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SHORT COMMUNICATION

Double pronuclei injection of DNA into zygotes increases yields of transgenic mouse lines

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Transgenic mice are increasingly used for gene function and regulation studies of mammalian genes. A major limitation is the necessity to produce a large number of founder animals to obtain one line with the desired expression pattern. We developed a method, the 'double pronuclei injection', that doubles the yield of transgenic mouse lines obtained from each injection session, thereby reducing the time, effort and costs of generating transgenic mice. Three transgenic vectors were microinjected into the male and female pronuclei of zygotes. Approximately half of the resulting born mice were transgenic. This represented a 60% increase in the yield of founders per injected zygote, and a 100% increase in the yield of transgenic mice per born animal, when compared to yields obtained using single pronucleus injection. This method should prove useful for generating large numbers of transgenic mice for gene regulation studies and for conditional gene ablation.

Keywords: transgenic; mouse; Cre recombinase; pronucleus; microinjection

Introduction

The use of transgenic mice has become a routine component of gene function and regulation studies in mammalian systems. They are used to analyse *cis*-acting regulatory elements of genes, for functional studies through the expression of dominant-negative products (Hermiston and Gordon, 1996) and more recently, in combination with the targeted gene ablation (knockout) technology, for providing spatiotemporal control of somatic mutations in mice (Furth *et al*., 1994; Kuhn *et al*., 1995).

A limiting factor in using transgenic mouse technology is that it requires the screening of several mouse lines to obtain the desired transgene expression pattern. A fraction, sometimes only one in ten, of transgenic mouse lines expresses the transgene. From these expressing lines, many express the transgene ectopically, or in a mosaic manner, or at inappropriate levels. We present here a method that doubles the yields of transgenic mouse lines over commonly used methods. This method is based on the alignment of the male and female pronuclei of the zygote with the holding and injecting pipette and consecutive introduction of DNA in both pronuclei. The procedure does not introduce major delays in the injection steps. The survival of zygotes is comparable to that using single pronucleus injection.

Materials and methods

Construction of transgenic vectors

MLCcre and MLCcreI2. The MLCcre and MLCcreI2 transgenic vectors contain the Cre recombinase cDNA under the control of the myosin light chain-II ventricular (MLCIIv) promoter, without or with an intron from the β-globin gene, respectively. The Cre recombinase cDNA was derived from pMCcre (Gu *et al*., 1993). It contains a nuclear translocation signal and the correct Kozak sequence. The 5′ end of the cDNA was subcloned using PCR into pCRII (Invitrogen), placed downstream of the 250 bp MLCIIv promoter (provided by Dr K. Chien, San Diego, USA) and subsequently cloned into pBluescript SKII (Stratagene) using *Hin*d III/*Sal* I digest. The remain-

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ing *Bam* HI-fragment from pMCcre was placed downstream to obtain the final MLCcre vector. The β-globin intron was inserted into the MLCcre vector upstream of the Cre recombinase cDNA to obtain the MLCcreI2 vector.

MMTVcre. The MMTVcre vector contains the Cre recombinase cDNA under the control of the MMTV promoter. The MMTV regulatory region was isolated from the pMMTV-SV40-Bssk plasmid (Guy *et al*., 1992) using a *Hin*d III/*Sal* I digest and was cloned into pBluescript SKII (Stratagene). The Cre recombinase cDNA was inserted downstream using a *Eco* RI/*Xba* I digest.

DNA injection into zygotes

The transgenes were separated from the plasmid sequences either by *Kpn* I/*Sac* II (MLCcre and MLCcreI2) or by *Sal* I/*Xba* I (MMTVcre). DNA fragments were isolated using the QIAquick Gel extraction kit (Qiagen) according to the manufacturer's instructions, dialysed overnight against injection buffer (10 mM Tris, 0.25 mM EDTA, pH 7.4), filtered through 0.45 μm filters (Millipore) and stored at a concentration of 1.5–2.0 ng μl^{-1} at −80 °C until further use.

E0.5 zygotes were collected from superovulated FVB/N female zygote donors, injected and transferred to pseudopregnant CD-1 recipients, according to established procedures (Hogan *et al*., 1994).

Identification of transgenic founders and progeny

Founders were identified using PCR of mouse tail DNA (Miller *et al*., 1988). DNAs were subjected to 30 cycles of

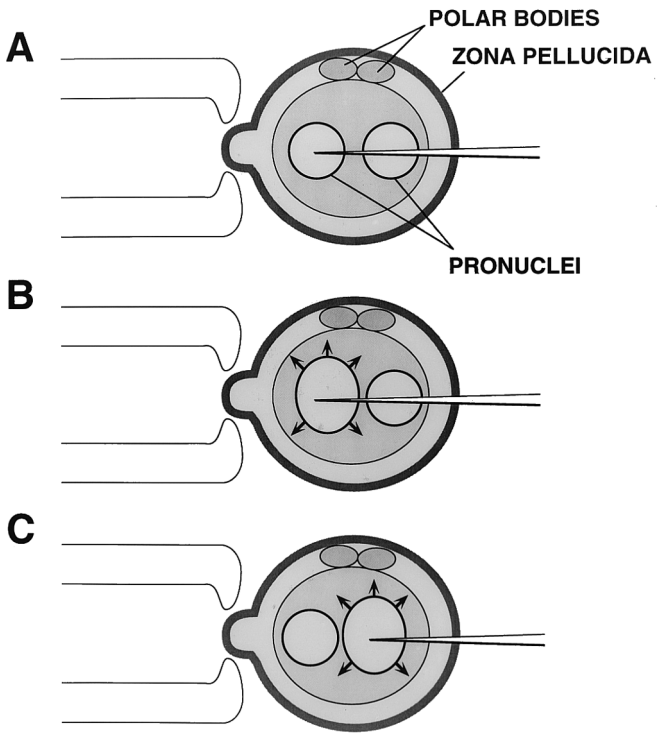

Fig. 1. Schematic representation of the double pronuclei injection method. Both pronuclei of the zygote are aligned to the injection and holding micropipettes. The injection pipette is first introduced through the proximal pronucleus and in the distal pronucleus (A). Second, DNA is injected into the distal pronucleus (B). The injection pipette is then retracted into the proximal pronucleus and DNA is injected (C). The injection pipette is removed, and the injected zygote is allowed to recover shortly prior to reimplantation in the oviduct of a foster mother.

Table 1. Yield of transgenic founders from single and double pronuclei DNA injection into zygotes

Transgenic vector	*Type of injection*	*Number of zygotes injected*	*% of lysed zygotes*	*Number of zygotes transferred*	*Corrected number of zygotes transferred*
MLCcre	1 pronucleus	148	27%	108	84
MLCcreI2	1 pronucleus	144	12%	127	108
MMTVcre	1 pronucleus	81	28%	56	44
Total	1 pronucleus	373	22%	291	236
MLCcre	2 pronuclei	164	17%	127	127
MLCcreI2	2 pronuclei	170	29%	116	116
MMTVcre	2 pronuclei	82	28%	55	55
Total	2 pronuclei	416	28%	298	298

Numbers represent the total of two injection sessions for each vector, MLCcre, MLCcreI2 and MMTVcre, for single and double pronuclei injection respectively. A total of twelve sessions are presented, i.e. six sessions per type of injection. The percentages of lysed zygotes from the total number of zygotes injected are indicated. Double pronuclei injection did not affect significantly the percentage of lysed zygotes relative to single pronucleus injection. The healthy-looking zygotes were transferred to the oviducts of pseudopregnant foster mothers. Two recipients, using the standard single pronucleus injection, failed to become pregnant and one recipient gave birth to two stillborn pups only. In the corrected number of zygotes transferred, the number of zygotes transferred to these three recipients were subtracted, as they most likely reflect the common failure of some recipients to support embryo development or nursing newborns, rather than an effect of the injection technique. The percentage of pups recovered at birth and the percentage of transgenic founders have been calculated with the corrected value of zygotes transferred as it represents a more accurate comparison of yields relative to the injection effort. All mice surviving to weaning have been analysed for the presence of the transgene. Despite a slightly lower percentage of pups recovered from the corrected number of injected zygotes, the percentage of transgenic founders was increased by 60%. The percentage of transgenic founders from the number of mice analysed was increased twofold when double pronuclei injection was used. The standard deviation (s.d.) on the percentage of transgenic founders from mice analysed are presented in the last column, as calculated by the formula, s.d. = $(npq)^{-2}$ for binomial distribution, where n is the size of the population analysed, p, the percentage of positive events and $q = 1 - p$, i.e. the percentage of negative events.

amplification (30 s at 94 °C, 30 s at 63 °C, and 1 min at 72 °C) using the primers cre4-5′ (5′ CCG GTC GTG GGC GGC CAT GGT GC 3′) and cre3-3′ (5′ GCG ATC GCT GCC AGG ATA TACG 3′). The PCR product was 330 bp in size.

Results and discussion

We routinely produced transgenic mouse lines using injection of DNA into the male pronucleus of FVB/N zygotes. We now have tested the effects of injecting simultaneously male and female pronuclei on the survival of zygotes and on the yield of transgenic founders. Figure 1 illustrates the double pronuclei injection technique. Both pronuclei were aligned with the holding and injection micropipettes, with either the female or the male pronucleus closer to the holding pipette. The injection pipette was introduced through the front and rear nuclear membrane of the proximal pronucleus, and through the front nuclear membrane of the distal pronucleus (Fig. 1A). A small amount of DNA solution was forced into the pronucleus until a visible swelling was observed (Fig. 1B). The pipette was then retracted in the proximal pronucleus and the introduction of DNA was repeated (Fig. 1C). After retraction of the pipette, the injected zygote was allowed to recover at 37 °C in a 5% CO_2 incubator for 2 h. Little additional time is involved in aligning the pronuclei and in the sequential injection.

Three transgenic vectors, named MLCcre, MLCcreI2 and MMTVcre, were used to produce transgenic mouse lines by single and double pronuclei injection methods (Table 1). The results from twelve injection sessions are presented. Each vector was injected on two separate days in one pronucleus and two days in both pronuclei. Our experience at producing transgenic mice routinely by standard methods, showed that yields of transgenic mouse lines vary among various DNA preparations, ranging from 0% to 30% of mice analysed, with an average of 20%. To eliminate variations introduced by the quality of DNA, the same preparation was used for all injection days of a given vector. In addition, each type of injection was performed on consecutive days to minimize other potential variations in handling of animals.

Approximately 400 zygotes were injected using each type of injection (Table 1). The proportion of lysed zygotes was determined by visual inspection under the light microscope. The proportion of lysed zygotes after double pronuclei injection was comparable to that of single pronucleus injection, i.e. between 20% to 25%. Lysed zygotes were discarded and nine to fourteen surviving zygotes were transferred per oviduct of recipient females. Two recipient females of the single pronucleus injection series failed to become pregnant, and one female of the same series gave birth to two dead pups. Failure of some recipient females to carry pregnancy is not unusual. Because it was seen only after single pronucleus injection, and not after double pronuclei injection, these miscarrages are unlikely to be due to the injection method. Consequently, to avoid underestimating the efficiency of the single pronucleus injection method, the number of zygotes transplanted in these three recipient females were subtracted in the

Number of newborn mice	*% of pups born from corrected number of zygotes transferred*	*Number of mice analysed*	*Number of founders*	*% of transgenic founders from corrected number of zygotes transferred*	*% of transgenic founders from mice analysed* [Standard deviation]
27	32%	27	4	5%	15%
44	41%	43	13	12%	30%
5	11%	3	0	0%	0%
76	32%	73	17	7%	23% [5%]
39	31%	39	16	13%	41%
25	22%	25	14	12%	56%
10	18%	10	4	7%	40%
74	25%	74	34	11%	46% [6%]

corrected number of zygotes transferred. The percentage of pups recovered at birth from the corrected number of transplanted zygotes was slightly higher when using single pronucleus injection, 33%, than when using double pronuclei injection, 23%, suggesting that injection in both pronuclei reduces slightly the viability of the zygotes.

Transgenic founders were identified by PCR. Two values were important in determining whether the double pronuclei injection method represents an improvement over the standard method. Firstly, the use of double injection resulted in a 60% increase in the number of founders per transferred zygotes, thereby reducing time, material and injection effort involved in generating transgenic mice. To avoid overestimating the efficiency of the method, this percentage was calculated with the corrected number of transplanted zygotes. Secondly, we observed a 100% increase in the number of transgenic mice per animal analysed, demonstrating that the use of double injection reduces the effort of preparing tail DNA and screening born progeny.

Overall, the use of the double pronuclei injection is more efficient to generate a large number of transgenic mouse lines. This new method is of considerable interest in view of the increasing number of transgenic mouse lines generated for conditional knockout experiments and for other gene function and regulation studies.

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