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Review

Production of functional transgenic mice by DNA pronuclear microinjection✪

Anna B. Auerbach✉

Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, NYU School of Medicine, New York, U.S.A.

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Successful experiments involving the production of transgenic mice by pronuclear microinjection are currently limited by low efficiency of random transgene integration into the mouse genome. Furthermore, not all transgenic mice express integrated transgenes, or in other words are effective as functional transgenic mice expressing the desired product of the transgene, thus allowing accomplishment of the ultimate experimental goal – *in vivo* analysis of the function of the gene or gene network. The purpose of this review is to look at the current state of transgenic technology, utilizing a pronuclear microinjection method as the most accepted way of gene transfer into the mouse genome.

Transgenic animals are providing a quintessential system to study various aspects of gene function *in vivo*, which is apparent more than ever before in the current era of functional genomics. Transgenic technology applications range from studying of the basic mechanisms of gene regulation to the generation of the models for human diseases to create systems utilized in pathophysiological and therapeutical studies. While similar genetic manipulations can be executed in tissue culture, the interaction of transgenes expressing

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✉Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, NYU School of Medicine, 540 First Avenue, New York, NY 10016, U.S.A.; tel: 212 263 7920; fax: 212 263 6585; e-mail: anauerba@saturn.med.nyu.edu

Abbreviations: BAC, bacterial artificial chromosome; CMV, cytomegalovirus; EU, endotoxin unit; LCR, locus control region; PAC, P1 artificial chromosome; YAC, yeast artificial chromosome.

hormones, oncogenes, neurotransmitters in the context of an intact organism provides a much more complete and physiologically relevant picture of the transgene's function than could be achieved in any other methods. Numerous examples can be given i.e. behavioral phenotypes, cognitive abilities, as well as other neurological defects. In some cases gene expression should be analyzed in multiple tissue and cell types to assess response to physiological stimuli and developmental signals. In the overexpression studies, constructs containing genes encoding transcription factors, cell surface receptors and structural proteins are used. Loss-of-function experiments might also be created by utilization of constructs encoding antisense RNA or dominant-negative genes, or by RNA interference to disrupt the function of endogenous genes. Transgenes containing toxin genes are used to ablate specific cells or markers to study a pattern of gene expression or to identify specific cell types. It is also possible to correct gene defects by adding a normal allele that can complement a mutant one allowing identification of functional genes within large chromosomal fragments.

HISTORICAL FOREWORD

Interest in manipulating the laboratory mouse genome has a long-standing history, beginning with selective breeding to the present use of mice for cloning. The route to transgenesis techniques can be traced to the convergence of gene-transfer methods developed in murine cell culture systems and methods to manipulate early mouse embryo. Development of protocols for removing preimplantation stages of mouse embryos, culturing them briefly *in vitro* and implanting them into foster mothers to allow normal embryogenesis (Brinster, 1972) lay down the ground for today's extensively used techniques of germ line transformation. The first permanent lines of mice containing foreign genes were produced by infecting preimplantation stages of embryos with murine leukemia virus (MuLV) (Jaenisch, 1976). Retrovirus-mediated transgenesis was limited by transgene's size and was introducing only a single copy of the transgene. Also produced highly mosaic animals with multiple insertions as a result of infecting embryos, which have already divided. Furthermore, the viral sequences can interfere with transgene expression, since silencing of the provirus during development results in low to undetectable levels of transgene expression (Jahner *et al.,* 1982). Therefore, techniques utilizing recombinant viruses did not evolve into widely used protocols for introducing foreign genes into the mouse germ line for more than thirty years, in spite of the potential. However, use of lentiviral vectors (Lois *et al.,* 2002) most recently allowed for some advances in transgenesis technique.

Nevertheless, the dominating technique leading to random integration of a transgene involves microinjection of DNA into the pronucleus of a developing zygote. Initially, techniques for injecting mRNA and then cloned genes were developed for much larger amphibian eggs (Gurdon & Melton, 1981) and for tissue culture cells. Parallel progress in developing assays for detecting gene expression (Graessmann *et al.,* 1978) was made. During late 1970s, it became possible to adapt these methods for microinjection of mRNA and finally DNA, into mouse zygotes (Brinster *et al.,* 1980; 1981a). Gordon and his colleagues published the first report describing transgenic mice resulting from pronuclear microinjection (Gordon *et al.,* 1980). Other groups also provided evidence for the stable integration of foreign DNA into the mouse genome (Brinster *et al.,* 1981b; Costantini & Lacy, 1981; Wagner EF *et al.,* 1981; Wagner TE *et al.,* 1981), with some of the first results suggesting that these genes could be expressed (Brinster *et al.,* 1981b; Wagner EF *et al.,* 1981; Wagner TE *et al.,* 1981), and even transmitted to offspring

(Costantini & Lacy, 1981; Gordon & Ruddle, 1981; Wagner TE *et al.,* 1981; Steward *et al.,* 1982), stably expressing foreign genes in biologically active amounts of product leading to dramatic phenotype(s) (Palmiter *et al.,* 1982).

This process of transforming mouse genome by DNA pronuclear microinjection was named "transgenic" by Gordon and Ruddle (Gordon & Ruddle, 1981). A "transgenic animal" is defined as an animal that has a foreign gene(s) stably incorporated into its genome through human intervention. This segment of recombinant double-stranded DNA is called a "transgene". Transgenes can be broken down into two categories: those that resulted from random insertion into the genome (usually by means of microinjection or viral infection) and those that are produced by homologous recombination as targeted events at a particular loci to express transgene in specific regulatory context (like in knock-in experiments). Microinjected DNA primarily integrates at random locations in the genome thus, usually is restricted to gain-of-function studies.

Following fertilization of a mouse egg, the male and female pronuclei remain separated for a few hours before they fuse to make the zygotic nucleus allowing a 3–5 hour period ideal for microinjection. These independent pronuclei can be visualized under a microscope and DNA can be injected into the larger male pronucleus. The nuclear membranes then break down and the pronuclei fuse. Viable eggs, which survived injections, are transferred on the same day or after overnight culture into the oviducts of pseudopregnant mice. F_0 generation mice that carry the transgene are called "founders" and can be identified by Southern blot or PCR analysis. Southern blot analysis allows for verification of transgene integrity, and in non-mosaic founders, for copy number determination, thus it is useful for genotyping F_0 generation. More than one integration site into the genome is also possible (Lacy *et al.,* 1983). The direct method for examining the number of integration sites in founders is by fluorescence *in situ* hybridization (FISH) analysis, or by an easier but less reliable Southern blot analysis of flanking genomic area. Segregation of independent integration sites becomes obvious in F_1 generation thus allowing establishing independent transgenic lines for each single integration site. This could be advantageous since independent integration sites usually contain different copy number of the transgene. Occasionally, phenotypes might be observed in the F_0 generation. A transgene DNA appears to integrate randomly on all autosomes as well as on sex chromosomes (Lo, 1986). Transgene integration usually occurs only on one chromosome thus, the resulting founders are hemizygous for the transgene.

The precaution of removing the plasmid vector sequences by restriction digestion before microinjecting is recommended, since the expression of several genes has been shown to be sensitive to the presence of prokaryotic DNA (Chada *et al.,* 1985; Hammer *et al.,* 1985; Krumlauf *et al.,* 1985; Townes *et al.,* 1985). Transgene expression and its phenotypic consequences can be analyzed in detail in subsequent generations of newly established permanent transgenic lines, for which faster PCR genotyping might be better suited. In any case, transgene detection strategy should be sensitive enough to detect transgene DNA at the single copy level (see http://www.med.umich.edu/tamc/tgoutline.html), thus positive animals will not be accidentally omitted. In experiments involving larger DNA fragments like YAC- or BAC-based constructs the probability of transgene rearrangement observed in the founder is higher and usually occurs before integration (see below). Most of the time plasmid-based transgenes are stably transmitted into progeny for many generations, although some exceptions have been observed, in which transgenes were rearranged, partially deleted or amplified (Brinster *et al.,* 1984; Palmiter *et al.,* 1982; 1983; Shani,

1986). Transgene expression in the progeny might also change as a result of transgene DNA methylation (Palmiter *et al.,* 1982).

MECHANISM OF THE TRANSGENE INTEGRATION

The precise mechanism of integration of injected DNA is not fully understood. Nevertheless, it might be broken down to two processes: concatamere formation and chromosomal insertion. Most of the time more than one copy of the transgene integrates into the genome and usually at the same chromosomal site. Multiple copies integrate as a tandem array usually forming a primarily linear direct head-to-tail array (Brinster *et al.,* 1981b; Gordon & Ruddle, 1985) called concatamere, but other arrangements have been reported (Brinster *et al.,* 1985; Wagner *et al.,* 1983). Concatamere formation is thought to occur before the integration. After a pool of identical linear molecules is introduced, initial circularization of those molecules (end-joining by ligation) is followed by their random cleavage (by cellular nucleases). These processes generate a population of circularly permuted monomers. At that point rounds of homologous recombination (HR) might form extrachromosomal concatameres (Bishop & Smith, 1989; Bishop, 1996). The chromosomal site of integration is randomly determined and may result from double-strand chromosomal breaks (Brinster *et al.,* 1985) present during S phase of cell cycle (Sonoda *et al.,* 2001; Petersen *et al.,* 2001). Non-repaired endogenous DNA lesions that block replication fork progression may result in dsDNA breaks. Lesions repaired by base excision repair mechanism could also lead to dsDNA breaks if they are located very close together. Thus, chromosomal breaks are naturally occurring during cell cycle and typically several breaks are observed per cell in S phase (Kunkel, 1999). The number of randomly generated chromosomal breaks might be a probable limiting factor for transgenesis. The theory that the ends of the transgene initiates integration at these breaks is in line with fivefold increase in integration efficiency of linearized DNA comparing with circular DNA for the traditional plasmid-based constructs (Brinster *et al.,* 1985).

Foreign DNA integrated into chromosomes of cultured cells has identical characteristics as DNA integrated in microinjected embryos. The list of those common characteristics includes presence of the multiple copies integrated predominantly at one integration site, the same direct arrangement of the copies in the concatamere and the presence of minor imperfections of input DNA at the junctions. The observation that irradiation of the transfected rodent cells increases the frequency of DNA integration (Perez *et al.,* 1985) also supports this view. The route of integration *via* homologous recombination is formally available when homology exists between the transgene and genomic DNA. Nevertheless, in cultured cells random integration of traditional constructs is 100 to 1000 times more frequent than homologous recombination, so such a theoretical event is much less probable. Thus, homologous recombination occurs between injected molecules into the nuclei of cultured cells to form tandem arrays (Folger *et al.,* 1982), but is not probable between transgene and chromosomal sequences. In addition, differences in chromatin structure between injected and chromosomal DNA make homologous recombination between two kinds of DNA less likely (Bishop & Smith, 1989). Three factors have an influence on success and timing of transgene integration after pronuclear microinjection: concentration of the DNA, the competency of the DNA repairs system as well as the replication and transcriptional activity of the embryo. Thus, the integration event is most likely to take place before or during DNA replication of the first and second cycle. Evidence for homologous recombination occurring between injected molecules comes

from co-injection of two or more different constructs with non-overlapping deletions. Transgenic mice can be generated that carry intact recombinant genes able to produce some amounts of functional product (Palmiter *et al.,* 1985). Homologous recombination in fertilized mouse eggs to generate large transgenes from injected overlapping genomic fragments was also reported (Keegan *et al.,* 1994). Furthermore, Wagner showed that three P1 clones carrying overlapping regions were able to recombine homologously to reconstitute a core human IgH locus in transgenic mice (Wagner *et al.,* 1996). When two different linear DNA molecules are co-injected they usually integrate together at the same site (Brinster *et al.,* 1985).

Analysis of junction fragments between the transgenes and chromosomal DNA revealed often deletions (Woychik *et al.,* 1985), duplications (Wilkie & Palmiter, 1987) or translocations of chromosomal DNA at the integration side (Gordon & Ruddle, 1985; Overbeek *et al.,* 1986). Some of those junctions contained short novel DNA sequences not originated from injected DNA or from neighboring DNA as described by Wilkie & Palmiter (1987). The later authors proposed a model that explains insertion-associated deletions and other rearrangements without the broken ends of the chromosome becoming dissociated from each other. The focus of their model is the theory that insertion of the transgene may involve the eye of the replication loop during chromosomal DNA replication. Two replication forks traveling away from the replication origin form the replication loop. Depending on how the sister chromatids are broken, the outcome is duplication, or deletion, or even an insertion of a DNA fragment originated from the remote region of the chromosome (Wilkie & Palmiter, 1987). Bishop and Smith (1989) suggested that extrachromosomal recombination and transgene insertion might have the same molecular basis and referred to it as an opportunistic repair-ligation accompanied by strand exposure and followed by complete repair. The frequencies of the extrachromosomal recombination and transgene insertion reflect both the frequencies with which different kinds of interactions occur between DNA molecules and their relative stability. The existence of rare head-to-head or tail-to-tail arrangements shows that end-joined molecules may contribute to some arrays.

If integration occurs during/after one or more rounds of replication or divisions then the transgene will be present only in some cells resulting in genetic mosaicism (Palmiter *et al.,* 1983; Wilkie *et al.,* 1986). Some of transgenic founders (about 15%) carry the transgene in all their cells including germ cells, but even those non-mosaic founders might be arising from mosaic founders as a result of the death of non-transgenic sister cell due to i.e. chromosomal damage (Whitelaw *et al.,* 1993; Ellison *et al.,* 1995). Occasionally, there is no contribution of the transgene to the germ cells at all, thus it is not possible to obtain germ line transmission from such animal.

LIMITATIONS OF DNA PRONUCLEAR MICROINJECTIONS

Protocols for performing this manipulation were optimized for mouse embryo within a few years of the first report. Transgenic technology is now practiced widely in flies, worms, fish, frogs, chicken and mammals including rats, cows, sheep, goats, rabbits and pigs. Transgenic plants were also created and are currently produced at the industrial levels.

One major disadvantage of DNA pronuclear microinjection is the low efficiency of transgenic mouse production, with an even lower number of transgenic founders generated, which express the transgene appropriately (with suitable level and correct spatial/temporal expression). Many studies have found dramatic differences in the expression of the

same transgene between individual founder siblings due to different integration loci. Some founders and their progeny show no expression at all. In expressing lines the level of expression in a given tissue varies greatly from line to line. Level of transgene expression rather than being dependent on the number of copies of the transgene, often is influenced by the genomic sequences flanking the integration site (Palmiter *et al.,* 1982; Overbeek *et al.,* 1986). Such a "position effect" can reduce or completely abolish transgene expression, because the transgene has inserted into a transcriptionally inactive region of the genome or through the action of specific silencing regulatory elements. On the other hand, flanking sequences may contain regulatory elements of neighboring genes that act on the transgene promoter as an enhancer resulting in the ectopic expression of the transgene (incorrect spatial expression). Thus, it is important to generate several independent founders/transgenic lines from the same construct, as well as check the levels of expression and the number of integration sites of the transgene in the founder, and also to compare the observed phenotypes of independent transgenic lines.

In the situation that a transgene inserts into transcriptionally active genetic sequences, an interruption of the normal expression of an endogenous gene can occur, and this might be from inconsequential to lethal. Insertional mutagenesis might be apparent when the insertion interferes with the expression of an endogenous developmentally active gene. These mutations are distinguishable from the transgenic phenotype since only a single transgenic line would exhibit the phenotypical change. For example, Woychik reported severe limb deformities after integration of MMTV-myc transgene into limb deformity (ld) locus (Woychik *et al.,* 1985), but an insertional mutation can affect virtually any system. About 5–10% of transgenic lines show an insertional mutation resulting in a pathological phenotype in mice homozygous for the transgene. The identification of the locus of transgene insertion can be of a great value, because it might define the locus of an important endogenous gene. Interpretation of transgenic phenotypes thus should be done cautiously and it may be best to analyze hemizygous animals. Gene dosage effects and compensatory alteration due to gene overexpression may also complicate the interpretation of results. In addition, not all of multiple copies of a transgene integrated at a single site can express.

TRANSGENE EXPRESSION CASSETTE

Generally speaking, transgenes contain two major components: the transcription unit of a gene of interest (including exons and often introns) and regulatory elements controlling the expression of a gene (Fig. 1). The consequences of expression of a gene can be investigated under control of its own or heterologous promoter. Such a promoter might be tissue-specific (see list in Erickson, 1999) or broadly expressed. No ideal regulatory sequences leading to ubiquitous expression are known, but several fusion genes have yielded fairly good and wide-spread expression for example the human cytomegalovirus (CMV) enhancer-chicken β-actin promoter linked to the cDNA of a studied gene. Other examples of such promoters are phosphoglycerate kinase (PGK), or β-actin (human: Nilsson & Lendahl, 1993; rat: Beddington *et al.,* 1989; chicken: Sands *et al.,* 1993), and ubiquitin promoters (Schorpp *et al.,* 1996). However,

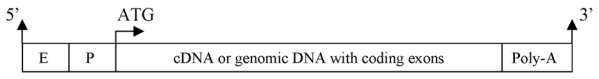

Figure 1. Schematic lay out of a typical transgene construct.

ATG, beginning of the transcriptional reading frame; E, enhancer; P, promoter; ↑, rare restriction enzyme recognition site.

use of those regulatory sequences lead to broad but scattered expression and in addition variable in different transgenic lines. The ROSA26 gene promoter – R26 (Soriano, 1999; Kisseberth *et al.,* 1999) directs generalized and uniform expression of reporter genes in a way that efficiently identifies transgenic cells in subpopulation of cells making this promoter suitable for i.e. transplantation studies.

The transgene can be tested *in vitro* for expression, when an appropriate cell culture system is available. Such experiments may identify potential problems with the transgene (i.e. 3' untranslated regions – UTRs can contain degradation signal or criptic splicing site introduced in construct may result in truncated or non-functional mRNAs) or problems resulting from detection of transgene expression. Nevertheless, expression in cell culture does not guarantee expression in a live mouse. It was demonstrated that efficient expression of a cDNA requires splicing (Brinster *et al.,* 1988) and a polyadenylation signal at its 3' end. To ensure accurate and efficient translation the start codon ATG should be preceded by a Kozak consensus sequence (Kozak, 1987). For tissue-specific expression it is better to include all naturally occurring intron elements that are involved in mRNA splicing and expression (Choi *et al.,* 1991; Palmiter *et al.,* 1991), since regulatory elements may also reside within intron sequences (usually first intron – Beermann *et al.,* 1990; Tanaka *et al.,* 1990; Ganss *et al.,* 1994). As already stated, it is also possible in some studies to use a shorter cDNA (a synthetic sequence representing only the protein encoding exon elements). Moreover, it is possible to add coding sequences not typical for specific gene or organism like coding sequences for nuclear localization signal (NLS) targeting protein synthesis into specific intracellular location. More compact vectors may be prepared using internal ribosome entry site (IRES) known to allow the translation of two adjacent cistrons when added between these coding sequences.

Several reporter genes have been successfully utilized to analyze regulatory elements or to mark distinct cell populations in transgenic mice. A transgene marker needs to be easily detectable with no background staining and should be developmentally neutral. The most widely used reporter gene is probably the *lacZ* gene allowing assaying enzyme β-galactosidase by rapid and sensitive detection of expression by whole-mount staining of embryos or organs as well as frozen sections. Unfortunately, there are some worries that the presence of bacterial *lacZ* coding sequences might result at least in cases when housekeeping genes are involved, in abnormal patterns of expression like silencing, variegation and ectopic expression (Montoliu *et al.,* 2000; Cohen-Tannoudji *et al.,* 2000). Another reporter gene, alkaline phosphatase (AP) might be used for staining to detect gene expression (i.e. Kisseberth *et al.,* 1999; DePrimo *et al.,* 1996). For detection in live animals, green fluorescent protein (GFP) from jellyfish or its enhanced version EGFP can be used (Okabe *et al.,* 1997; Kisseberth *et al.,* 1999), but only with a strong promoter like R26, since the sensitivity of GFP detection is much lower than β-galactosidase. Nevertheless, R26-EGFP transgene is detectable even in preimplantation embryos (Kisseberth *et al.,* 1999). The unique advantages of GFP over other reporter gene products are that it can be visualized in living cells, both *in situ* and in cell suspension without the need for any substrate which would have to penetrate tissue(s). Thus, GFP can be used to sort cells or for marking donor cells in transplantation studies. Since BAC-based constructs usually contain all native regulatory elements of the gene, recent use of modified BACs, in which a gene coding an EGFP substitutes gene of interest, allowed for functional genomics studies producing accurate patterns of *in vivo* expression in random integration transgenic

mice (Gong *et al.,* 2003). If quantitative analysis is needed the chloramphenicol acetyl transferase (CAT) assay or firefly luciferase gene are used for expression detection in tissue extracts of transgenic animals (Robinson *et al.,* 1989).

Variation in transgene expression resulting from integration into transcriptionally inactive regions can in some cases be overcome by the addition of *cis*-acting elements like locus control regions (LCR), insulators and scaffold/matrix attachment regions (S/MARs). LCR are regulatory sequences controlling accessibility of the DNA to transcription factors, which can be located tens of kilobases away from coding exons. The best example is the β-globin LCR, which gives position-independent and copy number-dependent expression to the human *β-globin* gene (Grosveld *et al.,* 1987; Ellis *et al.,* 1997) and other heterologous endogenous genes in transgenic mice. The mouse *tyrosinase* gene is another well-studied example with many different approaches undertaken to characterize LCR and its additional cooperative elements (Schedl *et al.,* 1993; Montoliu *et al.,* 1996). Many transgenes (from standard plasmid-based category) are not large enough to contain LCRs. Furthermore, heterologous LCRs do not always work reliably (Keegan *et al.,* 1994). Similarly, attempts to overcome position effects have been made by utilization of insulators. Such regulatory elements have been identified in a limited number of genes in *Drosophila* and some vertebrates (reviewed by West *et al.,* 2002) and proven to have the capacity to establish genomic barriers either by protecting gene sequences from the spreading of neighboring heterochromatin, or from the influence of distally located enhancers. The 5'HS4 element from the chicken β-globin locus, the first insulator identified in vertebrates (Chung *et al.,* 1993), did not fulfill the anticipated hopes to produce copy number-dependent transgene expression in numerous experiments (Giraldo *et al.,* 2003) in transgenic mice. However, inclusion of insulators in standard constructs seems to increase the probability of transgene expression (reviewed in Giraldo *et al.,* 2003). For potential benefits of inclusion of S/MARs see McKnight *et al.,* 1992; Gutierrez-Adan & Pintado, 2000.

CONDITIONAL AND INDUCIBLE SYSTEMS

The need for making gene function 'conditional' upon some other factors like presence or absence of drug, or expression of second transgene (binary system) in mouse has led to the development of inducible and conditional systems. There are two basic conditional systems utilizing site-specific DNA recombinases. The first of them utilizes bacteriophage P1 Cre recombinase recognizing *loxP* site and the second utilizes Flp recombinase from *Saccharomyces cerevisiae* recognizing *frt* site. Both transgene removal and transgene activation can be achieved utilizing Cre recombinase system (Fig. 2). Two mouse lines are required for conditional transgene removal. First, a conventional transgenic mouse line, also called a 'deleter' mouse, with Cre recombinase controlled by tissue-specific or cell-specific promoter and a second transgenic line containing a transgene flanked by two *loxP* sites in a direct orientation ('floxed gene'). Recombination (excision and consequently inactivation of the transgene) occurs only in those cells expressing Cre recombinase. Hence, the transgene remains active in all cells and tissues, which do note express Cre. The Cre's DNA excising capability can also be used to turn on a transgene by cutting out an intervening stop sequence between the promoter and the coding region. The ability of Cre recombinase to delete sequences between two *loxP* sites can be used to control variation in the copy number of a transgene (Lakso *et al.,* 1992). A transgenic mouse can be design in such a way that the transgene contains a single *loxP* site. Thus,

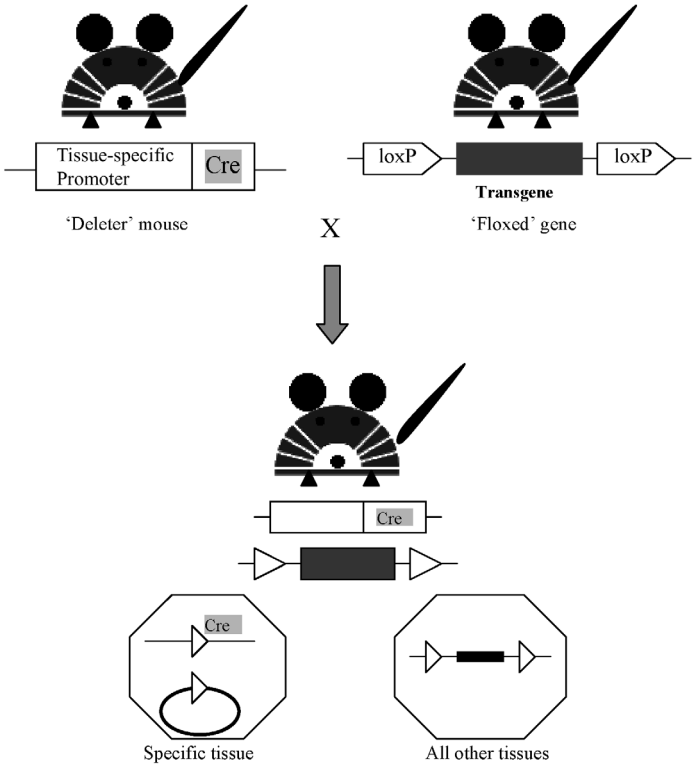

Figure 2. Conditional transgene removal utilizing Cre recombinase.

For conditional transgene removal breeding of two mouse lines is required. First is a conventional transgenic mouse line with Cre recombinase controlled by tissue-specific promoter. Such a mouse is also called 'deleter' mouse. A second transgenic line containing a transgene flanked by two *loxP* sites in a direct orientation ('floxed' gene). In the progeny, recombination (excision and consequently inactivation of the transgene) occurs only in those cells expressing Cre recombinase. The transgene remains active in all other tissues.

each copy of the transgene in a concatamere contains a single *loxP* site. If the mouse is mated to a 'deleter' transgenic mouse that expresses the Cre recombinase during germ cell development (Schwenk *et al.,* 1995) it will produce progeny with one copy or a reduced number of copies, allowing one to generate just by breeding several more transgenic lines expressing transgene at different levels.

Newer approaches allowing not only for spatial tissue-specific, but also for temporal control of onset of transgene expression independent from the endogenous regulatory elements have been developed with the Cre gene placed under the control of either a cell-specific or an inducible promoter, respectively (reviewed by Nagy, 2000). Conditional estrogen receptor fusion protein-inducible system Cre-ER takes advantage of the nuclear localization capacity of the estrogen receptor (ER) ligand-binding domain (LBD) in the presence of the ligand (Brocard *et al.,* 1998; Kellendonk *et al.,* 1999). In Cre-ER^{T} system, the CMV-driven site-specific Cre recombinase is fused to a mutant LBD, which has lost its ability to bind endogenous estrogen, but still binds tamoxifen – a synthetic estrogen antagonist. Introduction of a mutation into LBD allowed to achieve tighter than for Cre-ER system external control of the induction (Metzger & Chambon, 2001). A *loxP*-flanked (or floxed) STOP region separates the promoter and the coding sequence for the gene of interest. Ligand-activated site-specific recombination takes place in the presence (but not in the absence) of tamoxifen, when the Cre fusion protein translocates into the nucleus to excise the floxed alleles.

Another approach, the tetracycline-regulated system (Gossen & Bujar, 1992) utilizes a bacte-

rial tetracycline-inducible element. To achieve temporal control of transgene expression two transgenic lines must be obtained: activator and responder. The responder transgene is carrying a cDNA under the control of a minimal promoter and the tet operator (*tetO*), which can be controlled by a tetracycline-dependent transactivator (*tTA*). The second transgenic line – activator is expressing DNA-binding domain of tet-represor (*tetR*) fussed to transactivator domain from herpes simplex virus (Gossen *et al.,* 1995). Either, a tissue-specific or "ubiquitous" promoter drives the tetracycline-controlled transactivator. Inducible temporal expression is obtained by providing tetracycline in the water delivered to the mice. Fusion protein expressed from activator transgene binds to tetracycline and to the tet operator activating transcription. Unlike Cre-ER^T, this system is reversible, since tetracycline might be withdrawn when desired. The inducible systems are often applied to overcome lethal or compensatory effects, unexpected ectopic expression (leading to the problems with embryonic or juvenile survival) or gain-of-function during embryonic development. Effective inducible systems require tight external control of induction. Unfortunately, this requirement is yet to achieve. Albanese and his colleagues recently reviewed advances in inducible expression in transgenic mice (Albanese *et al.*, 2002).

GENERATING TRANSGENIC MICE FROM ARTIFICIAL CHROMOSOME-BASED CONSTRUCTS

Originally, DNA was isolated from plasmids; but recently much larger DNA fragments from bacterial or yeast artificial chromosomes are often used. Such vectors with a large cloning capacity usually make possible insertion of an entire locus* including all transcriptional and post-transcriptional regulatory elements resulting in correct spatial (tissue) and temporal (developmental) gene expression. As discussed above, regulatory information often is present in an intronic sequence or at a considerable distance from the gene. The best example of such remote sequences required for high level and correct expressions are previously mentioned LCRs. To achieve position-independent, copy number-dependent and an optimal expression level in transgenic mice, all regulatory elements associated with a specific expression domain should be included in the construct. In those situations, larger DNA fragments such as yeast artificial chromosomes (YAC) or bacterial (BAC) artificial chromosomes-based constructs are used (Peterson, 1997; Camper & Saunders, 2000; Giraldo & Montoliu, 2001; McCormick, *et al.,* 2003). Large insert clones can also be produced with the P1 bacteriophage and with P1 artificial chromosomes (PAC). An example is the ApoB100 structural gene of 45 kb. Elements required for its liver expression are located 5 kb upstream from the gene, but only inclusion of elements located 54 to 62 kb upstream allows for its appropriate intestinal expression. Presence of 19 kb of exon 1 containing intronic sequences and 17.5 kb of the polyadenylation site was needed to obtain physiological levels of expression in the liver, but still intestinal expression was lacking (Linton *et al.,* 1993; Callow *et al.,* 1994). Only large 145 kb BAC allowed for appropriate expression in both locations (Nielsen *et al.,* 1998). Transgenic mice produced with YACs and BACs often show copy number-dependent and position-independent gene(s) expression, since the risk of positional effects of the chromosomal sequences flanking the site of transgene integration is minimized, hence fewer independent transgenic lines are needed for conclusive results regarding phenotypes. For a comparison of types of vectors currently available see Table 1.

*The average gene is about 30–40 kb but a gene's size can range from less than 2 kb to more than 1 Mb.

Table 1. Comparison of technical aspects of plasmid-based and artificial chromosome-based transgenesis.

Technical aspect	Plasmid-based constructs	YACs	BACs/PACs
Host for cloning	*E. coli*	*S. cerevisiae*	*E. coli*
Form of DNA in host	Circular plasmid, multiple copies/cell	Single linear chromosome/cell	Circular plasmid, 1–2 copies/cell
Form of transgene molecule	Linear	Circular	Linear or circular
Cloning capacity	up to 20	Up to several Mb	100–300 kb
Resistance to shearing and fragmentation	High	Low (quite susceptible)	High (in supercoiled form)
Protocols for handling and isolation of DNA	Easy	Use wide-bore pipette tips, never vortex or freeze DNA samples Difficult	Moderate
DNA yield	High	Low	Medium (BACs), high (PACs)
Selection markers in host cell	Ampicillin Tetracycline Kanamycine	Complementation of auxotrophic mutants, resistance to drugs	Resistance to antibiotics Kanamycine (PACs) Chloramphenicol (BACs)
Modification capabilities	Ligation	Utilizing endogenous HR system	Utilizing HR systems normally absent in host cells
Number of molecules injected	Hundreds per pronucleus	Tens per pronucleus	Tens per pronucleus
Transgene expression	Often copy number-independent and integration-site dependent Reduced when cloning vector is present	Can be copy number-dependent and integration-site independent No evidence that vector sequences have effect on gene expression	
Efficiency of transgenesis	Similar ranges with slightly lower eggs survival for large transgenes		
Integrated copy number	Up to hundreds	1-10 (usually low)*	1-12
Insert rearrangements	Rare (many inserts intact, but also some abnormal)	Often	Less often than for YACs (related to transgene's size)

HR, homologous recombination. *As high as 50 copies were reported, see Table 3.

The most striking differences between artificial chromosome-based DNA constructs types are size and form related. Thus, the biggest limitation of YAC-based constructs is their fragility and tendency to shear. Large DNA inserts can be stabilized in microinjection buffer (MIB) by the addition of salt and polyamines. The presence of vector sequences in the microinjected large constructs does not seem to have adverse effects on expression of the transgenes as long as important locus regulatory sequences are present (Schedl *et al.,* 1992; 1993a; Antoch *et al.,* 1997; Gama Sosa *et al.,* 2002). Rearrangements and insertion of fragmented transgenes can occur (Schedl *et al.,* 1992; Antoch *et al.,* 1997), thus thorough analysis of the integrated large fragments of DNA is recommended to assure that the established mouse line(s) are carrying an intact transgene.

Additional advantage of artificial chromosome-based vectors is that all necessary modifications (like an introduction of a mutation or selectable marker) can be accomplished utilizing homologous recombination (efficient in both bacteria and yeast) instead of restriction enzyme digestion followed by ligation used to construct plasmid-based vectors. Methods to reduce the size of artificial chromosome-based constructs at a precise location (Montoliu *et al.,* 1996), to add a selectable marker or to delete regulatory sequences and other modifications were reviewed by Giraldo & Montoliu (2001) and Copeland *et al.* (2001). A BAC construct can be injected in two different forms: as a linearized DNA fragment (Probst *et al.,* 1998), or as a circular supercoiled DNA (Antoch *et al.,* 1997; Gama Sosa *et al.,* 2002).

Numerous mutant complementation studies have been performed in transgenic mouse (reviewed by Camper & Saunders, 2000) leading to the discovery of new genes (i.e. Antoch *et al.,* 1997; Smith *et al.,* 1997; Majumder *et al.,* 1998; Probst *et al.,* 1998; Zhu *et al.,* 2000). Much improved models for human genetic diseases have been established using artificial chromosome-based approaches. YAC fragments as large as 1000 kb containing the entire human *PS-1* gene were used to study Alzheimer's disease (Lamb *et al.,* 1999). A YAC-based transgene of 1300 kb containing hu-

man *Ig light chain* was used to explore production of human antibodies in transgenic mice (Davies *et al.,* 1993).

EFFICIENCY OF STANDARD PLASMID-BASED TRANSGENESIS

Brinster and his colleagues in their paper from 1985 discussed factors affecting the efficiency of transgenic mouse production by pronuclear microinjections. They considered the influence of concentration of injected DNA, form and size of DNA fragments. They optimized microinjection buffer and compared the sites of injection on the efficiency of DNA integration rather than transgene expression. The study showed that the frequency of integration improves as the DNA concentration is increased until the level becomes toxic. They compared results of injection of supercoiled DNA *versus* linearized DNA with "sticky" ends, blunt ends or dissimilar ends for the same plasmid. Their results indicated that linear molecules with similar or different ends gave an integration efficiency of 24 to 31 percent of transgenic fetuses compared to about 8% for blunt-ended linear or supercoiled molecules. Their data also suggested that DNA integration following injection into the male pronucleus might be slightly more efficient than injection into smaller female pronucleus. Finally, they compared the efficiency of transferring genes into inbred C57BL/6 genetic background with that of a hybrid and concluded that nearly every aspect of working with inbred mice was less efficient. The size of DNA molecule ranging from 0.7 to 50 kb did not seem to be an influential parameter. The authors also pointed out that the overall efficiency of transgenic production is not measure solely by achieving a high percentage of positive fetuses, but it should account for the ability to produce good quality zygotes, skills of the person performing microinjections, ability of eggs to tolerate manipulation and to continue development after implantation into the foster moms. All the above factors affecting transgenic mouse production by DNA microinjection are strain-dependent. Additional factors not dependent on egg donor genetic background in optimized experimental conditions are purity and concentration of the DNA. Surprisingly, only a handful of papers have since attempted to address factors that influence the efficiency of transgenic mouse generation and specifically the choice of genetic backgrounds. The results reported in those publications and some of our recently published results are summarized in Table 2. Overview of this summary leads to several conclusion. Typically, mouse egg survival (portion of injected eggs suitable for transfer) ranges from 60 to 90 percent depending on donor strain. The fraction of injected eggs that result in born mice is in the range of 10 to more than 20 percent with typically only 2 to 4 percent of injected eggs resulting in transgenic founders.

Zygotes microinjection to produce transgenic mouse is a complex process consisting of a number of sequential steps controlled by many factors. Recently, we considered strain-dependent factors: the response of egg donor females to superovulation, fertilization rate, egg survival after injection, ability of manipulated embryos to implant and develop to term (Auerbach *et al.,* 2003) for four different egg donor mouse strains. We found significant influences of genetic background on the efficiency of different steps of the production (Auerbach *et al.,* 2003) with FVB/N mice consistently yielding the highest efficiency of transgenic mouse production at each step of the process with the exception of egg production.

We showed that there were no significant differences in the percentage of transgenics from the pups born for the four genetic backgrounds tested. This suggests that the frequency of DNA integration is the same in different strains, probably resulting from a universal mechanism of chromosomal integration common for different mouse strains. On

the other hand, embryo survival is influenced by the method of DNA purification and is strain-dependent. When the efficiency of transgenic mouse production is expressed as a percentage of eggs injected, a significant difference was seen between all four genetic backgrounds. Thus, expressing transgenic production efficiency, as a percentage of the eggs injected for the same strain is more informative for comparing the results obtained by different laboratories or from different protocols. In a paper comparing two methods of DNA preparation (Wall *et al.,* 2000), the authors concluded that one purification technique resulted in a higher embryo survival rate to term, but neither of two protocols of purification had a significant influence on transgene integration rate.

TRANSGENE QUALITY AFFECTS EFFICIENCY OF TRANSGENIC MOUSE PRODUCTION BY ZYGOTE MICROINJECTION

The quality of transgene DNA has a major influence on transgenic production. Toxicity of DNA can be a major cause of variability in transgenic production efficiencies. We found a substantial improvement in overall transgenic production efficiency after introduction of a uniform transgene purification protocol along with additional quality assurance and concentration determination.

DNA characteristics affecting efficiency of transgenic mouse production (formal considerations)

- Construct type (plasmid or artificial chromosome-based constructs, lentiviral or transposone vectors);
- Faulty construction (preventable by checking size of construct, sequencing, or restriction mapping);
- Size and form of DNA (linearized, circular or supercoiled);
- Contamination (phenol, ethidium bromide, ethanol, agarose, bacterial endotoxin generated during alkaline lysis, bacterial genomic DNA, proteins or dust particles);
- Integrity (can be influenced by storage temperature and buffer's composition);
- Accurate evaluation of the concentration (can be influenced by method of evaluation and by the presence of contamination from bacterial DNA or RNA and/or proteins).

Note: Eggs from different donor strains have different tolerance for injected DNA.

DNA transgenes purified according to the same protocol in some cases produced different efficiencies of transgenic production (Auerbach *et al.,* 2003). This likely reflects a difference in the purity of the DNA, since the lower efficiencies were associated with a high rate of egg lysis and 1-cell blocks. Poor quality DNA might affect every step of production with the cumulative lowering of the overall transgenic production efficiency.

It is not simple to evaluate levels of chemical contaminants in transgene sample. Some practical conclusions might be drawn from spectroscopy. Contamination with proteins or phenol might be detected in such a way. Another possible contaminant, bacterial endotoxin is a lipopolysaccharide, which is a major component of the outer membrane of most Gram-negative bacteria (*Escherichia coli*). It remains associated with the cell wall until disintegration of the bacteria. Endotoxin serves as a potent pattern recognition molecule that is detected by the innate immune system of multicellular organisms (Opal & Gluck, 2003). Concentrations of endotoxin higher than 10 EU/ml have a biological effect at the intracellular level (by binding to the cytoskeletal elements and affecting calcium channel as well as having an effect on eukaryotic signal transduction). We measured levels of endotoxin in DNA samples prepared by different protocols. For four out of five constructs higher than average rate of transgenic mice correlated with lower (below 0.02 EU/ml) level of endotoxin in corresponding DNA sample.

Table 2. Previously published efficiencies of transgenic mouse production with plasmid-based constructs

Donor Strain	# Eggs Injected	% Transferred/Inj. (Eggs Survival)	% Born/ Inj.	% TGs/ Inj.	Reference
C 57Bl/6	1463	70	5^	1^	Brinster *et al.*, 1985
B6SJL/F1	1201	79	9^	3.2^	
FVB/N	407	74	23	3.7*	
C57BL/6	195	72	7	0.5	Taketo *et al.*, 1991
B6SJL/F1	248	70	13	3.2*	
B6CBA/F1	1941	90	28	11.4*	Mann and McMahon, 1993
B6C3/F2	702	67	6	0.7^	
SWD2/F1	690	57	10	1.3^	Osman *et al.*, 1997
SWR	852	55	8	1.3^	
FVB/N	2 997	43-58	8-10^	< 0.2^	
B6SJL/F1	1 266	67	11^	0.2^	Paris *et al.*, 1995
B6CBA/F1	1 059	49	8^	< 0.2^	
CD-1	500	91	7^^	2.5^^	Canseco *et al.*, 1994
FVB/N	28 608	80	16	3	
B6D2/F1	30 369	72,5	13	2,1	Auerbach *et al.*, 2003
SW	54 027	61	12	1.7	
C57BL/6	7 139	71	9	1.2	
Typical		60 to 90	10 to >20	2 to 4	

^Note: Some data were not directly available from the paper, but calculation was possible. ^^ Calculated for 18.5 dpc only.
*A small portion of pups were not genotyped.

Even the same purification protocol produced different levels of endotoxin in purified DNA samples (not shown). Our observation is in line with previously made observations from the comparison of compiled results of transgene injection for samples purified according to the same protocol and producing different average production efficiencies for different laboratories (Auerbach *et al.,* 2003). Transgenic production experiments with expected lower egg survival like microinjection of artificial chromosome- based transgenes, might particularly benefit from the inclusion of an endotoxin removal step in the purification protocol.

Influence of the size of the DNA on transgenic mouse production efficiency may be analyzed by comparing results from microinjection of linear plasmid-based constructs (Table 2) with efficiencies obtained for microinjections using BAC-based DNA (Table 3). Our data from microinjection of 18 constructs into FVB/N eggs show that injection of supercoiled BAC DNA can produce almost the same rates of transgenic mouse production per egg injected (about 2%) as linear plasmid-based constructs (about 3%), indicating that the size of a DNA construct can have a lesser influence on the overall efficiency of transgenic production than the purity of the DNA sample. We observed somewhat lower egg survival with BACs compared to plasmid-based constructs. In our opinion, this reflects differences in composition and resulting final osmolalities of injected DNA solutions.

The amount of DNA transferred into the pronucleus is variable and depends on the volume, concentration and number of molecules injected. Typically, the volume of the pronucleus is doubled as 1-2pls of the solution are injected (Hogan *et al.,* 1986). Injecting a larger volume could lead to egg lysis, but injection of a smaller volume of more concentrated DNA could produce still acceptable number of founders. Injection of too many molecules of DNA occasionally may produce

very small litters with an incredibly high percentage of transgenic pups (up to 75%). However, the total number of transgenic offspring obtained from one experiment might be lower when too much of DNA is injected. Accurate DNA concentration in the range of 1–5 ng/μl is relatively hard to determine. Thus, it is useful to draw conclusions from two independent methods of its evaluation (e.g. gel electrophoresis and fluorometer). The number of molecules transferred depends on the construct size and ranges from hundred(s) of molecules for constructs smaller than 10 kb to just a few molecules for large DNA fragments of several hundred kilobases changing with used concentration and injected volume. The DNA concentration in the range of 0.25–0.5 ng/μl, thus much lower than typically used for standard construct microinjections, might help to control integrated copy number, when very

Table 3. Previously published efficiencies of transgenic mouse production with artificial chromosome-based constructs.

Cloning Vector	Egg Donor Strain	Transgene Size (kb)	Conc. Inj. ng/ml	Vector sequences	DNA Form	% Born /Inj.	% TGs /Born	%TGs /Inj.	Copy No.	Expressing founders	Reference
YAC	NMRI	35	2	Present	Circular	23.8	26	6.1	1 to 50	5 out of 9	Schedl *et al.*, 1992
			4			10.2	3.45	0.35			Schedl *et al.*, 1993a
YAC	NMRI	250		Present	Circular				1 to 8	5 out of 5	
			5			3.75	20.8 [1]	0.8			Schedl *et al.*, 1993b
YAC			1			n/a	11 to 31	n/a			Smith *et al.*, 1995
	FVB/N	up to 670		Present	Circular				1 to 9	4 out of 4	
P1			1			n/a	9 to 35	n/a			Smith *et al.*,1997
YAC	FVB/N	600	1	Present	Circular	6.2	14.8	0.9	1 to 2	3 out of 3	Hodgson *et al.*, 1996
		350				8.1	39.3	3.2	1 to 5	9 out of 9	
YAC	B6CBA/F1	420	5	Present	Circular	n/a	5.6	n/a	<1 to 10	4 out of 4	Schedl *et al.*, 1996
YAC	FVB/N	200	1	Present	Circular	7.4	33.3	2.5	5 to 10	3 out of 3	Majumder *et al.*,1998
YAC	B6CBA/F1	380	n/a	Present	Circular	n/a	9.3	n/a	2 to 22	3 out of 3	Sun *et al.*, 2000
BAC	C57BL/6	128	3	Present	Linear	no born					Yang *et al.*, 1997
			0.6			n/a	13.3	n/a	1 to 3	1 out of 1	
BAC	CD1 and	140	1	Present	Circular	n/a	7.8	n/a	1 to 10	4 out of 5	Antoch *et al.*, 1997
BAC	CD1 x (BALB7c x B6)	100	1	No	Linear	n/a	5.6	n/a	2 to 12	n/a	
BAC		160	1	Present	Circular	n/a	16.7	n/a	1 to 5	n/a	
BAC	n/a	140	n/a	No	Linear	15.7	12.5	2	n/a	1 out of 1	Probst *et al.*, 1998
BAC	B6SJL/F1 and FVB/N	68 to 124	0.5 to 3	No	Linear	n/a	15 [1]	n/a	1 to 4	22 out of 22 [2]	Nielsen *et al.*, 1998
BAC	C3H x C57BL/6	100	1	No	Linear	n/a	8.8	n/a	1 to 6	8 out of 9	Kaufman *et al.*,1999
BAC	B6SJL/F1	210	2	No	Linear	12	12	1.4	1 to 2	2 out of 3	Chrast *et al.*, 1999
BAC	B6D2/F1	125	1 to 2	Present	Circular	30.5	13	4	1 to 2	2 out of 2	Means *et al.*, 2001
BAC	C57BL/6 x KM	110	1	No	Linear	n/a	8.6	n/a	1 to 2	3 out of 3	Feng *et al.*, 2001
PAC	B6D2/F1	80	1	Present	Circular	n/a	37	3.5 - 4.7	n/a	n/a	Gama Sosa *et al.*,
BAC	and	143	1	Present	Circular	n/a	37	1.5 - 2	n/a	n/a	2000; and personal
BAC	B6C3/F1	120	1	Present	Circular	n/a	41	3.0 - 4	n/a	n/a	communication
PAC		204	1	Present	Circular	n/a	23	2- 2.7	n/a	n/a	
BAC	FVB/N, B6D2/F1 and C57BL/6	110 to 300	2 to 4	Present	Circular and linear	14.3	14.5	2	3 to 5	n/a	Injected at NYUSM [4]
	ICR		3			n/a	n/a	5 [1]	1 to 6	total:	Linton *et al.*, 1993
P1	B6SJL/F1	79,5	n/a	No	Linear	n/a	n/a	2.7 [1]	6 to 42	16 out of 20	
	B6SJL/F1		n/a			n/a	n/a	8.5 [1]	1 to 10		
P1	FVB/N	90	n/a	Present	Both	n/a	28.3	n/a	1 to 15	11 out of 13	Callow *et al.*, 1994
Typical						10 to 16		2 to 3		Majority [3]	

[1]Data from the expression assay; [2]Out of 43 founders, 21 were mosaic and not check for expression; [3]Unless not analyzed/rearranged/mosaic/fragmented transgene; [4]Results not published: 18 constructs injected into FVB/N eggs, 4 constructs injected into B6D2/F2 eggs and 1 injected into C57Bl/6 eggs. Only 2 out of 23 constructs were linearized and vector was removed; n/a, data not available.

low number or one copy integration is desired (Ellis *et al.,* 1997).

EFFICIENCY OF ARTIFICIAL CHROMOSOME-BASED TRANSGENESIS

Some of the results of previously published microinjection experiments with generating transgenic mice from artificial chromosome-based constructs for BAC, PAC, P1 and YAC cloning vectors are summarized in Table 3. The highest overall efficiency (percentage of injected eggs leading to transgenic mice) is reported for B6SJL/F1 hybrid strain when DNA was purified with a protocol containing $CsCl_2$ step (Linton *et al.,* 1993). For YAC- and BAC-based constructs usually fewer than ten copies of the transgene integrate with numerous founders having only one copy inserted into the genome. The presence of vector sequences in microinjected artificial chromosome-based constructs does not seem to have an overt effect on the expression of transgene. While it is possible in some instances that an effect does occur, there is no evidence to support this. When the integrated transgene is intact, the majority of analyzed founders express the transgenes. The transgenic mice described in Table 3 contain large, up to 670 kb, segments of foreign DNA.

FINAL COMMENTS

It may be best to analyze transgenes in hemizygous transgenic mice to avoid confusion resulting from an unexpected phenotype caused by an insertional mutation as already discussed. Mutations can also have different phenotypes or different phenotype severity in different genetic backgrounds (Smithies & Maeda, 1995; Barthold, 2000; Bullard & Weaver, 2002) due, for example, to the influence of modifier and compensatory genes. Interestingly, homozygote epithelial growth factor receptor (EGFR) knockout mice on CF-1 (outbred Swiss) background die in the preimplantation stage due to the inner cell mass degeneration, whereas on CD-1 (also outbred) background they live to the weaning age, but die due to multiple organ defects (Threadgill *et al.,* 1995). Those findings represent apparent difference between phenotypes in two closely related strains emphasizing the importance of background strain genetics. In addition, some characteristics like the strain's susceptibility to certain types of tumors might preclude its suitability for the specific experimental design. In behavioral studies, learning ability of the strain, or its inclination to explore its environment might be influential (reviewed in Ward *et al.,* 2000). Thus, the decision of genetic background in which transgenic lines would be maintained and analyzed ought to be thoroughly considered. Typically, founders produced in inbred background are bred with mice of the same inbred strain. If possible even the same sub-strain should be used (Barthold, 2002), or inbred mice of the same origin, since genetic drift can quickly introduce substantial genetic variation. When hybrid donors are used, founders need to be backcrossed for many generations if an inbred background is required. In those cases, it may be worthwhile making transgenics in C57BL/6 eggs in the first place, rather than using hybrid mice and subsequently backcrossing (Auerbach *et al.,* 2003). There is a possibility of problems arising with establishing appropriate controls for more and more complex experimental systems (Smithies & Maeda, 1995; Barthold, 2002), like crossing random-insertion transgenic mouse with targeted mutant and Cre recombinase transgenic, where three or four genetic backgrounds may contribute to the system. Available published data relevant to the experimental design referring to the specific genetic backgrounds will also be crucial for interpretation of the obtained results.

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REFERENCES

Albanese C, Hulit J, Sakamaki T, Pestell RG. (2002) Recent advances in inducible expression in transgenic mice. *Semin Cell Dev Biol.*; **13**: 129–41.

Antoch MP, Song EJ, Chang AM, Vitaterna MH, Zhao Y, Wilsbacher LD, *et al.* (1997) Functional identification of the mouse circadian clock gene by transgenic BAC rescue. *Cell.*; **89**: 655–67.

Auerbach AB, Norinsky R, Ho W, Losos K, Guo Q, Chatterjee S, Joyner AL. (2003) Strain-dependent differences in the efficiency of transgenic mouse production. *Transgenic Res.*; **12**: 59–69.

Barthold SW. (2002) "Muromics": genomics from the perspective of the laboratory mouse. *Comp Med.*; **52**: 206–23.

Beddington RS, Morgernstern J, Land H, Hogan A. (1989) An *in situ* transgenic enzyme marker for the midgestation mouse embryo and the visualization of inner cell mass clones during early embryogenesis. *Development.*; **106**: 37–46.

Beermann F, Ruppert S, Hummler E, Bosch FX, Muller G, Ruther U, Schultz G. (1990) Rescue of the albino phenotype by introduction of a functional tyrosinase gene into mice. *EMBO J.*; **9**: 2819–26.

Bishop JO, Smith P. (1989) Mechanism of chromosomal integration of microinjected DNA. *Mol Biol Med.*; **6**: 283–98.

Bishop JO. (1996) Chromosomal insertion of foreign DNA. *Reprod Nutr Dev.*; **36**: 607–18.

Brinster RL. (1972) Cultivation of the mammalian embryo. In *Growth, Nutrition and Metabolism of Cells in Culture*. Rothblat G, Cristafalo V, eds, **2**, pp 251–86. Academic Press, New York.

Brinster RL, Chen HY, Trumbauer ME, Avarbock MR. (1980) Translation of globin messenger RNA by the mouse ovum. *Nature.*; **283**: 499–501.

Brinster RL, Chen HY, Trumbauer ME. (1981a) Mouse oocytes transcribe injected *Xenopus* 5S RNA genes. *Science.*; **211**: 396–8.

Brinster RL, Chen HY, Trumbauer ME, Senear AW, Warren R, Palmiter RD. (1981b) Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell.*; **27**: 223–31.

Brinster RL, Chen HY, Messing A, van Dyke T, Levine NJ, Palmiter RD. (1984) Transgenic mice harboring SV40 T-antigene genes develop characteristic brain tumors. *Cell.*; **37**: 367–79.

Brinster RL, Chen HY, Trumbauer ME, Yagle MK, Palmiter RD. (1985) Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc Natl Acad Sci U S A.*; **82**: 4438–42.

Brinster RL, Allen JM, Behringer RR, Gelinas RE, Palmiter RD. (1988) Introns increase transcriptional efficiency in transgenic mice. *Proc Natl Acad Sci. U S A.*; **85**: 836–40.

Brocard J, Feil R, Chambon P, Metzger D. (1998) A chimeric Cre recombinase inducible by synthetic, but not by natural ligands of the glucocorticoid receptor. *Nucleic Acids Res.*; **26**: 4086–90.

Bullard DC, Weaver CT. (2002) Cutting-edge technology: IV. Genomic engineering for studies of the gastrointestinal tract in mice. *Am J Physiol Gastrointest Liver Physiol.*; **283**: G1232–7.

Callow MJ, Stlotzfus LJ, Lawn RM, Rubin EM. (1994) Expression of human apolipoprotein B and assembly of lipoprotein(a) in transgen-

ic mice. *Proc Natl Acad Sci. U S A.*; **91**: 2130–4.

Camper SA, Saunders TL. (2000) Transgenic rescue of mutant phenotypes using large DNA fragments. In *Genetic manipulation of Receptor Expression and Function.* Accili D, ed, pp 1–22. Wiley, New York.

Canseco RS, Sparks AE, Page RL, Russell CG, Johnson JL, Velander WH, Pearson RE, Drohan WN, Gwazdauskas FC. (1994) Gene transfer efficiency during gestation and the influence of co-transfer of non-manipulated embryos on production of transgenic mice. *Transgenic Res.*; **3**: 20–5.

Chada K, Magram J, Raphael G, Radice E, Lacy E, Costantini F. (1985) Specific expression of a foreign beta-globin gene in erythroid cells of transgenic mice. *Nature.*; **314**: 377–80.

Choi T, Huang M, Gorman C, Jaenisch R. (1991) A generic intron increases gene expression in transgenic mice. *Mol Cell Biol.*; **11**: 3070–7.

Chrast R, Scott HS, Antonarakis SE. (1999) Linearization and purification of BAC DNA for the development of transgenic mice. *Transgenic Res.*; **8**: 147–50.

Chrast R, Scott HS, Madani R, Huber L, Wolfer DP, Prinz M, *et al.* (2000) Mice trisomic for a bacterial artificial chromosome with the single-minded 2 gene (Sim2) show phenotypes similar to some of those present in the partial trisomy 16 mouse models of Down syndrome. *Hum Mol Genet.*; **9**: 1853–64.

Chung JH, Whiteley M, Felsenfeld G. (1993) A 5 element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila. *Cell.*; **74**: 505–14.

Cohen-Tannoudji M, Babinet C, Morello D. (2000) *LacZ* and ubiquitously expressed genes: should divorce be pronounced? *Transgenic Res.*; **9**: 233–5.

Copeland NG, Jenkins NA, Court DL. (2001) Recombineering: a powerful new tool for mouse functional genomics. *Nat Rev Genet.*; **2**: 769–79.

Costantini F, Lacy E. (1981) Introduction of rabbit β-globin gene into the mouse germ line. *Nature.*; **294**: 92–4.

Davies NP, Rosewell IR, Richardson JC, Cook GP, Neuberger MS, Brownstein BH, *et al.* (1993) Creation of mice expressing human antibody light chains by introduction of a yeast artificial chromosome containing the core region of the human immunoglobulin kappa locus. *Bio/Technology.*; **11**: 911–4.

DePrimo SE, Stambrook PJ, Stringer JR. (1996) Human placental alkaline phosphatase as a histochemical marker of gene expression in transgenic mice. *Transgenic Res.*; **5**: 459–66.

Ellis J, Pasceri P, Tan-Un KC, Wu X, Harper A, Fraser P, *et al.* (1997) Evaluation of beta-globin gene therapy constructs in single copy transgenic mice. *Nucleic Acids Res.*: **25**: 1296–302.

Ellison AR, Wallace H, Al-Shawi R, Bishop JO. (1995) Different transmission rates of herpes virus thymidine kinase reporter transgenes from founder male parents and male parents of subsequent generations. *Mol Reprod Dev.*; **41**: 425–34.

Erickson RP. (1999) Use of antisense to study gene expression. *Methods.*; **18**: 304–10.

Feng D-X, Liu D-P, Huang Y, Wu L, Li T-C, Wu M, *et al.* (2001) The expression of human α-like globin genes in transgenic mice mediated by bacterial artificial chromosome. *Proc Natl Acad Sci U S A.*; **98**: 15073–7.

Folger KR, Wong EA, Wahl G, Capecchi MR. (1982) Patterns of integration of DNA microinjected into cultured mammalian cells: evidence for homologous recombination between injected plasmid DNA molecules. *Mol Cell Biol.*; **2**: 1372–87.

Gama Sosa MA, De Gasperi R, Wen PH, Gonzalez EA, Kelley RA, Lazzarini RA, *et al.* (2002) BAC and PAC DNA for the generation of transgenic animals. *BioTechniques.*; **33**: 51–3.

Ganss R, Montoliu L, Monaghan AP, Schutz G. (1994) A cell-specific enhancer far upstream of the mouse tyrosinase gene confers high level and copy number-related expression in transgenic mice. *EMBO J.*; **13**: 3083–93.

Giraldo P, Montoliu L. (2001) Size matters: use of YACs, BACs and PACs in transgenic animals. *Transgenic Res.*; **10**: 83–103.

Giraldo P, Rival-Gervier S, Houdebine L-M, Montoliu L. (2003) The potential benefits of insulators on heterologous constructs in transgenic animals. *Transgenic Res.*: **12**: 751–5.

Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra, *et al.* (2003) A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature.*; **245**: 917–25.

Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH. (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc Natl Acad Sci U S A.*; **77**: 7380–4.

Gordon JW, Ruddle FH. (1981) Integration and stable germ line transmission of genes injected into mouse pronuclei. *Science.*; **214**: 1244–6.

Gordon JW, Ruddle FH. (1985) DNA-mediated genetic transformation of mouse embryos and bone marrow — a review. *Gene.*; **33**: 121–36.

Gossen M, Bujard H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A.*; **89**: 5547–51.

Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science.*; **268**: 1766–9.

Graessmann A, Graessmann M, Guhl E, Mueller C. (1978) Quantitative correlation between simian virus 40 T-antigen synthesis and late viral gene expression in permissive and nonpermissive cells. *J Cell Biol.*; **77**: R1–8.

Grosveld F, van Assendelft GB, Greaves DR, Kollias G. (1987) Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell.*; **51**: 975–85.

Gurdon JB, Melton DA. (1981) Gene transfer in amphibian eggs and oocytes. *Annu Rev Genet.*; **15**: 189–218.

Gutierrez-Adan A, Pintado B. (2000) Effect of flanking matrix regions on the expression of microinjected transgenes during preimplantation development of mouse embryos. *Transgenic Res.*; **9**: 81–9.

Hammer RE, Brinster RL, Palmiter RD. (1985) Use of gene transfer to increase animal growth. *Cold Spring Harbor Symp Quant Biol.*; **50**: 379–87.

Hodgson JG, Smith DJ, McCutcheon K, Koide HB, Nishiyama K, Dinulos MB, *et al.* (1996) Human huntingtin derived from YAC transgenes compensates for loss of murine huntingtin by rescue of the embryonic lethal phenotype. *Hum Mol Genet.*; **5**: 1875–85.

Hogan B, Costantini F, Lacy E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual.* 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Jaenisch R. (1976) Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. *Proc Natl Acad Sci U S A.*; **73**: 1260–4.

Jahner D, Stuhlmann H, Stewart CL, Harbers K, Lohler J, Simon I, Jaenisch R. (1982) De novo methylation and expression of retroviral genomes during mouse embryogenesis. *Nature.*; **298**: 623–8.

Kaufman RM, Pham CT, Ley TJ. (1999) Transgenic analysis of a 100-kb human beta-globin cluster-containing DNA fragment propagated as a bacterial artificial chromosome. *Blood.*; **94**: 3178–84.

Keegan CE, Karolyi IJ, Burrows HL, Camper SA, Seasholtz AF. (1994) Homologous recombination in fertilized mouse eggs and assessment of heterologous locus control regions function. *Transgenics.*; **1**: 439–49.

Kellendonk C, Tronche F, Casanova E, Anlag K, Opherk C, Schutz G. (1999) Inducible

site-specific recombination in the brain. *J Mol Biol.*; **285**: 175–82.

Kisseberth WC, Brettingen NT, Lohse JK, Sandgren EP. (1999) Ubiquitous expression of marker transgenes in mice and rats. *Dev Biol.*; **214**: 128–38.

Kozak M. (1987) An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.*; **15**: 8125–48.

Krumlauf R, Hammer RE, Brinster RL, Chapman VM, Tilghman SM. (1985) Regulated expression of α-fetoprotein genes in transgenic mice. *Nucleic Acids Res.*; **14**: 9667–78.

Kunkel TA. (1999) The high cost of living. *Trends Genet.*; **15**: 93–4.

Lakso M, Sauer B, Mosinger B, Lee EJ, Manning RW, Yu SH, *et al.* (1992) Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc Natl Acad Sci U S A.*; **89**: 6232–6.

Lamb BT, Bardel KA, Kulnane LS, Anderson JJ, Holtz G, Wagner SL, *et al.* (1999) Amyloid production and deposition in mutant amyloid precursor protein and presenilin-1 yeast artificial chromosome transgenic mice. *Nat Neurosci.*; **2**: 695–7.

Lacy E, Roberts S, Evans EP, Burtenshaw MD, Costantini FD. (1983) A foreign beta-globin gene in transgenic mice: integration at abnormal chromosomal positions and expression in inappropriate tissues. *Cell.*; **34**: 343–58.

Linton MRF, Farese RV, Chiesa G, Grass DS, Chin P, Hammer RE, *et al.* (1993) Transgenic mice expressing high plasma concentrations of human apolipoprotein B100 and lipoprotein(a). *J Clin Invest.*; **92**: 3029–37.

Lo C. (1986) Localization of low abundance DNA sequences in tissue sections by *in situ* hybridization. *J Cell Sci.*; **8**: 143–62.

Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science.*; **295**: 868–72.

Majumder K, Shawlot W, Schuster G, Harrison W, Elder FF, Overbeek PA. (1998) YAC rescue of downless locus mutation in mice. *Mamm Genome.*; **9**: 863–8.

Mann JR, McMahon AP. (1993) Factors influencing frequency production of transgenic mice. *Methods Enzymol.*; **225**: 771–81.

McCormick SP, Liu CY, Young SG, Nielsen LB. (2003) Manipulating large insert clones for transgenesis. In *Methods in Molecular Biology: Transgenic Mouse Methods and Protocols.* Hofker MH, van Deursen J, eds, vol. **209**, pp 105–23. Humana Press Inc.

McKnight RA, Shamay A, Sankaran L, Wall RJ, Hennighausen L. (1992) Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. *Proc Natl Acad Sci U S A.*; **89**: 6943–7.

Means GD, Boyd Y, Willis CR, Derry JM. (2001) Transgenic rescue of the tattered phenotype by using a BAC encoding Ebp. *Mamm Genome.*; **12**: 323–5.

Metzger D, Chambon P. (2001) Site- and time-specific gene targeting in the mouse. *Methods.*; **24**: 71–80.

Montoliu L, Umland T, Schütz G. (1996) A locus control region at –12kb of the tyrosinase gene. *EMBO J.*; **15**: 6026–34.

Montoliu L, Chavez S, Vidal M. (2000) Variegation associated with *lacZ* in transgenic animals: a warning note. *Transgenic Res.*; **9**: 237–9.

Nagy A. (2000) Cre recombinase: the universal reagent for genome tailoring. *Genesis.*; **26**: 99–109.

Nielsen LB, Kahn D, Duell T, Weier HU, Taylor S, Young SG. (1998) Apolipoprotein B gene expression in a series of human apolipoprotein B transgenic mice generated with *recA*-assisted restriction endonuclease cleavage-modified bacterial artificial chromosomes. *J Biol Chem.*; **273**: 21800–7.

Nilsson E, Lendahl U. (1993) Transient expression of a human β-actin promoter/*lacZ* gene introduced into mouse embryos correlates

with a low degree of methylation. *Mol Reprod Dev.*; **34**: 149–57.

Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. (1997) Green mice as a source of ubiquitous green cells. *FEBS Lett.*; **407**: 313–9.

Opal SM, Gluck T. (2003) Endotoxin as a drug target. *Crit Care Med.*; **31**: S57–64.

Osman GE, Jacobson DP, Li SW, Hood LE, Liggitt HD, Ladiges WC. (1997) SWR: an inbred strain suitable for generating transgenic mice. *Lab Anim Sci.*; **47**: 167–71.

Overbeek PA, Lai S-P, van Quill KR, Westphal H. (1986) Tissue-specific expression in transgenic mice of a fused gene containing RSV terminal sequences. *Science.*; **231**: 1574–7.

Palmiter RD, Chen HY, Brinster RL. (1982) Differential regulation of metallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. *Cell.*; **29**: 701–10.

Palmiter RD, Wilkie TM, Chen HY, Brinster RL. (1983) Transmission distortion and mosaicism in an unusual transgenic mouse pedigree. *Cell.*; **36**: 869–77.

Palmiter RD, Hammer RE, Brinster RL. (1985) Expression of growth hormone genes in transgenic mice. In *Banbury Report. 20. Genetic Manipulation of the Early Embryo.* Costantini F, Jaenisch R, eds, pp 123–32.

Palmiter RD, Sandgren EP, Avarbock MR, Allen DD, Brinster RD. (1991) Heterologous introns can enhance expression of transgenes in mice. *Proc Natl Acad Sci U S A.*; **88**: 478–82.

Paris D, Toyama PM, Sinet PM, Kamoun P, London J. (1995) A comparison between an inbred strain and hybrid lines to generate transgenic mice. *Mouse Genome.*; **93**: 21–3.

Perez CF, Botchan MR, Tobias CA. (1985) DNA-mediated gene transfer efficiency is enhanced by ionizing and ultraviolet irradiation of rodent cells *in vitro*. *Radiat Res.*; **104**: 200–13.

Petersen S, Casellas R, Reina-San-Martin B, Chen HT, Difilippantonio MJ, Wilson PC, *et al.* (2001) AID is required to initiate Nbs1/gamma-H2AX focus formation and mutations at sites of class switching. *Nature.*; **414**: 660–5.

Peterson KR. (1997) Production and analysis of transgenic mice containing east artificial chromosomes. In *Genetic Engineering.* Setlow JK, ed, vol. **19**, pp 235–55. Plenum Press, New York.

Probst FJ, Fridell RA, Raphael Y, Saunders TL, Wang A, Liang Y, *et al.* (1998) Correction of deafness in shaker-2 mice by an unconventional myosin in a BAC transgene. *Science.*; **280**: 1444–7.

Robinson MO, McCarrey JR, Simon MI. (1989) Transcriptional regulatory regions of testis-specific PGK2 defined in transgenic mice. *Proc Natl Acad Sci U S A.*; **86**: 8437–41.

Sands AT, Hansen TN, Demayo FJ, Stanley LA, Xin L, Schwartz RJ. (1993) Cytoplasmic β-actin promoter produces germ cell and preimplantation embryonic transgene expression. *Mol Reprod Dev.*; **34**: 117–26.

Schedl A, Beermann F, Thies E, Montoliu L, Kelsey G, Schutz G. (1992) Transgenic mice generated by pronuclear injection of a yeast artificial chromosome. *Nucleic Acids Res.*; **20**: 3073–7.

Schedl A, Montoliu L, Kelsey G, Schutz G. (1993a) A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice. *Nature.*; **362**: 258–61.

Schedl A, Larin Z, Montoliu L, Thies E, Kelsey G, Lehrach H, *et al.* (1993b) A method for the generation of YAC transgenic mice by pronuclear microinjection. *Nucleic Acids Res.*; **21**: 4783–7.

Schedl A, Ross A, Lee M, Engelkamp D, Rashbass P, van Heyningen V, Hastie ND. (1996) Influence of PAX6 gene dosage on development: overexpression causes severe eye abnormalities. *Cell.*; **86**: 71–82.

Schorpp M, Jager R, Schellander K, Schenkel J, Wagner EF, Weiher H, Angel P. (1996) The human ubiquitin C promoter directs high ubiquitous expression of transgenes in mice. *Nucleic Acids Res.*; **24**: 1787–8.

Schwenk F, Baron U, Rajewsky K. (1995) A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res.*; **23**: 5080–1.

Shani M. (1986) Tissue-specific and developmentally regulated expression of a chimeric actin/globin gene in transgenic mice. *Mol Cell Biol.*; **6**: 2624–31.

Smith DJ, Zhu Y, Zhang J, Cheng J-F, Rubin EM. (1995) Construction of a panel of transgenic mice containing a contiguous 2-Mb set of YAC/P1 clones from human chromosome 21q22.2. *Genomics.*; **27**: 2425–34.

Smith DJ, Stevens ME, Sudanagunta SP, Bronson RT, Makhinson M, Watabe AM, *et al.* (1997) Functional screening of 2 Mb of human chromosome 21q22.2 in transgenic mice implicates minibrain in learning defects associated with Down syndrome. *Nat Genet.*; **16**: 28–36.

Smithies O, Maeda N. (1995) Gene targeting approaches to complex genetic diseases: atherosclerosis and essential hypertension. *Proc Natl Acad Sci U S A.*; **92**: 5266–72.

Sonoda E, Takata M, Yamashita YM, Morrison C, Takeda S. (2001) Homologous DNA recombination in vertebrate cells. *Proc Natl Acad Sci U S A.*; **98**: 8388–94.

Soriano P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet.*; **21**: 70–1.

Stewart TA, Wagner EF, Mintz B. (1982) Human β-globin gene sequences injected into mouse eggs, retained in adult, and transmitted to progeny. *Science.*; **217**: 1046–8.

Sun T, Jayatilake D, Afink GB, Ataliotis P, Nister M, Richardson WD, Smith HK. (2000) A human YAC transgene rescues craniofacial and neural tube development in *PDGFRα* knockout mice and uncovers a role for PDGFRα in prenatal lung growth. *Development.*; **127**: 4519–29.

Taketo M, Schroeder AC, Mobraaten LE, Gunning KB, Hanten G, Fox RR, Roderick TH, Stewart CL, Lilly F, Hansen CT. (1991) FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc Natl Acad Sci U S A.*; **88**: 2065–9.

Tanaka S, Yamamoto H, Takeuchi S, Takeuchi T. (1990) Melanization in albino mice transformed by introducing cloned mouse tyrosinase gene. *Development.*; **108**: 223–7.

Threadgill DW, Dlugosz AA, Hanson LA, Tennenbaum T, Lichti U, Yee D, *et al.* (1995) Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science.*; **269**: 230–4.

Townes TM, Lingrel JB, Chen HY, Brinster RL, Palmiter RD. (1985) Erythroid-specific expression of human β-globin genes in transgenic mice. *EMBO J.*; **4**: 1715–23.

Wagner EF, Stewart TA, Mintz B. (1981) The human β-globin gene and a functional thymidine kinase gene in developing mice. *Proc Natl Acad Sci U S A.*; **78**: 5016–20

Wagner TE, Hoppe PC, Jollick JD, Scholl DR, Hodinka RL, Gault JB. (1981) Microinjection of a rabbit β-globin gene in zygotes and its subsequent expression in adult mice and their offspring. *Proc Natl Acad Sci U S A.*; **78**: 6376–80.

Wagner EF, Covarrubias L, Stewart TA, Mintz B. (1983) Prenatal lethalities in mice homozygous for human growth hormone gene sequences integrated in the germ line. *Cell.*; **35**: 647–55.

Wagner SD, Gross G, Cook GP, Davies SL, Neuberger MS. (1996) Antibody expression from the core region of the human IgH locus reconstructed in transgenic mice using bacteriophage P1 clones. *Genomics.*; **35**: 405–14.

Wall RJ, Paleyanda RK, Foster JA, Powell A, Rexroad C, Lubon H. (2000) DNA preparation method can influence outcome of transgenic animal experiments. *Anim Biotechnol.*; **11**: 19–32.

Ward JM, Anver MR, Mahler JF, Devor-Henneman DE. (2000) Pathology of mice commonly used in genetic engineering (C57BL/6; 129; B6, 129; and FVB/N). In *Pathology of Genetically Engineered Mice.* Ward JM, Mahler JF, Maronpot RR, Sundberg JP,

eds, pp 161–179, Iowa State University Press, Ames.

West AG, Gaszner M, Felsenfeld G. (2002) Insulators: many functions, many mechanisms. *Genes Dev.*; **16**: 271–88.

Whitelaw CB, Springbett AJ, Webster J, Clark J. (1993) The majority of G0 transgenic mice are derived from mosaic embryos. *Transgenic Res* **2**.; 29–32.

Wilkie TM, Brinster RL, Palmiter RD. (1986) Germline and somatic mosaicism in transgenic mice. *Develop Biol.*; **118**: 9–18.

Wilkie TM, Palmiter RD. (1987) Analysis of the integrant in Myk-103 transgenic mice which failed to transmit the integrant. *Mol Cell Biol.*; **7**: 1646–55.

Woychik RP, Stewart TA, Davis LG, D'Eustachio P, Leder P. (1985) An inherited limb deformity created by insertional mutagenesis in a transgenic mouse. *Nature.*; **318**: 36–41.

Yang XW, Model P, Heintz N. (1997) Homologous recombination based modification in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nat Biotechnol.*; **15**: 859–65.

Zhu Y, Jong MC, Frazer KA, Gong E, Krauss RM, Cheng JF, *et al.* (2000) Genomic interval engineering of mice identifies a novel modulator of triglyceride production. *Proc Natl Acad Sci U S A.*; **97**: 1137–42.