Evaluation of technical approaches to pronuclei injection.

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Introduction

A transgenic animal is generated by introducing specific alterations into the genome, either by the injection or the embryonal stem (ES) cell technique (Hogan et al. 1986, Hooper 1992). In the injection technique purified DNA fragments are physically injected into the pronuclei of fertilized mouse eggs. The injected DNA integrates into one of the chromosomes in an apparently random fashion and replicates together with the genomic DNA. The ES cell technology, in contrast, allows a functional gene to be removed from the genome. An engineered, non-functional version of the gene is introduced into undifferentiated, totipotent ES cells and replaces the endogenous gene by homologous recombination. The targeted ES cells can be identified and are introduced into blastocysts, which are transplanted into fostermothers, in which they develop to term. The resulting chimeric animals are then bred further and analysed for transmission of the mutated ES cells to the next generation (Hogan et al. 1986, Hooper 1992).

The transgenic technology is the only means by which the functions of specific genes can be analysed in vivo in mammals and has had a large impact in research areas like developmental biology, immunology, cancer research and to generate animal models for various genetic diseases (for review see Hanahan 1989). Neurobiology has also benefited from the transgenic technology. The nervous system originates at the dorsal side of the embryo by the transition of ectoderm to neuroectoderm. The neuroectoderm, i.e. the neural plate, folds and forms the neural tube (for review see Jacobson 1991) and at this stage there is a transient burst in cellular proliferation. The cell progeny of the central nervous system (CNS) progenitor cells migrates out from the neural tube and forms neural and glial cells in the CNS, i.e. the brain and spinal cord. From the dorsal aspect of the neural tube the neural crest cells migrate out to form the peripheral nervous system (PNS).

We have previously characterized a gene, nestin, which is predominantly expressed in the CNS progenitor cells. The nestin gene encodes a novel intermediate filament protein, which is part of the cytoskeleton in these cells (Lendahl et al. 1990, Dahlstrand et al. 1992). Upon differentiation to neurons and glial cells nestin expression is downregulated and replaced by expression of other intermediate filaments, neurofilaments and GFAP, respectively (Dahlstrand et al. 1995). Outside the nervous system nestin is expressed in developing muscle (Sejersen & Lendahl 1993). Nestin is also reexpressed in various cell types that resemble CNS progenitor cells, i.e. cells in CNS tumors (Dahlstrand et al. 1992), PNS tumors (Florenes et al. 1994), in immortalized cell lines (Redies et al. 1991) and after CNS injury (Frisén et al., in prep.).

More recently we have identified three mammalian Notch genes (Lardelli & Lendahl 1993, Lardelli et al. 1994, Larsson et al. 1994). In the fruit fly, Drosophila melanogaster, the Notch gene encodes a large transmembrane receptor, which is important in the decision of cell fates in many tissues including the nervous system (Artavanis-

Tsakonas & Simpson 1991). Absence of the Drosophila Notch gene is lethal at the larval stage and results in an embryo that develops an excess of neural cells at the expense of epidermal cells. The Drosophila Notch protein contains 36 epidermal growth factor like repeats (EGF repeats) at the extracellular side and six cdc10/SWI6 repeats (ankyrin repeats) at the intracellular side. The mode of signal transduction into the cell is presently unknown. The three mammalian Notch homologues, Notch 1, 2 and 3, are strikingly similar to Drosophila Notch (approximately 50% amino acid identity over the entire coding region) and the only major structural difference is that Notch 3 lacks two of the 36 EGF repeats (Lardelli et al. 1994).

In this report we analyse some paramaters influencing the efficiency by which transgenic offspring is generated. We also discuss the dissection of regulatory elements in the nestin gene and an approach to ectopically express mutated Notch genes in the developing CNS.

Materials and Methods

Superovulation and animal care

All animals (Bommice, Denmark) were housed and cared for in compliance with the NIH guidelines for animal experiments. Superovulation was carried out in the following way: F1(C57BL6 X CBA) or C57BL6 females (4-6 weeks of age) were injected intraperitoneally with 0.1 ml of 5 U/ml PMS (pregnant mare serum) at noon and with 0.1 ml of 5 U/ml hCG (human chorion gonadotropin) at 9 AM two days later. The same day the females were housed with fertile males (F1(C57BL6 X CBA) and C57BL6 males, respectively) and checked for mating by the presence of a copulation plug the following morning. The light/dark cycle was: 3 AM, light on; 5 PM, light off.

Purification of DNA for injection

The plasmid used for DNA injection was prepared by banding in a cesium chloride (CsCl) gradient followed by extraction with butanol extraction and precipitation with ethanol (Maniatis et al. 1989). Alternatively, in some experiments the plasmid was purified by a commercial DNA purification kit, Qiagen, according to the manufacturer's suggestions (Diagen). After purification, the plasmid DNA was cleaved with appropriate restriction enzymes to remove plasmid sequences and directly subjected to gel electrophoresis in standard agarose gels in 1 X TAE buffer (Maniatis et al. 1989). The correct DNA fragment was then excised from the gel and purified by Geneclean (Bio 101), according to the manufacturer's suggestion. In the final step the DNA fragment was dissolved in a small volume of injection buffer (10 mM Tris, 0.2 mM EDTA, pH 7.4) and the concentration estimated by gel electrophoresis with DNA fragments of known concentrations run in parallel. Finally, the DNA fragment was diluted to 2-3 ng/µl, filtered over a 0.22 µm filter (Millex, Millipore) and stored at +4°C until use.

Injection procedure

Fertilized eggs were surgically removed from females that had been mated to fertile males during the night. The eggs were kept in M2 medium (Hogan et al. 1986) and the cumulus cells were removed by treatment with 10 µl of 10 mg/ml hyaluronidase in 160 µl M2 medium for 1 minute. The eggs were then rinsed 5 times in M2 medium and stored in M16 medium (Hogan et al. 1986) in a 37°C incubator with 5% CO_2. After identification of fertilized eggs, i.e. presence of visible pronuclei under a dissection microscope (Nikon SMZ-U), 15 eggs at the time were put in a microdrop of M2 at an inverted microscope stage (Nikon) and injected with a sufficient volume of DNA (2-3 ng/µl) to cause a transient swelling of the pronucleus. The injection pipette was made of TF10 glass (Clark) and the holding pipette of G-1 glass (Narishige). After injection the eggs were transfered back to M16 medium and transplanted to pseudopregnant fostermothers either the same day at the 1-

cell stage or the following day, at the 2-cells stage (Hogan et al. 1986).

Transplantation of injected eggs

Pseudopregnant fostermothers were generated by mating 6-10 week old F1(C57BL6 X CBA) females with sterile males (vasectomized Tif $Bom^{ag/ag}$). The following day females with a copulation plug were anestetized by Avertin (0.15 ml/gram body weight of a 40 X dilution in H_2O from a stock solution containing 10 gram of tribromoethyl alcohol in 10 ml of tertiary amyl alcohol). Normally, 25 eggs (at the 1-cell or 2-cell stage) were transplanted to the oviduct on the right side. Alternatively, when there was a shortage of pseudopregnant females, 25 eggs were operated to each of the two oviducts.

DNA preparation and test for transgenicity

Offspring from injection experiments was tested for transgenicity either at various embryo stages or after birth. At embryo stages DNA was prepared from the most caudal parts of the embryo and the extraembryonic tissues; in postnatal animals from tail biopsies (when the animal was at least three weeks old). DNA was prepared as previously described (Laird et al. 1991). Briefly, the tissue was incubated in lysis buffer (100 mM Tris, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg proteinase K/ml) overnight on a rocking platform at 55°C. The next day the sample was centrifuged in a microcentrifuge for 10 minutes at 13 000 rpm and the supernatant transfered to a new tube. One volume of isopropanol was added and the stringy precipitate was attached to a micropipette tip, quickly washed in 70% EtOH, briefly air dried and dissolved in 200 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer for four hours at a rocking platform at 55°C. An aliquot of the DNA was then tested for transgenicity by PCR or Southern blot analysis by standard technology (Maniatis et al. 1989). In the PCR analysis a positive control corresponding to one integrated copy of the construct per genome was always included (10 femtogram of the injected fragment mixed with 10 nanogram of wildtype mouse genomic DNA). In Southern blot experiments the positive control corresponding to one integrated copy per genome was composed of 10 picogram of the injected DNA mixed with 10 µg of wildtype mouse genomic DNA.

lacZ immunohistochemistry

Mice transgenic for the lacZ gene were identified by a PCR assay as previously described (Nilsson & Lendahl 1993). To analyse lacZ expression embryos were fixed in 0.2% glutaraldehyde in PBS for 10-15 minutes (8.5-10.5 days post coitum (dpc) embryos). After three rinses in PBS embryos were permeabilized in PBS with 2 mM $MgCl_2$, 0.02% NP40 and 0.01% Na-deoxycholate for 20 minutes and then incubated at 37°C overnight in the same buffer supplemented with 5mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 1 mg/ml 5-bromo-4-chloro-3-indole-β-D-galactoside (X-gal).

Results and Discussion

The transgenic facility

We have established a facility for the production of transgenic mice at the Karolinska Institute. The facility produces transgenic mice by the injection technology both for our own research needs and for a number of other reserach groups. Since the start in 1991 we have generated stable lines of transgenic mice from 37 different DNA constructs. The projects include analysis of regulatory elements for immunoglobulin genes (Arulampalam et al. 1994), expression of a toxicity gene in cells of the immune system (Erlandsson et al. 1994), analysis of hybrid class I MHC transplantation antigens (Sentman et al. 1994), expression of a viral homologue to a thyroid hormone receptor (Barlow et al. 1994), analysis of the human β-actin promoter (Nilsson & Lendahl 1993), and analysis of regulatory regions active in the early nervous system (Zimmerman et al. 1994). In addition, we have recently established the gene target-

ing technology and generated germ line chimeras after inactivation of a thyroid receptor gene by homologous recombination in ES cells (Wikström et al., in prep.).

Parameters influencing the frequency of transgenic offspring

The 37 injected constructs range in length from 2 to 14 kb. This size span gives us the oppurtunity to analyze if the length of the construct may influence the frequency of transgenic offspring. Since the large majority of constructs does not appear to affect the survival of the transgenic offspring it can be assumed that the observed frequency of transgenicity indeed reflects the integration frequency. Our data show that the frequency of transgenic offspring appears to be independent of the size of the injected DNA (Table 1). For example, a number of the 2-3 kb long insertes generated transgenic mice with a frequency of only 7-25%, while two different 13 kb inserts produced 31 and 44% transgenic offspring. It is thus more likely that the sequence composion affects the frequency of integration, although we have observed differences in transgenic frequency also between closely related constructs (data not shown).

Two different procedures to purify the DNA for injection were used: CsCl gradient centrifugation and purification by a commercial DNA purification resin (Qiagen). In Table 2 we compared if the DNA purification procedure influences the frequency of transgenic offspring. The average frequency for CsCl was 22% and for the commercial resin 12%. This suggests that CsCl purification is superior, although it should be kept in mind that we have not yet compared the efficiency of introducing exactly the same DNA construct after purification with the two methods and that the data material using the commercial resin is limited.

For genetic reasons three of the transgenic strains were generated in the C57BL6 genetic background while 34 strains were made in the F2 intercross between CBA and C57BL6. It has previously been suggested that it is more difficult to generate transgenic mice in inbred strains (Hogan et al. 1986) and we wished to compare to what extent this was true in our material and at which steps in the process problems may be encountered. From data presented in Table 3 it is apparent that the overall efficiency was lower in the C57BL6 background and there are two major reasons for this. First, the yield of fertilized eggs was much lower, 46% versus 72%, despite the fact that all C57BL6 males were tested for fertility. Second, there were relatively fewer offspring born after transplanting C57BL6 eggs back to fostermothers, 4.5% versus 25%. This suggests an increased death rate in utero and early postnatally, due to small litter sizes. In contrast, the proportion of transgenic offspring was not dramatically different from that of the F2 intercross, suggesting that the integration frequences may be relatively similar in the two genetic backgrounds.

CNS development and transgenic technology

The transgenic technology has already made important contributions to our understanding of gene regulation and gene function in the nervous system. Dramatic effects on CNS development are seen after targeting of a number of genes, e.g. Hox genes, Pax genes, the MASH 1 gene, neurotrophin genes, trk receptor genes and the wnt-1 gene. The phenotypes from some of these gene targetings are the subject of a recent review (Joyner & Guillemot 1994). It is also important to chracterize regulatory regions for genes expressed at various stages of CNS development, both to learn more about gene regulation in the CNS per se, and to define promoter elements which can direct expression of a heterologous gene to a particular developmental state or cell type. Promoter elements are generally identified by linking putative regulatory regions to a reporter gene and assay for expression. The most commonly used

reporter genes are lacZ (β-galactosidase), CAT (chloramphenicol acetyl transferase) and luciferase. Each has distinct advantages and disadvantages. For example, lacZ provides excellent cellular resolution, but is not as sensitive as CAT and luciferase. A more detailed review of reporter genes in transgenic mice has recently been published (Cui et al. 1994).

We were interested in identifying regulatory regions that are active in the CNS progenitor cells and therefore decided to dissect the nestin regulatory regions. A number of transgenic constructs were made by fusion various parts of the rat nestin gene and flanking sequences to the lacZ gene (Zimmerman et al. 1994). It was observed that the upstream regions had little effect on expression, but that a fragment containing the genomic region of the nestin gene directed expression of lacZ to the developing CNS and muscle. A more detailed mapping revealed that the CNS and muscle expression patterns are controlled independently, by enhancer elements in the second and first intron, respectively (Zimmerman et al. 1994). More recently, we have established permanent lines of the nestin promoter/lacZ mice and the expression pattern has been stable for at least three generations (data not shown). We have also compared the sequences of the second intron from rat and man and identified certain regions that are highly conserved and thus may be important for the enhancer function (data not shown). Finally, CNS injury appears to upregulate expression both of the endogenous nestin gene and the lacZ gene in the nestin promoter/lacZ transgenic mice (Frisén et al., in preparation).

Data from Drosophila (Lieber et al. 1993), Xenopus (Coffman et al. 1993) and analysis of tumors in mouse (Robbins et al. 1992) and man (Ellisen et al. 1991) strongly indicate that the intracellular domain of the Notch proteins is involved in cellular signalling. To test this idea for the Notch 3 protein we have engineered a DNA construct which carries the Notch 3 region encoding the intracellular domain under the control of the nestin promoter. Preliminary data indicate that this affects CNS development, possibly by inducing hyperplasia in the transgenic mice (Lardelli et al., in preparation). It is our hope that ectopic expression of interesting developmental control genes in the early CNS may contribute to a better understanding of the molecular mechanisms governing the construction of the mammalian nervous system.

Acknowledgements
We thank Marie-Louise Alun and Mona Schröder for excellent technical assistance in the animal facility. This work was supported by the Swedish Cancer Society, Margaret och Axel Ax:son Johnsons Stiftelse, Kjell och Märta Beijers Stiftelse, Knut och Alice Wallenbergs Stiftelse, and Magn. Bergvalls Stiftelse. This work is dedicated to the memory of our beloved friend and colleague Jonas Dahlstrand.

Abstract
The transgenic technology makes it possible to introduce specific genetic alterations into the genome of all cells in an organism. This has opened entirely new possibilities to analyse the regulation and function of individual genes in a living animal. We have established a facility for the production of transgenic mice and have generated 37 different transgenic mouse strains. Here we analyse if the frequency of transgenic offspring correlates with the length of the introduced transgene and/or with the genetic background of the injected eggs. Our data suggest that the transgenic frequency is relatively independent of the length of the construct. The genetic background is more important and we find that specific steps in the process of generating transgenic mice are considerably more efficient in an F2 intercross between C57BL6 and CBA than in inbred C57BL6 mice. Finally we discuss how we have used the transgenic technology to analyse the regulation and function of genes in the developing nervous system.

Skandinavisk (svensk) sammanfattning:
I denna artikel utvärderar vi olika parametrar som kan tänkas påverka frekvensen transgen avkomma efter pronukleär injektion av DNA i befrukatade musägg. Vi finner att DNA-preparationsmetoden är viktig och att cesiumklorid-renat DNA generellt ger en högre frekvens transgen avkomma än andra preparationsmetoder. Däremot förefaller längden på det injicerade DNAt ej nämnvärt påverka integrationsfrekvensen. Slutligen diskuterar vi de resultat vi erhållit när det gäller att definiera promotorsekvenser som är aktiva i det tidiga nervsystemet hos transgena möss.

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