Comparative Medicine
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Vol 53, No 2
April 2003
Pages 126-139

Overview

Transgenic Animal Technology: Alternatives in Genotyping and Phenotyping

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Over the past decade, breakthrough technologies in transgenic animal technology and functional genomics have played a central role in the explosive growth of rodent modeling and in scientific innovation. Various noninvasive alternatives to routine surgical biopsy have been described for genotypic and phenotypic analyses of laboratory animals. A number of options are available to refine or replace potentially painful and invasive procedures ranging from tissue biopsies (including tail biopsies and toe docking) to several blood sampling techniques. Unfortunately, adoption of many non- or minimally invasive alternatives has proven difficult on a number of fronts ranging from historical reservations to procedural expectations and actual experimental productivity. Similarly, a variety of phenotyping considerations have addressed throughput efficiencies and the health and well being of research animals. From an animal welfare perspective, marked increases in laboratory animal populations have accompanied rapid advancements spanning the life sciences. As described for rodent modeling, but with applications across many laboratory animal species, diverse procedural refinements are available that will readily aid in the analysis of whole animal models. Ultimately, non-invasive technologies and complementary refinements have bearing on the quality and reproducibility of data that are reported, as well as of critical importance to the well being and ethical management of animals at all developmental stages: from fetal existence, to the neonatal period, and on through adulthood.

In 1996, Professor F. M. Loew stated that significant strides in animal well being would follow the adoption of minor modifications of existing procedures in the often-contentious realm of animal welfare (1). From an ethical standpoint, he went on to illustrate the development of various techniques that are commonplace in laboratory animal medicine today. Most importantly, improvements in various procedural processes in animal research and the daily lives of research animals have readily emerged as logical and humane extensions of our various scientific pursuits.

The techniques for generating genetically modified animals have evolved considerably over the past 25 years (Fig. 1). In contrast, many of the techniques for characterizing animals at a genetic or molecular level, since polymerase chain reaction analysis was adopted, have changed little. The application of recombinant DNA techniques to whole-animal systems through the creation of genetically modified "transgenic" animals (including gain-of-function or "over-expression" models created by DNA microinjection, and loss-of-function or gene-targeted events using embryonic stem [ES] cell transfer) allows precise development of experimental models to address specific hypotheses. The laboratory mouse, in turn, is presently the most widely used, available, and economical animal model used in biomedical research and in transgenic research, in particular.

In the early 1980s, success at gene transfer and the development of transgenic mice focused on gain-of-function models generated, using DNA microinjection or viral transfection methods. In gene transfer, animals receiving new genes (foreign DNA sequences integrated into their genome) were referred to as

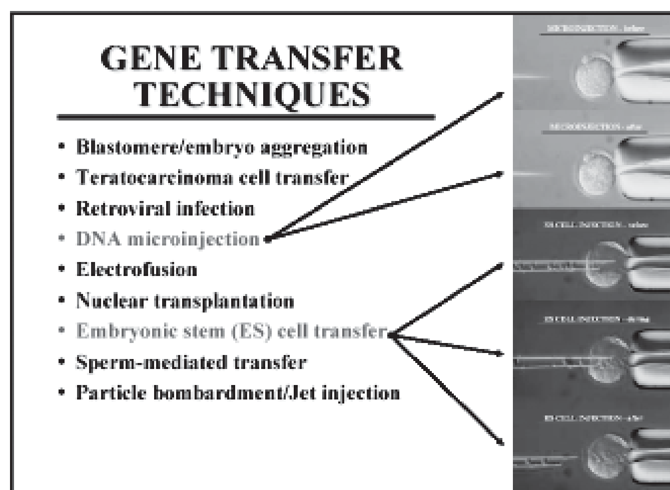

Figure 1. Gene transfer techniques. A number of gene transfer techniques have been used in the generation of genetically modified mice. Blastomere/embryo aggregation and teratocarcinoma cell transfer have been used to introduce whole genomes into developing embryos. In contrast, the remaining technologies have the advantage of modifying discrete gene sequences (i.e., for gain-of-function, loss-of-function, or conditional modeling). Microinjection of DNA and embryonic stem (ES) cell transfer are currently the two most practiced techniques for genetic engineering of mice, and are depicted on the right.

Received: 11/01/02. Revision requested: 1/07/03. Accepted: 1/28/03.
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"transgenic," a term first coined by Gordon and Ruddle in 1981 (2). In the 1990s, the term "transgenic" was extended to include chimeric or "knock-out" animals in which gene(s) have been selectively removed from the host genome, as well as nuclear transfer-derived models (3-5).

Production of genetically modified animals marked the convergence of previous advances in the areas of recombinant DNA technology and manipulation and culture of animal germplasm. Transgenic animals provide powerful models for exploration of the regulation of gene expression as well as the regulation of cellular and physiologic processes. Various experimental designs have taken advantage of our ability to direct specific (e.g., cell, tissue, organ specificity) as well as ubiquitous (whole-body) expression in vivo. From embryology to virology, transgenic technology provides unique animal models for studies in various disciplines that would otherwise be all but impossible to develop spontaneously (4-11). With advances in genomics and in the characterization of factors that control gene expression (including promoter-enhancer elements and transcription-regulatory proteins), gene transfer technologies have become proven assets in dissecting mechanisms involved in gene regulation and developmental pathways in vivo.

Many of us are interested in developing greater understanding of gene regulation and function in the context of the complex interactions of multiple cell types in the entire mammalian organism. Unfortunately, in vitro systems that mimic these complex and critical events are not available, necessitating use of intact animals. Any search of the literature by use of Medline or PubMed databases confirms this conclusion. Procedures for producing transgenic animals represent well developed systems for in vivo modeling in the life sciences. Therefore, the methods used to characterize these animals become critical and an integral component of the research process. Developing technologies that minimize trauma to animals, while having sufficient sensitivity to best characterize the developmental consequences of various manipulations, can go hand in hand. Consequently, the speed, cost (time, labor, and supplies), and reproducibility of the analyses remain the major obstacles to implementing changes in deference to techniques that enhance the well being of the experimental animal. It should become apparent that the gaps in these objectives are not mutually exclusive. Concerns for the welfare of animals used in research and in development of optimal experimental efficiencies can be resolved in a complementary rather than contrasting fashion.

Development of Transgenic Laboratory Animals

The relative importance of using particular strains or species of animals in gene transfer experimentation will vary markedly according to the model under consideration. Probably the most complex system is encountered in the production of transgenic mice, simply because so much work has been done with representative murine species. Well documented differences in reproductive productivity, behavior, related husbandry requirements, and responses to various experimental procedures influence the efficiency and degree of effort associated with production of transgenic founder animals. A general discussion of these factors and specific technologies, therefore, serves as an appropriate starting point for understanding the many processes and procedures that must be evaluated and monitored when considering genotypic and phenotypic characterization of subsequent animal models.

Microinjection of DNA. This procedure generally involves methods to physically inject a given DNA construct in solution into zygotes (mouse eggs shortly after fertilization has taken place). Virtually any cloned DNA construct can be used. With few exceptions, microinjected gene constructs integrate randomly throughout the host's genome, but usually only in a single chromosomal location (the "integration site"). This fact can be exploited to simultaneously co-inject more than one DNA construct into a zygote, where the constructs co-integrate together at a single, randomly located, integration site.

The integration process itself also is poorly understood, but apparently does not involve homologous recombination. During integration, a single copy or multiple copies of a transgene (actually as many as a few hundred copies of the particular sequence) are incorporated into the genomic DNA, predominantly as a number of copies in head-to-tail concatemers. Regulatory elements in the host DNA near the site of integration, and the general availability of this region for transcription, appear to play major roles in affecting the level of transgene expression. This "positional effect" is presumed to explain why the levels of expression of the same transgene may vary markedly between individual founder animals as well as their offspring (or "lines"). It is, therefore, prudent to examine transgene expression in offspring from at least three or four founder animals (or lineages) to determine what might be a result of the integration location, and what might reflect the activity of the transgene.

Host DNA near the site of integration frequently undergoes various forms of sequence duplication, deletion, or rearrangement as a result of transgene incorporation. Such alterations, if sufficiently drastic, may disrupt the function of normally active host genes at the integration site and constitute "insertional mutagenesis," wherein an aberrant phenotype may result. Such events cannot be purposefully designed, but have led to the serendipitous discovery of previously unsuspected genes and gene functions. Because DNA microinjection is usually accomplished in ova at the one-cell stage, transgene incorporation should occur in essentially every cell that contributes to the developing embryo (and in some instances, multiple integration events may occur within the zygote). Yet, as we learned in some of the earliest transgenic animal experiments, the integration event could also occur beyond the first cleavage division (resulting in transgene mosaicism at birth; 12). However, incorporation of a transgene into cells that will eventually contribute to development of germ cells (spermatozoa or ova) is a common occurrence associated with this method, and makes heritability of the transgene by offspring of founder animals likely within one generation. In such instances, the transgene has been said to be germline or the animals are referred to as "germline-competent." In contrast, integration of the microinjected DNA construct into the host's genome occasionally may be inexplicably delayed. In such a case, if cells of the early embryo (blastomeres) undergo mitosis before the transgene-integration event occurs, some but not all of the cells will contain the transgene, and the founder animal, although still considered to be transgenic, will be a mosaic, with a transgene-expression profile that may not be representative of the lineage. With the advent of exquisitely sensitive qualitative as well as quantitative PCR analyses, founder transgenic animals could be identified where the relative pro-

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portion of genetically modified cells were by far in the minority, thereby preventing the loss of potential valuable founders (13).

Retrovirus-mediated gene transfer. Transfer of foreign genes into animal genomes has also been accomplished, using viral transfection procedures. Although embryos can be infected with retroviruses up to midgestation, ova usually at the four- to 16-cell stage are used for infection, with one or more recombinant retroviruses harboring an insert of interest. Immediately following infection, the retrovirus produces a DNA copy of its RNA genome, using reverse transcriptase. Completion of this process requires that the host cell undergoes the S phase of the cell cycle; therefore, retroviruses effectively transfect only mitotically active cells. Procedures for stably transfecting a host of cell types are still an active area of investigation. Modifications to the retrovirus frequently consist of removal of structural genes, such as *gag*, *pol*, and *env*, which support viral particle formation. Additionally, most retroviruses and complementary lines are ecotropic in that they infect only rodents, and rodent cell lines rather than humans. Although it is a boon to the safety of such experiments to the researcher or technician as well as the utility in developing animal models, such specificity belies the usefulness of such ecotropic vectors in developing vehicles for human gene therapy.

Embryonic stem cell technology. The ES cell transfer techniques have been used to produce random and targeted insertions; the latter also are used for modification or ablation of discrete DNA sequences in the mouse genome. Comparatively, when using DNA microinjection for targeted insertion, the gene-targeting efficiency is extremely low (14, 15). The use of ES cell transfer into mouse embryos has been quite effective in allowing one to identify a specific genetic modification (a targeted event), via homologous recombination, at a precise chromosomal position. This selection capability has led to the production of mice that have incorporated a particular gene randomly within their genome: “knock-in” mice that carry modified endogenous genes, and knockout models that lack a specific endogenous gene following targeting techniques. Indeed, technologies involving ES cells, and primordial germ cells (PGCs), have been used to produce a host of useful and exciting mouse models (15-17).

Pluripotential ES cells are derived from early pre-implantation embryos and are maintained in culture for a sufficient period for one to perform various in vitro manipulations. The genome of ES cells can be manipulated in vitro by introducing foreign genes or foreign DNA sequences by use of techniques, including electroporation, microinjection, precipitation reactions, transfection, or retroviral insertion. The use of ES cells to produce transgenic mice faced a number of procedural obstacles before it became competitive with DNA microinjection as a standard technique in mouse modeling. Within the last few years, the addition of coculture techniques involving tetraploid host embryos (eight-cell stage to morula), has resulted in founders that can be derived completely from the cocultured ES cells (18; Fig. 1). Hence, the founders are no longer chimeras, as all the cells come from the same progenitor cells, and the founder animals will breed true (and faithfully transmit the genetic modification in the first generation offspring).

Yet, although ES cell lines have been identified for species other than the mouse, to the author’s knowledge, the production of germline-competent ES cell-derived/chimeric farm animals has not been reported. With the advent of nuclear transfer-related technologies, the need to identify and use ES or PGCs to effect genetic change may become of lesser consequence.

From spermatozoa to nuclear transfer. In contrast to progress in embryo manipulation, a completely different route to transgenesis was taken with the advent of spermatozoal-related transfer procedures. In 1989, spermatozoa-mediated gene transfer was reported but was quite controversial when it was unrepeatable by others (19-21). Yet, the initial spermatozoa-mediated gene transfer story generated sufficient interest that led to the development of spermatogonial cell transplantation procedures as feasible alternatives for in vivo gene transfer (22, 23). However, whole-animal and somatic cell techniques (including liposome-mediated gene transfer, particle bombardment, and jet injection), coupled with novel vector systems, will continue to evolve to genetically engineer animals in an efficient and effective manner.

Similarly, in the early 1980s, a number of laboratories reported on nuclear transfer experiments in laboratory animals. Although initial studies were also controversial (and unrepeatable), interest in nuclear transfer did not abate and has now taken on greater emphasis as a vehicle for genetic engineering, particularly as the jump from mouse ES cells to those of other species did not progress as originally anticipated.

In either stem cell-based or nuclear transfer techniques, the power of gene transfer technology is catapulted forward because such processes allow targeted sequence insertions into the genome. Such targeting is extremely important, as previous technologies were best suited to generation of random integration events.

From the nucleus to the mitochondrion. Until recently, in vivo mitochondrial transfer remained a technologic hurdle in the development of mitochondrial-based gene transfer and therapies. In humans, metabolic and cellular pathologic changes exist due to mutations arising exclusively within the mitochondrial genome, yet until recently, a directed means of modifying the mitochondrial genome in vivo was not available. Various diseases have been associated with mitochondrial DNA (mtDNA) point mutations, deletions, and duplications, as well as age-associated changes in the functional integrity of mitochondria. Therefore, for agricultural and biomedical research efforts, the ability to manipulate the mitochondrial genome and to regulate the expression of mitochondrial genes would provide one possible mode of genetic manipulation and therapy. Studies revolving around mitochondrial transfer and techniques to produce animals harboring foreign mitochondrial genomes have been initiated to date (24). The creation of heteroplasmic transmitochondrial mice represents a new model system that will provide a greater understanding of mitochondrial dynamics, leading to the development of genetically engineered production animals and therapeutic strategies for human metabolic diseases affected by aberrations in mitochondrial function.

Analytical Technologies

Analysis of genetic manipulations: transgenes and other modifications. There are a number of strategies in the development of transgenic mouse models, including systems designed to study: dominant gene expression, homologous recombination/gene targeting, and use of ES cells; efficiency of transformation of eggs or cells; disruption of gene expression by anti-sense transgene

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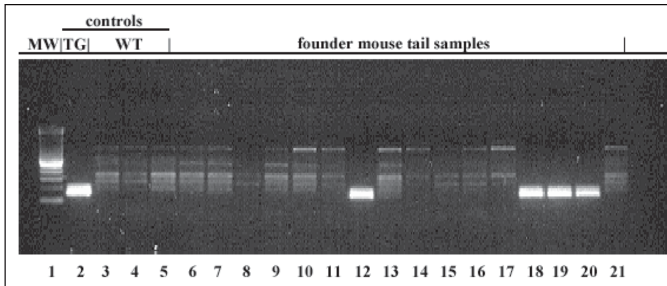

Figure 2. Polymerase chain reaction (PCR) analysis of founder mice. Analysis of tail biopsy specimens from founder mice where four of 16 offspring harbor the DNA sequences of interest. Lanes: 1, 100-bp ladder (MW, molecular weight marker). 2–21, polymerase chain reaction amplification products of: 2, positive control (TG; transgenic mouse DNA); 3–5, negative controls (WT; wild-type normal mouse DNA); 6–21, amplified DNA samples from tail biopsies of 16 founder pups, with the foreign DNA sequence identified in mouse specimens in lanes 12, 18, 19, and 20. Although non-specific or mispriming of endogenous sequences is observed with normal mouse DNA, the strong bands in lanes representing the positive control and four genetically modified offspring are clear (74).

constructs; gene ablation or knockout models; reporter genes; and marking genes for identification of developmental lineages.

To identify transgenic mice or for routine genetic monitoring procedures, tissue biopsy specimens or blood are commonly used as sources of cells for DNA analysis (Fig. 2; 6, 13, 25). Alternatively, phenotypic evidence of gene expression may be used to simplify the screening process (Fig. 3; 26, 27).

In routine nucleic acid analyses, DNA samples are qualitatively and/or quantitatively analyzed first by use of polymerase chain reaction (PCR; Fig. 2) analysis, and the results are subsequently verified by use of hybridization analysis (e.g., Southern blotting), and possibly, gene expression analyses. The PCR procedure can be completed within one day, while the more labor-, time-, and cost-intensive procedures may take three days or longer to complete.

Generally, PCR or DNA hybridization analyses (from dot/slot blot to Southern blot analyses) are used to identify ova and mice harboring genetic modifications. Reverse transcriptase-PCR (RT-PCR) analysis, and RNA slot blot and northern blot analy-

Figure 3. Phenotypic characterizations. Coat color, growth rate, and fluorescence of a GFP (7) gene product are representative of the alternatives used in evaluating founder mice. (A) Offspring resulting from an F1 × F1 cross where coat color segregates randomly. This combined with identification of the sex of the pups can usually be used as an intermediate identification of specific mice in a litter, minimizing the need for other permanent identification procedures. (B) Following activation of an oncogene that limited the growth of founder mice, the clear growth differential could be readily gauged. (C) A chimeric founder derived by ES cell transfer, when mated to a C57BL/6 (black coat color mouse) gave rise to one agouti pup in the first generation litter, indicative of germline transmission of the injected ES cells (and later verified by DNA analysis, using PCR analysis or hybridization protocols). (D) Targeting ubiquitous expression of a GFP marker gene to study whole-animal gene expression, UV light can be used to analyze for the presence or absence of whole body, tissue specific, or developmentally regulated gene expression.

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ses are techniques used to confirm transgene-encoded mRNA expression and to analyze developmental- and tissue-specific dynamics associated with the given genetic manipulation. The minimal number of animals necessary to characterize a particular genetic modification is another contentious issue faced by Institutional Animal Care and Use Committees. In identifying power calculations and minimal numbers of animals necessary to characterize a given modification, it has been well documented that some animals within lineages will not accurately represent the genetic modification at hand or even the earlier characterized model (e.g., rearrangements, deletions, non-functional transgenes, integration events in transcriptionally silent loci, as well as various considerations associated with genetic background of animals and mutations elsewhere within the genome).

Although tail or other specific biopsy procedures may be standardized in a laboratory, analysis of a single tissue may or may not provide the information necessary to identify founders. Beyond founder identification as well as in the absence of germline transmission when transgenics are bred, analysis of more than one tissue type in a given individual may aid in identifying potentially mosaic founders. Additionally, such analyses go beyond characterization of the integration event, toward the study of episomal or extra-nuclear maintenance of genetic material as well (16, 18, 24, 27, 28).

For DNA microinjection, each founder animal, as well as offspring from any given line, likely will be unique, with an uncharacterized inheritance pattern/distribution. From transfer and evaluation of nuclear transgenes, full sibs have been documented where logarithmic-fold differences in transgene expression or function were observed (29-31). Due to these differences in initial characterizations, multiple sampling may be needed to better explore and illustrate gene function. As such, sequential tissue biopsies may be necessary beyond an initial tail and or tissue biopsy, and may also include partial organ resection (e.g., partial hepatectomy or splenectomy). Therefore, availability of a noninvasive and repeatable procedure would alleviate the associated pain and discomfort attributable to the given tissue sampling procedure.

Presence of the genetic modification of interest. Representative tissues for analysis of transgene integration and expression can be obtained at birth. For integration analysis, blood or tail tissue typically is obtained. Preliminary determination of transgene integration by use of PCR analysis can be useful when the target sequence (the transgene) has unique sequences (not endogenous to the genome of the animal). However, given the extreme sensitivity of PCR analysis, other more informative techniques (either DNA slot blot or Southern blot hybridization) should be used to confirm the presence of a transgene. Southern blotting is the most useful, as it can indicate transgene copy number, length of a given target sequence, and possible sequence mutation. Additionally, a transgene can be constructed to include a molecular "tag"— a unique sequence that is easily detected and has minimal similarity to any endogenous sequence. This strategy is especially useful when the transgene itself is similar or nearly identical to an endogenous gene. In such instances, another strategy that may be helpful is the introduction of new restriction sites into the transgene without perturbing function, so that restriction fragment length polymorphism (RFLP) analysis can be used to distinguish the genetic modification from its endogenous counterpart. In some instances, phenotypic screening is possible for gain-of-function and loss-of-function studies, if the genetic modification leads to an identifiable change in the appearance of the animal. Phenotypic screening by co-transfer of a second transgene or marker that gives rise to a specific phenotype also can be useful, particularly in models where the marker co-integrates with the sequence of interest in DNA microinjection, or the marker can be associated with the particular cell line used in nuclear transfer. In either scenario, the integration of the desired genetic modification must subsequently be confirmed by use of other direct means. The utility of phenotypic expression for training purposes is undeniable, particularly as it minimizes the actual numbers of animals needed. Yet the confounding effects of co-injected genes and impact on subsequent expression profiles may otherwise limit the usefulness of such procedures (12, 26, 27).

Other concerns related to analysis of the genetic modification vary as to gain- or loss-of-function models. In gain-of-function models or where a suppressor gene is used to induce loss of function, it is important to characterize copy number of the transgene per cell, orientation of tandemly arranged copies, the presence of multiple integration sites, and possibly methylation state (or identifying other posttranslational events). These questions can be addressed by use of Southern blot hybridization following digestion of the genomic DNA with appropriate restriction enzymes. In contrast, in loss-of-function studies where a targeted disruption or conditional knock-out is predicated on a secondary event (e.g., Cre-lox targeting vectors where a particular sequence is excised from the genome), analyzing the modified chromosomal locus is warranted to characterize the specific targeting event. Lastly, the critical question of whether the gene modification is stably heritable must be answered by analysis of offspring.

Polymerase chain reaction analysis is used for the identification of transgenic founder mice or their offspring (Fig. 2). Numerous sources of information are available that deal with the theory, performance, and optimization of the PCR procedure in a variety of applications (6, 8, 13, 25, 32, 33). Rather, this brief overview is intended to address some of the general considerations for the PCR procedure and some of the particular concerns when using this technique to detect transgene integration in experimental animals (especially laboratory mice). Although standard PCR analysis is exceedingly useful for integration analysis, it has limitations. For example, PCR analysis can detect integration of the transgene only, and, in its basic form, provides no information on expression of the transgene. Reverse transcriptase-PCR analysis can be useful in detecting and quantifying gene expression. However, RT-PCR protocols are somewhat more complex than is conventional PCR analysis, as might be anticipated, and pose a number of practical considerations (34).

Real-time PCR methods are gaining considerable attention in genomic analyses due to their power as quantitation tools (Fig. 4; 13, 35, 36). Although PCR techniques have often replaced labor-intensive hybridization analyses, real-time PCR analysis is not as commonplace at this time, principally due to cost and time constraints. Otherwise, what distinguishes real-time PCR analysis from conventional methods is that the amplification product is measured after each extension/cycle, rather than at completion of all of the cycles (endpoint determination).

The high sensitivity and quantitative nature of real-time PCR analysis makes it especially useful in transgene analysis.

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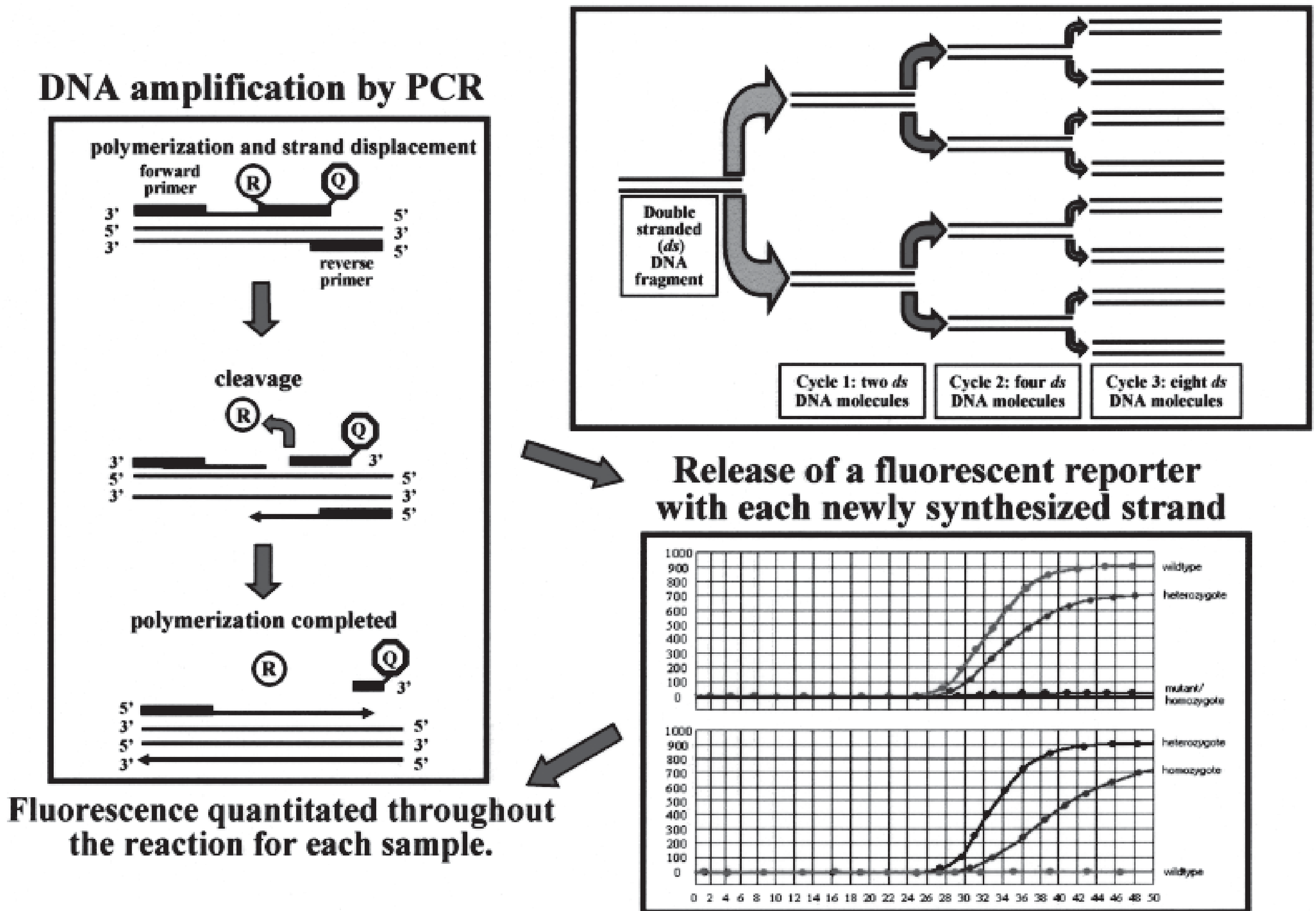

Figure 4. Real-time PCR analysis. The advantage that real-time PCR analysis gains over conventional PCR analysis relates to use of a fluorescent reporter during each amplification cycle to quantify relative amounts of substrate. This allows extrapolation of the sample's initial concentration and at each cycle throughout the analysis. As noted in the lower right panel, the sensitivity and quantitative nature of real-time PCR analysis can be used to discriminate between a wild-type, single copy (hetero- or hemizygote), and two-copy (homozygote) insertion. R = reporter molecule; Q = quencher molecule. (DNA amplification adapted from <http://views.vcu.edu/dnalab/about_RTPCR.htm>; real-time PCR trace adapted from <http://www.epochbiosciences.com/>, Epoch Biosciences, Bothell, Wash.).

The technique can identify and quantify insertion events and can characterize sequence stability over time (37, 38). Additionally, determination of copy number and zygosity is possible: whether an organism is hetero/hemizygotic or homozygotic for a targeted allele. This is especially useful for low-copy integrations and differentiation from wild-type offspring when endogenous genomic sequences are introduced into the genome (39). Real-time methods can also be combined with RT-PCR analysis of mRNA products, allowing one to monitor gene expression profiles with exquisite sensitivity (13, 34).

Lastly, beyond individual sample preparation or batch handling, all of the techniques, from PCR to hybridization analyses are amenable to high throughput genomic analyses, as described for microarray and proteomic technologies. Undeniably, the use of multi-well formats (with accommodation of 96- to 1,536-well plates) provides an economy of scale and subsequent rapid completion of multiple analyses, facilitating characterization of genetic modifications, genetic monitoring across the entire genome, and animal health status.

Transgene-encoded mRNA expression. Although absence of mRNA expression is likely for loss-of-function models and may only represent a confirmatory step of minor importance, the analysis of expression of the transgene is absolutely essential in determining the usefulness of a particular transgenic animal. The most critical step in analyzing transgene-encoded mRNA expression is the isolation of intact RNA. Care must be taken to avoid contamination of RNA preparations with ribonucleases (enzymes that degrade RNA). The presence of a specific mRNA is usually determined by use of RNA slot-blot or northern blot hybridization. Northern blotting is more informative, as it confirms not only the presence, but also the size of the mRNA transcript of interest.

Additional techniques exist for determining the presence of relative amounts of mRNA transcripts from transgenic animals. In a nuclease protection assay, a labeled probe is allowed to hybridize to the RNA in solution, followed by nuclease digestion of non-hybridized RNA. The sample is then resolved on a polyacrylamide gel. This technique is useful in determining the steady-state amounts of RNA in a given tissue. In the RT-PCR assay, the RNA of interest is transcribed into a cDNA molecule by use

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of a specific, random, or oligo d(T) primer and the enzyme, reverse transcriptase. Standard PCR amplification is then performed. The advantage of RT-PCR analysis lies in its extreme sensitivity; theoretically, as few as a single mRNA molecule can be amplified to a quantity sufficient for visualization on an agarose gel. Alternatively, in an in situ hybridization technique, a labeled probe is hybridized to a target mRNA transcript in sections of tissue so that individual cells containing the transcript can be identified. This technique is particularly useful in identifying gene expression in a small subset of cells within a given tissue, which might prove difficult to detect by other means. Lastly, although laser dissection and RT-PCR analysis provide an interesting and sensitive combination, various constraints and expenses associated with initiating such analyses are still quite prohibitive at this time (40-42).

Transgene-encoded protein expression. The first phenotypic characterization of genetically modified mice was greatly stimulated by the initial experiments where body size and growth rate were markedly affected in transgenic mice expressing growth hormone transgenes driven by a metallothionein enhancer/promoter (43). Use of such constructs was intended to allow tight regulation of individual transgene expression by dietary supplementation. However, although resulting phenotypes included enhanced growth performance, increased productivity was accompanied by undesirable developmental sequellae (44, 45).

Various techniques are often used to identify the unique protein itself or, perhaps, a specific enzyme activity. The immunoblotting technique, in which proteins are resolved on a polyacrylamide gel, transferred to a membrane, and detected by use of a labeled antibody, is useful in verifying the appropriate molecular weight of the protein product of interest. To identify which cells within a tissue contain the protein product of the transgene, immunohistochemical staining of tissue sections with a labeled antibody can also be used. Additionally, the use of reporter genes can often simplify determination of expression levels by producing a protein that is easily and unequivocally detectable.

In analyzing the expression of any transgene, it is always important to evaluate at least two or more separate lines of transgenic animals (or use of more than one clone of cells in ES cell studies) to document that the expression patterns and or phenotype are consistent and reproducible. It is quite common for the site of integration in a given line of transgenic animals to have a profound influence on transgene expression, independent of any transcriptional control sequences in the transgene itself.

Reporters and markers. A number of vector systems, including transgene constructs, can be used to identify genotypic incorporation and phenotypic expression in expeditious and noninvasive fashion. A number of transgene constructs were identified in the late 1980s and early 1990s that could be used as reporters in generating founder animals (12, 27). Some transgene constructs allowed assessment of factors affecting integration efficiency while minimizing the confounding losses during in vivo development. After microinjection of an elongation factor 1-α promoter (46) driving a *lacZ* reporter gene (*EF-GAL*) into pronuclear eggs, the eggs could be cultured in vitro or in vivo through the blastocyst stage and stained for β-galactosidase activity at any time during subsequent development (12).

The EF-GAL construct also was used to analyze the effect of DNA concentration and the type of DNA ends on foreign gene integration. For example, transgene integration frequency increased rapidly with increasing concentrations of DNA in the injection aliquot (nanograms of DNA/microliter of injection buffer); however, egg viability diminished in direct opposition to increased DNA concentrations (12).

In addition, an elastase-EJ *ras* fusion construct was used (26, 47) for training purposes, as well as foreign gene integration studies. This construct produces a visible phenotype (abdominal enlargement, pancreatic tumor formation, and ascites accumulation) at days 18 to 20 of gestation. While obviating the need for biochemical or molecular analyses, this construct affords the trainee an opportunity to evaluate the entire spectrum of procedures necessary to produce transgenic mice. In turn, substantial variation in transgene expression between individuals, litters, or generations can doom a transgenic model at several stages. It can complicate the initial characterization of the model if the genetic background severely influences the transgene expression. Alternatively, as the model is bred through several generations, inbreeding can alter transgene expression slowly or bring out new recessive phenotypes unrelated to the transgene.

Using genes that code for reporter proteins (e.g., oncogenes, growth factor genes, *lac Z*/β-galactosidase, luciferase, or fluorescent protein genes; Fig. 3), analysis of transgenic animals has revealed the importance of various regulatory factors in determining developmental timing, tissue distribution, and relative efficiency of gene expression. Additionally, transgenic animals have also proven quite useful in determining in vivo artifacts of other model systems or techniques.

Phenotypic analysis can include analysis of developmental characteristics, such as coat color, eye pigmentation, growth rate, tumor formation, or other altered developmental characteristics (Fig 3; 27). Histologic evaluation and behavioral characteristics can complement the wide range of identified marker genes in the identification of genetically modified animals. From a behavioral standpoint, Crawley's 2000 text on behavioral phenotyping extends the realm of phenotypic characterizations in the fields of behavioral neuroscience and genetics in a manner most conducive to understanding complex traits that may be affected in genetically modified animals (48). Beyond ruling out health status concerns unrelated to a given genetic modification, the author explores motor functions, sensory abilities, learning and memory traits, and hypothalamic/higher brain control (from feeding and drinking analyses to reproductive, social, and emotional behaviors). The categorization of various domains useful in behavioral phenotyping addresses what would otherwise be complex and confounding genetic traits. In the end, it is not just the genes, but other genetic modifiers as well that lead to the combined action of many genes and differential patterns of transcription and translation that influence the development and potential usefulness of a genetic modification in vivo.

Development of Non-invasive Methods

Conventional tail biopsy and DNA extraction. Conventional tail biopsy (amputation or tissue excision) is usually done when mice are weaned at 18 to 22 days of age, although other times have been described in literature, from shortly after birth through adulthood (6, 13, 25). Generally, the distal one centimeter (or less) of the tail is excised, using a sterile razor blade or scalpel against a hard surface (disinfected or sanitized stainless steel or Plexiglas surface) or excised using sanitized or sterile

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scissors. The wound may then be cauterized, and each mouse is returned to a freshly prepared cage. The excised/amputated tail specimen is then enzymatically digested, and the DNA is extracted. Microgram quantities of total DNA are needed for techniques, such as Southern blot hybridization and DNA dot blotting. In contrast, PCR analysis requires only picogram to nanogram quantities of DNA. For established and well-characterized lineages, testing by PCR analysis alone is usually sufficient to confirm a given genotype.

For mice four weeks of age or older, biopsy, including that of the tail, is performed after mice are first anesthetized or given a local analgesic agent (6, 12). The basis for anesthesia or analgesia for tail biopsy relates principally to the ossification of the caudal vertebrae and resulting pain perception or sensitivity (49, 50). Alternatively, mice with developmental abnormalities, including blood clotting or wound healing defects, or skeletal deformities (including those lacking tails or distal segments) would require additional attention and procedural handling or other sampling techniques.

Identification of biopsied mice. After biopsy is performed, if color and sex of animals in a litter are not sufficient to discriminate between specific animals in a cage (e.g., many experiments involving use of hybrid strains result in offspring where coat color segregation is helpful in animal identification; Fig 3A [5]), ear punch or toe amputation identification techniques are still routinely used in many laboratories (Fig. 5A). For short-term and less painful (or painless) identification, a waterproof, non-toxic, permanent felt-tip marker (e.g., Sharpie brand fine point to broad tip markers; Fig. 5) may also be used to write a number or code on the ear or on the proximal portion of the tail head prior to analysis completion and/or use of other identification methods (e.g., physical modifications, identification tags, or implanted transponders). This identification has been used for mice, from neonates to adults. Although the markings may be maintained for two or three days, if longer time might be necessary, it is also quite simple to re-mark the animal.

Non-invasive alternatives. By the late 1980s, a variety of sampling techniques appeared in the literature for analysis of genetic modifications or in genetic screening efforts (e.g., monitoring for genetic drift within a characterized genetic background). With the advent of PCR technologies, many laboratories started downsizing the sample size necessary for analysis and streamlining animal usage (4, 6, 25, 51, 52).

The rate at which humane care and use of animals impacted on the analysis of genetically modified animals progressed rather slowly in the 1990s. In 1990, a method of using ear identification samples (the portion of ear tissue remaining after notching) was reported (53). The authors made use of usually discarded ear clippings for PCR analyses. Thus, a byproduct of a mouse identification scheme was better used, obviating an additional biopsy procedure. Yet, only a single specimen was usually obtained, and the amount of specimen recovered was not sufficient for additional hybridization analyses.

The analysis of a variety of tissues for transgene integration analysis include tail, toe, and ear specimens for either initial characterization of transgenic animals or for sex determination (6). Toe lysates were later identified in detail for analysis of genomic DNA sequences (54). Here, the authors described use of toe tissue for PCR analysis. Again, the tissues were usually obtained as a byproduct of a mouse identification scheme. Additionally, the procedure might provide more total tissue (and DNA) than that obtained from ear notch specimens. However, one was again limited to a single sampling. This was one of the first reports to discuss the use of toe lysates specifically for genotyping mice. Nevertheless, a number of IACUCs have found this procedure to be controversial, either for animal identification or transgene analysis.

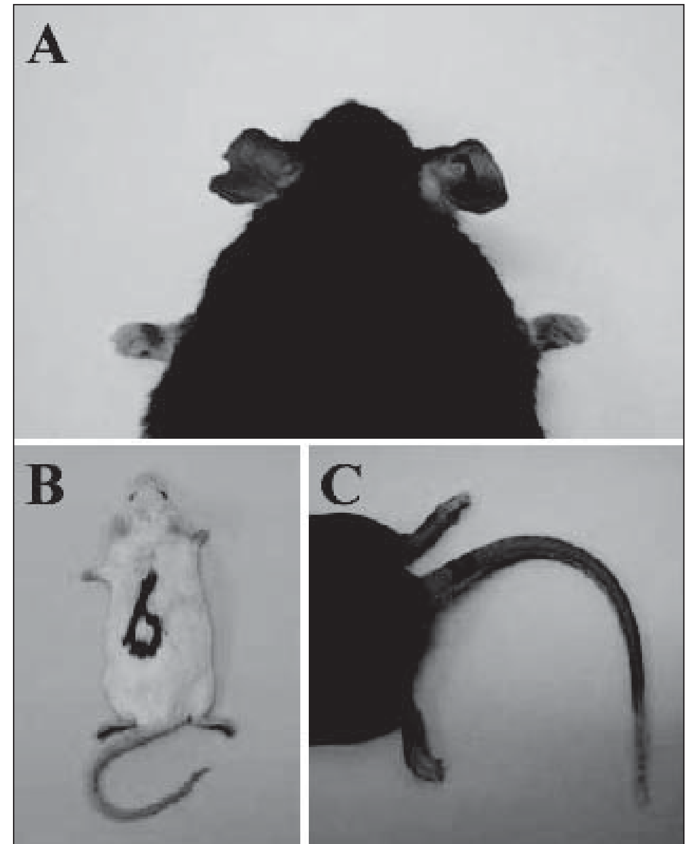

Figure 5. Temporary identification of mice. (A) On left, an example of an ear notch at a given location on the mouse ear provides a quick and permanent identification system. In contrast, if the analysis will be completed, and the duration needed is only two to three days, use of a "permanent," non-toxic marker can be used as noted on the right ear, and in (B) where a broad-tip marker provides a visible identifier, or (C) where the tail head is used for identifying information (e.g., letters, numbers, symbols, bands, or various colored markers may be used).

Use of "plucked" hair follicles (55, 56), tissue from newborn mice (50, 57), and blood samples among various sources for genomic DNA recovery (6, 8, 9, 33), was described in detail. Interestingly, many of these procedures, with the exception of blood and skin sampling, have not benefitted from widespread use. Unfortunately, collection of blood, skin, and hair follicles is discomforting, and multiple sampling can prove problematic without accompanying anesthesia or analgesia.

By the late 1990s, two additional non-invasive procedures were identified. Polymerase chain reaction analysis of rectal epithelial cell lysates was identified as a means of transgene analysis (57), followed by a timely study that illustrated the use of PCR analysis of fecal pellets to identify transgenics (58). Although intestinal mucosa sampling and fecal pellet analyses have become commonplace in the field of pathogen analysis (59-65), the fecal pellet model truly simplified analytical requirements.

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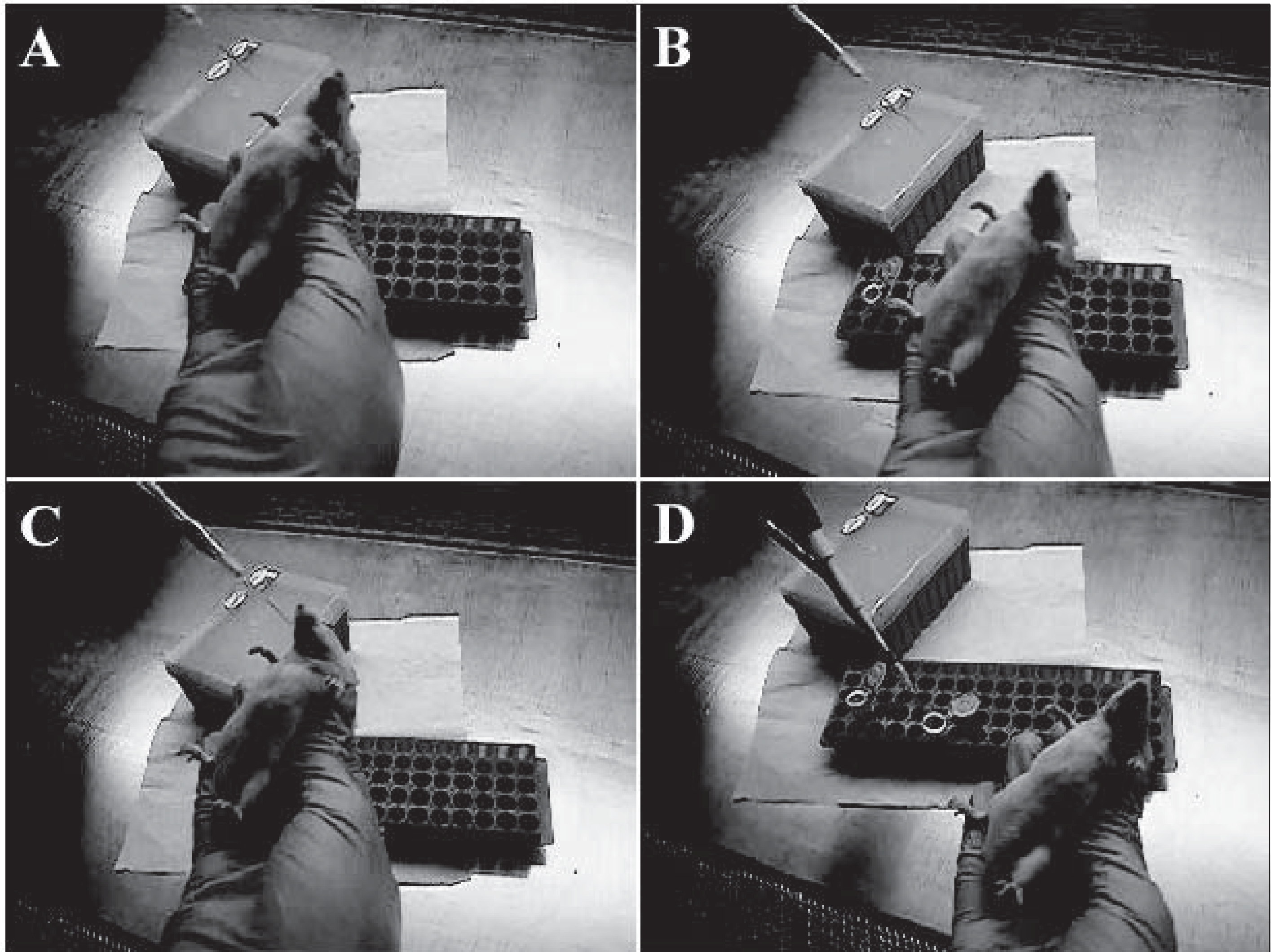

Figure 6. Saliva collection from a mouse. (A–D) illustrate the saliva sampling procedure in sequential images, from holding the mouse, to inserting the pipette tip into the oral cavity, to withdrawing the sample, and placing it in a microcentrifuge tube. The sampling procedure takes but a few seconds to complete.

However, specific genetic sequences had to be characterized against prokaryotic and other DNA sources/contaminants. Early on in pathogen analyses, it was documented that ingested foreign DNA sequences could be found in fecal pellets, in amounts not only detectable by PCR analysis, but by use of hybridization analyses as well (66). Other contaminants may or may not be problematic; however, sampling of control littermates or sentinels should help obviate such problems. Additionally, as noted in various bacterial and viral screening protocols involving use of fecal samples, careful consideration of substances inhibitory to the PCR analysis itself should be identified (67). Indeed, pretreatment by use of gel filtration or boiling failed to inactivate or remove inhibitors of PCR amplification in various species, including mouse. Yet, in the aggregate, the use of fecal pellets for genotyping illustrates a reproducible and sensitive screening technique for selected sequences (58).

Similarly, from a non-invasive sampling standpoint, our efforts in 1996 first illustrated the use of saliva in determining presence of a given DNA sequence, including transgene integrations (68). The PCR analysis of DNA obtained from trace amounts of human saliva was successfully used for several years by forensic laboratories in DNA fingerprinting applications (69-72), and in diagnostic laboratories for viral DNA detection (73). Studies related to these techniques pointed to the possibility of a unique application—extraction of DNA from mouse saliva for PCR analysis to confirm transgenic status or for routine genetic monitoring. After initial attempts at developing a collection technique, using oral swab specimens or scrapings, similar to human forensic analyses (74), it proved rather simple to obtain cells from the oral cavity of mice (Fig. 6 and 7A and 7B [68]). For weanling mice, a 10-μl aerosol-resistant tip was attached to an adjustable pipetter loaded with five microliters of sterile water. The water was pipetted back and forth into the oral cavity three to four times. For adult mice, the pipetter was used to remove approximately five microliters of saliva without the need for an oral wash (Fig. 6). During these procedures, the pipet tip was gently positioned underneath the tongue, and pipetting was performed rapidly. This technique had proven less traumatic to the mouse, compared with conventional sampling, as there was no surgical intervention or wounding. In addition, local or gen-

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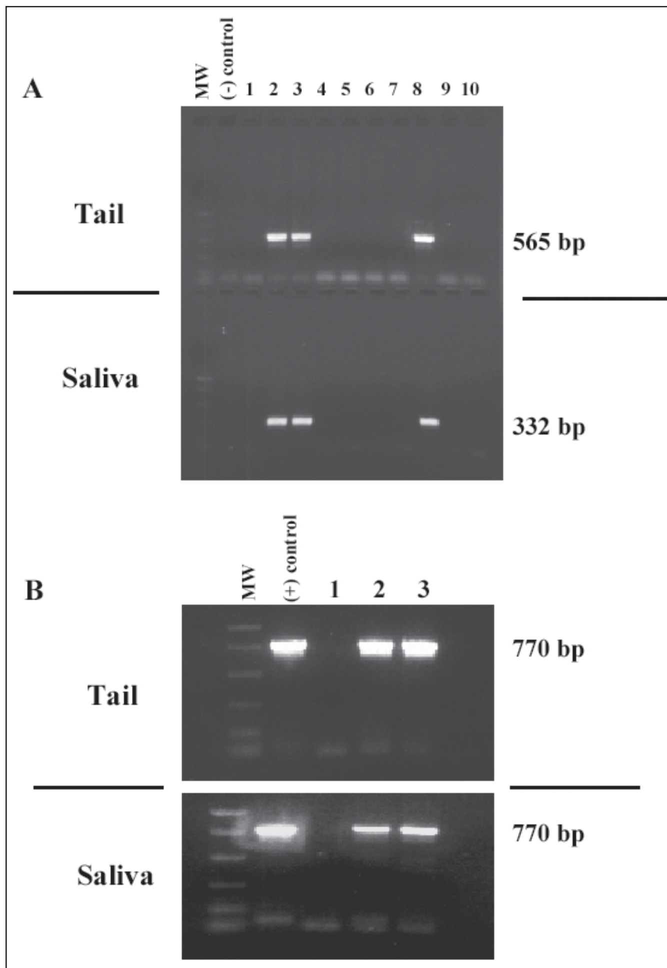

Figure 7. Polymerase chain reaction analysis of specimens from weanling and adult mice. (A) Identification of transgenic mice by use of agarose gel electrophoresis of PCR products amplified from DNA obtained from tail tissue (upper panel) and saliva (lower panel) of weanlings. Standard PCR analysis of 100 ng of DNA from tail tissue amplified a 565-bp product. Nested primer PCR analysis of DNA from saliva obtained by oral flushing with sterile water amplified a 332-bp product. Lanes: 1, size markers (50, 150, 300, 500, 750, and 1,000 bp); 2, polymerase chain reaction analysis of control (non-transgenic) mouse DNA; 3–12, PCR analysis of DNA from 10 weanling (3-week-old) mice from litters of two individual females, both harboring the same transgene (anti-K99 *E. coli* Ig gene). Of these 10 pups, three were identified as transgenic by results of PCR analysis using DNA from tail tissue and saliva. (B) Polymerase chain reaction analysis of DNA from tail tissue (upper panel) and saliva (lower panel) from adult mice harboring a carcinoembryonic antigen (CEA) transgene. Analysis of 100 ng of DNA isolated from tail tissue obtained at weaning amplified a 770-bp product. Analysis of DNA from five microliters of saliva obtained from adult mice amplified the same 770-bp product. Lanes: 1, polymerase chain reaction size markers (50, 150, 300, 500, 750, and 1,000 bp); 2, amplification of control (transgenic) tail DNA; 3–5, amplification of DNA from littermates showing two of three positive for the transgene by results of PCR analysis of DNA from either tail tissue or from saliva (reprinted with permission, 68).

eral anesthesia was not necessary, and sampling could be repeated as often as needed without apparent distress to the mouse. Unfortunately, this procedure was best used when repeat sampling was required, or in genetic monitoring protocols where older animals were analyzed. The concern that was found challenging was in relation to relative sensitivity in sampling weanling mice, and the total amount of DNA obtained from the saliva sample (57, 58, 68, 75). Although these procedures could be repeated many times with minimal stress to the animals, to ensure that a sufficient sample was obtained for unequivocal results, a nested PCR protocol was preferred for mice less than four weeks of age, without repeated sampling. In the end, the nested PCR protocol was more time consuming, with approximately twice the attendant net costs (75).

In our original experiments, PCR analysis of potential transgenic weanling mice harboring a rearranged immunoglobulin heavy chain transgene (76, 77) included analytical comparisons from samples obtained from saliva and tail biopsy specimens. Using 100 ng of tail DNA, a prominent and appropriate fragment was amplified. With DNA obtained from an oral flushing, nested PCR analysis was required to obtain sufficient product for visualization on agarose gels. Amplification of normal mouse DNA and DNA from non-transgenic littermates was consistently negative in standard and nested PCR analyses. Amplification of DNA obtained from five microliters of saliva from adult mice, harboring a carcinoembryonic antigen transgene (78) further illustrated that standard PCR analysis, without the need for nested primer reactions, was sufficient to generate sufficient PCR product for visualization on agarose gels. Although nested PCR analysis was best reserved for very young mice, conventional PCR analysis has been used for analyzing older animals. Interestingly, of late, real-time PCR analysis, with an opportunity to quantify amplicons, has proven desirable in the analysis of young mice (13). Obviously, less DNA was obtained from weanlings than from adults, as evidenced by a lack of visible product on agarose gels following first-round PCR analysis for virtually all weanling mice tested. The addition of a second round of amplification added about four hours to the procedure, which compares favorably to the time one would spend digesting tail tissue with proteinase K and purifying DNA, even if phenol/chloroform extraction were replaced by any of the rapid DNA purification kits currently available. Saliva sampling lends itself to: study of developmentally influenced or induced modification of transgene insertions, mutagenesis analyses, multiple sampling over time for qualitative and quantitative analyses, verifying analyses from other laboratories, comparisons between founders and subsequent offspring collected and prepared at a particular time point, genetic monitoring of multiple loci, and contamination or loss of specific samples where additional sampling becomes necessary.

In contrast to sampling young mice, if a given animal is beyond four weeks of age, per our Institutional Animal Care and Use Committee guidelines, administration of an anesthetic or analgesic prior to biopsy is required. Similar requirements exist where multiple analyses are needed for either specific scientific endpoints or when procedural problem arise. For instance, in optimizing the protocols described in 1996, some mice were subjected to saliva collection three or four times (68). Compared with multiple biopsies in the design or refinement of other procedures (e.g., minimizing false priming of PCR primers or optimizing primer conditions), the minimal stress associated with saliva sampling was and still is consistent with our desire to safeguard the welfare of animals used in our research efforts while obviating the need for multiple biopsies and associated trauma.

As such, further acceptance of non-invasive technologies may be realized by other laboratories that currently use routine tis-

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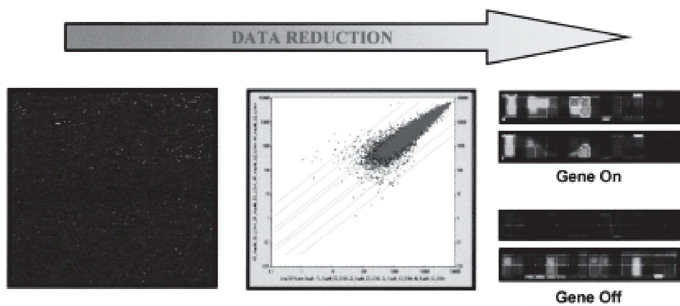

Figure 8. Microarray technology. Until recently, gene discovery was performed studying individual genes, one gene at a time. With microarray technology, standard molecular biological principals could be used on an industrial scale, allowing one to generate fully quantitative and qualitative gene expression profiles for thousands of genes simultaneously. Gene expression profiles can now be used to decipher molecular mechanisms that underlie targeted biological perturbations or comparative development in a host of biological systems (reprinted with permission, 80).

sue biopsy or blood sampling for genomic analyses, thus eliminating the need for perhaps hundreds of thousands of surgical procedures performed on laboratory animals annually. In reviewing the literature on PubMed, it was surprising to see that fewer than a dozen reports had made use of non-invasive analyses, unless there was a phenotypic marker included (reporters including fluorescent protein, luciferase, or tyrosinase genes). However, unless the reporter was an integral component of the inserted transgene, co-injection experiments provided a degree of inaccuracy in initial characterizations and may have influenced gene expression profiles (79).

Additional Tools

From microarray technology to proteomics. A number of tools amenable to high throughput technologies and in establishing functional genomics technologies include microarray analyses (Fig. 8). The DNA microarray technology has a number of applications, including identification of specific genes or gene mutations, and determination of expression level or relative abundance of specific genes or clusters of genes (80). A survey of relative DNA or mRNA abundance for specific genes can be a powerful tool for helping to understand the relevance of known as well as uncharacterized genes in terms of spatial and temporal regulation.

Gene expression can be quantitatively analyzed by hybridizing labeled mRNA to targets on a DNA microarray. The DNA microarrays are printed on glass or nylon slides or "gene chips." The microarrays can be printed with hundreds or thousands of cDNA or oligonucleotide samples in an ordered two-dimensional grid. Generally, two samples of mRNA can be differentially labeled, and their fluorescence scanned. The differential expression of these spots is then compared, generally by use of sophisticated data mining/analysis software. The relative significance of the generated data can be examined in the context of either total gene product or complex expression patterns (there are a host of tutorials on the web including those at http://www.gene-chips.com/, http://www.tigr.org/tdb/microarray protocolsTIGR.shtml, and http://industry.ebi.ac.uk/~alan/MicroArray).

Beyond genomics characterizations, the emerging field of proteomics centers on the study of proteins and protein interactions to understand cell metabolism and behavior. Modern proteomics technology can be used to determine all of the post-translational modifications that proteins undergo and potentially determine what differentiates proteins influencing a cascade of developmental consequences within a cell or organism. As noted for microarray techniques, without the capability to amass substantial amounts of data in performing experiments and analyzing subsequent outcomes, these powerful technologies would be of little use. The ability to store and use a genomic or proteomic database relies on new algorithms and bioinformatics infrastructure. Such revolutionary technologies are influencing the life sciences in dramatic fashion as we develop means to better capture, interpret, and categorize data.

Gene mapping of animals could be considered to have originated with the development of a recombination map of the *Drosophila* X chromosome by Sturtevant in 1913 (81). Since that time, concerted efforts have led to the mapping of the human genome as well as a number of strain-specific mouse genomes. Today, databases and bioinformatics-based resources are conveniently found on the World Wide Web and relate directly to transgenic animal modeling. Recently, various resources available to researchers were summarized in detail (82). Many of the mouse-related and modeling resources provide well documented phenotypic and genotypic characterizations of genetically engineered mouse models, contain extensive bibliographic references, and include active external links to other sites. A variety of mouse-related databases includes a vast array of information [e.g., The European Mouse Mutant Archive (EMMA), http://www.emma.rm.cnr.it/; Gene eXpression Database (GXD), http://www.informatics.jax.org/menus/expression_menu.shtml; Hereditary Hearing Impairment in Mice (HHIM), http://www.jax.org/research/hhim/documents/models/html; Mouse Genome Resource (MGR) http://www.ncbi.nlm.nih.gov/genome/guide/mouse/; Induced Mutant Resource (IMR), http://www.jax.org/resources/documents/imr/; Mouse Genome Database (MGD), http://www.jax.org/research/hhim/documents/models/html; Mouse and Rat Homology Map, http://www.informatics.jax.org/reports/homologymap mouse_rat.shtml; Mouse Genetic Resources—Shared Information of Genetic Resources (SHIGEN), http://www.shigen.nig.ac.jp/mouse/mouse.default.html; The Mouse Heart, Lung, Blood, and Sleep Disorders Center, http://www.jax.org/hlbs/index.html; Mouse Knockout and Mutation Database (MKMD), http://biomednet.com/db./mkmd; Mouse Models of Human Cancers Consortium (MMHCC), http://web.ncifcrf. gov/researchresources/mmhcc/default/asp; The Mouse Phenome Database, http://www.jax.org/phenome; NeuroMouse, http://www.mshri.on.ca/molec/henderson/neuro mouse.htm; RIKEN Mouse Encyclopedia Index, http://genome.rtc.riken.go.jp; TBASE, http://tbase.jax.org; TIGR Mouse Gene Index, http://www.tigr.org/tdb/mgi/index.html; University of California Genetically Engineered Mouse Research, http://ccm.ucdavis.edu/tgmouse/; University of California Resource of Gene Trap Insertions, http://socrates.berkeley.edu/~skarnes/resource.html; and the Whole Mouse Catalog, http://www.rodentia.com/wmc/]. Indeed, many important and useful databases and Internet resources have been omitted from this abbreviated list. In addition to rodent-related resources, databases related to human disease, and animal genomics are reviewed in detail and regularly updated at http://tbase.jax.org/docs/databases.html. Additionally, as mitochondria play central roles in cellular metabolism, and there is increasing evidence of mitochondrial involvement in a broad variety of human diseases, the Human Mitochondrial Protein Database

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(HMPDB, http://bioinfo.nist.gov:8080/examples/servlets/index.html) contains information including human nuclear and mitochondria encoded genes and proteins. This database consolidates information from SwissProt, LocusLink, Protein Data Bank (PDB), GenBank, Genome Database (GDB), Online Mendelian Inheritance in Man (OMIM), Human Mitochondrial Genome Database (mtDB), MITOMAP, Neuromuscular Disease Center, and other resources.

Conclusions

A number of methods to genotype and phenotype laboratory animals are available. Although the options are varied, most non-invasive or minimally invasive techniques described herein are favored by a limited few laboratories. Although procedures outlined here could appreciably enhance the well being of laboratory animals, a number of caveats exist and are related to specific use and training that must be addressed. The welfare of animals used in research from a humane standpoint is of paramount importance, as is efficient and representative data collection and analysis. In the end, it is clear that various techniques applied appropriately will ensure, rather than hinder greater experimental throughput. The challenges that we face relate to how and in what light the technologies are perceived by the bench-level scientist, coupled by the added benefits to the animals used and experimental outcomes.

Acknowledgments

The author apologizes to colleagues whose studies were not discussed or cited due to review limitations. The author thanks C. A. Cassar, D. A. Dunn, R. L. Howell, C. A. Ingraham, W. K. Pogozelski, C. A. Lerner, L. W. Johnson, M. H. Irwin, W. A. Bates, and R. B. Baggs for their assistance and critical comments. Some of the figures were included within the program of the 2002 ACLAM Forum and were cited in the text. K. A. Bussey, C. A. Cassar, D. A. Dunn, C. A. Ingraham, L. W. Johnson, and C. A. Lerner are acknowledged for aid in figure production and A. I. Brooks is recognized for providing Fig. 8. Development of mouse models at URMC and UAB, analyses, and reagent production were supported by funding from NCRR, NICHD, NIDCR and NCI. Gratitude is extended to the Doerenkamp Zbinden Foundation for recognizing the non-invasive saliva collection technique for identification of transgenic mice in 1997. All animals mentioned in this manuscript were cared for according to NIH and the Office of Laboratory Animal Welfare guidelines for appropriate animal husbandry (OLAW assurances: URMC, A3292-01; UAB, A3255-01). The University of Rochester and the University of Alabama at Birmingham have been accredited by AAALAC, International since 1966 and 1971, respectively.

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