of foreign DNA into fertilized eggs.

Table 3. Integration of DNA following injection into the cytoplasm of one-cell eggs or the nuclei of two-cell eggs

Injection site	DNA, kb*	Dose†	Survival (%)‡	Integration frequency (%)§
Cytoplasm	4.8 (D)	400	58/167 (35)	2/58 (3.4)
Cytoplasm	5.6 (D)	3000	60/119 (50)	0/60 (0)
Cytoplasm	9.1 (S)	1–20 × 10^3	106/604 (17)	0/106 (0)
Two-cell nuclei	3.5 (D)	241	14/149 (9.4)	2/14 (14)

*DNA samples were either supercoiled (S) or linear with dissimilar staggered ends (D).
†Approximate number of DNA molecules injected per egg.
‡Number of fetuses that developed divided by number of injected eggs transferred.
§Fraction of the fetuses that retained the injected DNA.

Table 5. Integration frequency of a variety of linear and supercoiled DNA molecules

DNA*	Number of experiments†	Size, kb	Integration frequency (%)‡
Linear (S)	24	4.8–50.0	239/941 (25.4)
Linear (D)	14	0.7–30.0	139/543 (25.5)
Supercoiled	8	4.3–14.0	24/458 (5.2)

*DNA molecules were either supercoiled, linearized with one enzyme to leave similar staggered ends (S), or linearized with two enzymes to leave dissimilar staggered ends (D).
†A different DNA construct was used for each experiment. The DNA concentrations for injection were all between 1 and 2 ng/μl.
‡Fraction of fetuses or mice that retained the injected DNA.

The data substantiate the effectiveness of injecting DNA into the male pronucleus of one-cell eggs, although injection into the smaller, female pronucleus is nearly as efficient (Table 4). However, the efficiency of integration following cytoplasmic injection is very low.

The basic technique involves impaling the pronucleus with a glass needle (≈2 μm outside diameter and 1.3 μm inside diameter) and then forcing buffer containing DNA into the pronucleus. Swelling of the pronucleus (to about twice normal volume) in response to the pressure is an essential sign of successful penetration. It is not unusual for some of the pronuclei to burst in response to injection. The volume of injection fluid remaining in the pronucleus (≈2 pl) is estimated from the increase in pronuclear volume after the needle is withdrawn. Low concentrations of either $MgCl_2$ or EDTA could be included in the injection buffer, but higher concentrations were toxic (Table 2).

The two most critical parameters affecting DNA integration are the concentration and form of the DNA. The data in Table 1 indicate that the integration efficiency improved as the DNA concentration in the injection fluid increased to ≈1 ng/μl (equivalent to several hundred copies of DNA injected into the pronucleus). Poor survival was observed with higher DNA concentrations and the integration frequency did not increase in those fetuses that did survive. These observations contrast with those obtained in tissue culture cells, in which stable integration frequency reaches a plateau with a few molecules of microinjected, linear DNA (23).

Table 6. Efficiency of producing transgenic mice with C57 vs. C57 × SJL hybrid eggs

Mice	C57		C57 × SJL*	
	Exp. A	Exp. B	Exp. A	Exp. B
Donor females†	181	180	36	49
Pregnant donors	65	59	24	27
Eggs collected	904	763	672	684
Eggs injected‡	788	675	600	601
Eggs transferred§	501	529	474	472
Eggs per donor¶	2.8	2.9	13.2	9.6
Pups born	39	38	62	49
Pups with growth hormone gene	4	11	17	22
Pups that express growth hormone gene	4	8	11	12
Pups with growth hormone gene/female donors	15/361 (4.2%)		39/85 (45.9%)	
Pups that express growth hormone gene/female donors	12/361 (3.3%)		23/85 (27.1%)	

In experiment A, 600 copies of a linear DNA containing the metallothionein rat growth hormone gene (21) were injected; in experiment B, 1100 copies of a linear DNA containing a metallothionein human growth hormone gene (22) were injected.
*F_2 hybrid mice.
†Females primed for superovulation.
‡Only those eggs that were fertilized and looked healthy were used.
§Eggs that survived microinjection.
¶Number of transferred eggs per donor female.

Our previous data suggested that integration of circular plasmids was as efficient as linear molecules (9). However, it is now clear from a much larger collection of data that integration of linear molecules is about five times more efficient than circular ones (Tables 4 and 5). The overall efficiency for integration of linear molecules (compiled from Tables 1–6) was 718/2775 or 25.8%. Linear DNA molecules with either similar or dissimilar staggered ends integrated with essentially the same efficiency and they may be superior to linear molecules with blunt ends, although the data base for blunt ends is not as extensive. Long single-stranded ends did not improve the integration frequency compared to those generated by common restriction enzymes. The size of the DNA molecules (ranging from 0.7 to 50 kb) does not seem to be an important parameter.

To gain insight into the events that occur prior to integration, we have analyzed the status of the injected DNA 24 hr after microinjection by Southern blot. For these experiments it was necessary to inject high concentrations of DNA (≈50,000 copies) and pool many eggs to obtain enough DNA for analysis; thus, the results may not accurately represent what occurs at the much lower concentrations used for stable integration. When supercoiled molecules were injected, the total amount of DNA did not change, suggesting that extrachromosomal replication did not occur, but there were some changes in the distribution of supercoiled, nicked-circular, and linear molecules during the 24-hr incubation (data not shown). When blunt-ended linear molecules were injected, a faster migrating species (presumably supercoiled) and a ladder of ligated or recombined oligomers were observed. Even when linear molecules with heterologous ends (e.g., *Eco*RI and *Kpn* I) were injected, a large fraction of the molecules migrated in the position of supercoiled molecules after 24 hr. An attempt to prevent circularization by injecting "endless" linear molecules with hairpin structures at their ends failed, as judged by the appearance of molecules migrating as supercoils after 24 hr of incubation (unpublished observations). Thus, mouse eggs have an abundance of enzyme activities that can modify the ends of linear DNA molecules and ligate them together.

The mechanism of DNA integration into mouse chromosomes following microinjection is unknown. Some of the pertinent observations are that (*i*) linear molecules integrate more efficiently than circular molecules, (*ii*) linear molecules are readily circularized, (*iii*) usually all of the molecules that integrate are on the same chromosome and at the same site (24), (*iv*) those integrated molecules are usually arranged in a tandem, head-to-tail array (5, 9), and (*v*) when two different linear DNA molecules are coinjected they usually integrate together (data not shown). As a working hypothesis, we assume that the ends of linear molecules are somehow involved in the integration process because linear DNA molecules integrate more efficiently than circular ones. Although linear molecules are readily circularized, staggered ends may retard the process. One possibility is that spontaneous breaks occur in chromosomes, possibly exacerbated by the microinjection technique, and these breaks are sites for integration of linear DNA by ligation reactions. Other molecules that may have circularized probably recombine with each other and with the integrated copies to generate a tandem array. Recombination among coinjected molecules is probably favored because of a high local concentration and special properties, such as absence of normal chromatin structure. The number of chromosomal breaks is presumably limiting, which would explain why the number of integration

sites is low (usually one) and why different DNA molecules are usually integrated into the same site. Introduction of DNA into eggs by iontophoresis has been reported to increase the number of unit length integrations (25).

Although variations in the basic techniques described here may increase the integration frequency, a radically different approach that allowed single-copy integration at a defined site within the introduced DNA would be a great improvement for many of the biological studies that are envisaged. A circular plasmid containing a *Drosophila* P element integrated with low efficiency and retained the vector sequences (data included in Table 5), indicating that this transposable element does not function in the mouse nucleus. However, the integration mechanisms of retroviruses or transposable elements might well be adapted to allow more control over the integration process.

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