

Genomics techniques: An overview of methods for the study of gene expression

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ABSTRACT: A variety of methods to identify differences in gene expression exist, including subtractive hybridization, differential display, serial analysis of gene expression, and microarray hybridization. Some of these techniques have been used to investigate changes in gene expression in livestock, and important

resources are being developed that will facilitate the use of others. The objectives of this review are to provide a general overview of techniques for evaluating gene expression in livestock and to identify resources currently available and in development for animal science researchers.

Key Words: Gene Expression, Laboratory Methods, Livestock, Techniques

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Introduction

The ability to sequence entire genomes has stimulated research directed not only at producing DNA sequence, but also at defining the function of genes on a genome-wide level. Given that genes with related functions are likely to be regulated together, techniques that evaluate global gene expression provide a mechanism for the initial identification and clustering of novel gene sequences with related functions. The organization of sequences into functional groups based on expression will provide a basic framework to direct additional experiments aimed at defining the precise function of the final gene product. In the last two decades, techniques for the evaluation of gene expression have progressed from methods developed for the analysis of single, specific genes (e.g., Northern, slot, and dot blotting; semiquantitative and quantitative reverse transcription and PCR; and nuclease protection assays) to techniques focused on identifying all genes that differ in expression between or among experimental samples (e.g., subtractive hybridization, differential display, sequencing of expressed sequence tags, serial analysis of gene expression, and hybridization to microarrays). The objectives of this review are to provide a general overview of the methodology of the latter techniques and to identify supporting resources currently available to animal science researchers.

Methods for the Study of Gene Expression

Subtractive Hybridization

Subtractive hybridization methods were first described in the early 1980s for the purposes of creating cDNA libraries (Sargent and Dawid, 1983) and generating probes (Davis et al., 1984) of differentially expressed genes. Differentially expressed genes were isolated by hybridizing cDNA representing one sample (tester) to an excess of mRNA representing a second sample (driver). Transcripts expressed in both the tester and driver would form a mRNA/cDNA hybrid molecule, whereas a cDNA sequence unique to the tester would remain single-stranded. Single- and double-stranded molecules were separated using hydroxylapatite chromatography, and the eluted single-stranded cDNA represented genes expressed only in the tester. Differentially expressed cDNA could then be cloned or used directly as probes for screening libraries. Two significant limitations of the original protocol were the requirement of large quantities of mRNA (e.g., 270 μ g were used by Sargent and Dawid, 1983) and a bias against the identification of rare transcripts.

Modifications to the original protocol include the production of cDNA with biotin tags (Welcher et al., 1986) or oligo(dT)30-latex (Hara et al., 1991) to improve the separation of single- and double-stranded molecules. Amplification of selected cDNA by PCR has also been incorporated to decrease the initial amount of mRNA required and to improve the cloning efficiency of selected transcripts (Hara et al., 1991). Additional modifications have been developed to alleviate bias against rare transcripts. Gurskaya et al. (1996) and Diatchenko et al. (1996) described similar protocols, termed *sup-*

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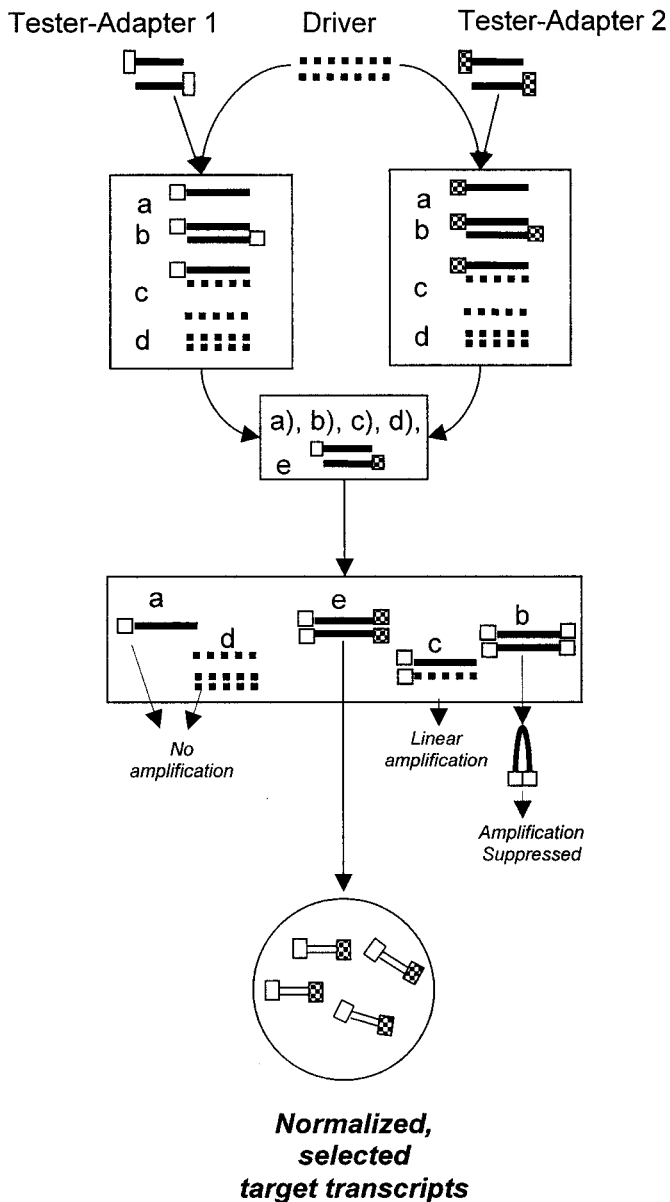


Figure 1. Suppression subtractive hybridization (SSH). Messenger RNA is extracted from a reference sample, or driver, and from an experimental sample, or tester. The mRNA of the driver and tester are reverse-transcribed to cDNA. Tester cDNA is prepared by ligating two separate aliquots to different adapter oligonucleotides (Tester-Adapter 1 and Tester-Adapter 2). Subtractive hybridization is carried out by combining an excess of driver with tester-adapter 1 and tester-adapter 2 cDNA in separate reactions, denaturing the mixtures, and allowing the single-stranded molecules to re-hybridize. During hybridization, four types of products may result: a) single-stranded tester transcripts that are not present in the driver sample, b) double-stranded tester transcripts formed by re-annealing of tester molecules, c) hybrids of tester and driver transcripts representing transcripts present in both samples, and d) double-stranded driver transcripts. Normalization of transcript abundance is achieved because abundant differentially expressed transcripts will be more likely to form b) molecules than rare transcripts, based on hybridization kinetics. A second hybridization is carried out from a mixture of the original two hybridization products, plus additional denatured driver cDNA. Resulting products are the same as for the first hybridization, except that the a) products from the original two tester hybridizations may anneal to each other to form e), a double-stranded, differentially expressed transcript with two different adapters. The ends of all double-stranded molecules are filled in to facilitate PCR using primers complementary to adapters 1 and 2. Enrichment of the e) molecules is achieved by PCR amplification because a) and c) contain a single primer site and will only amplify linearly; amplification of b) will be suppressed due to complementarity of long inverted terminal repeats in the adapter sequence (Siebert et al., 1995); and d) does not contain primer binding sites. The resulting PCR products will primarily contain normalized, subtracted transcripts initially present in the tester but absent from the driver mRNA samples. Figure 1 is adapted from Diatchenko et al. (1996).

pression subtractive hybridization (SSH). The SSH method is designed to selectively amplify differentially expressed transcripts while suppressing the amplification of abundant transcripts, thus eliminating the need to separate single- and double-stranded molecules. In addition, SSH normalizes target transcripts to approximately equal abundance. The SSH method as originally described by Diatchenko et al. (1996) is described in Figure 1 and is currently available in the form of a commercial kit, PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA).

Subtractive hybridization is the first technique to be widely used for the purpose of identifying differentially expressed genes on a global scale. Advantages of the technique include the ability to isolate genes with no

prior knowledge of their sequence or identity and the use of common molecular biology techniques that do not require specialized equipment or analyses. Several limitations of the original protocols, such as requirements of large quantities of RNA and bias toward abundant genes, have been overcome by incorporation of PCR into the SSH technique. However, SSH remains applicable only to pair-wise treatment comparisons and must be replicated with the tester and driver reversed to identify gene expression changes in both directions. Additionally, subtractive hybridization does not provide a quantitative measure of expression differences and is most efficient at identifying genes that are completely absent, rather than expressed less abundantly, in the driver sample.

Differential Display

Techniques known in general as RNA fingerprinting include differential display (Liang and Pardee, 1992) and RNA fingerprinting by arbitrary primed PCR (**RAP-PCR**); Welsh et al., 1992). Both methods were introduced in 1992 and are based on PCR amplification of random subsets of genes from two or more RNA samples (Figure 2). Since the introduction of these techniques, differential display has been more widely applied than RAP-PCR. The first step of either differential

display or RAP-PCR is to reverse-transcribe random subsets of mRNA to cDNA. In differential display, this is done using an anchored primer, which is typically a polyT oligonucleotide with one or two additional bases (e.g., T₁₂AC). These primers anneal to the polyA tail of mRNA, anchoring the resulting cDNA to the 3' end of the RNA transcript. In contrast, RAP-PCR uses arbitrary primers in reverse transcription. These primers are typically 10 bp in length and may anneal to complementary sequence and prime reverse transcription from any point along an RNA transcript.

Following reverse transcription of firststrand cDNA, segments of the cDNA transcripts are amplified using multiple PCR primer pairs. For both differential display and RAP-PCR, the forward PCR primer is an arbitrary primer, typically 10 bp in length, that couples with the anchored (differential display) or arbitrary (RAP-PCR) primer used to produce the cDNA. It has been estimated that PCR products from 240 unique primer pair combinations (e.g., all combinations of 20 arbitrary and 12 anchor primers) would represent all mRNA that were present in the original RNA sample (Liang and Pardee, 1992). The PCR products may be labeled by incorporation of a radiolabeled nucleotide such as 33P-dCTP or by a fluorescently labeled primer (see Ito et al., 1994). Visualization of PCR products is achieved by electrophoresis on polyacrylamide gels followed by appropriate imaging. Images are then evaluated by comparing the relative intensities of bands produced from different experimental samples. Bands that are present in one sample and absent in another or bands that are present at different relative intensities across experimental treatments represent potentially differentially expressed mRNA transcripts. Typically, bands are evaluated only if they are amplified consistently in duplicate PCR reactions from the same experimental sample.

The final phase of differential display is to identify the sequence of the transcript represented by the differentially displayed PCR product and to confirm that the transcript is truly differentially expressed. These steps are accomplished by physically locating and excising the acrylamide gel containing the PCR product of interest, based on alignment of the imaged PCR products with the dried acrylamide gel. The PCR products are purified from the gel and reamplified by PCR. Various strategies and techniques have been used to confirm differential expression, including the use of reamplified PCR products as a probe for Northern hybridizations, spotting multiple reamplified PCR products on membranes for reverse dot- or slot-blot analysis, and cloning and sequencing reamplified PCR products so that gene specific primers may be designed for use in semiquantitative PCR of cDNA. Regardless of the strategy followed to confirm differential expression, sequences of differentially expressed gene fragments will ultimately be required to begin to understand the function of a gene.

Differential display and RAP-PCR have been used in a variety of experiments to identify differentially expressed genes in livestock. Examples include the ex-

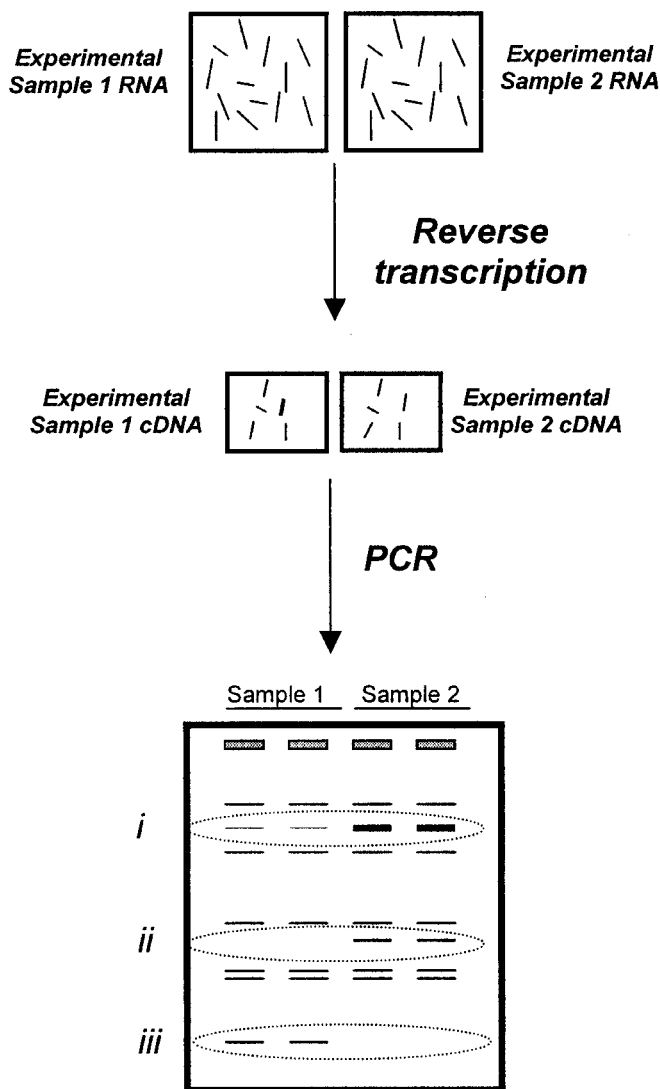


Figure 2. Differential display. Total RNA is extracted from two experimental samples, 1 and 2. An anchored primer, such as T₁₂G, is used to reverse-transcribe a subset of the mRNA to cDNA. Random fragments of the cDNA transcripts are amplified in duplicate PCR reactions using different combinations of forward and reverse primers. The resulting PCR products are evaluated to identify gene fragments expressed in sample 1 but not 2 (iii), expressed in 2 but not 1 (ii), and expressed in both 1 and 2 but at different intensities (i).

amination of gene expression changes following induced regression of bovine corpora lutea (Sayre et al., 2000), changes associated with the peri-implantation uterus in pigs (Green et al., 1996), and changes associated with different breeds (Li et al., 1996) and genetic lines (Janzen et al., 2000) of pigs.

Two important advantages of RNA fingerprinting methods relative to subtractive hybridization are the abilities to compare multiple experimental samples simultaneously and to identify genes that are either up- or down-regulated in one sample relative to another. However, RNA fingerprinting shares with SSH the limitation that it is not a quantitative method. Additionally, RNA fingerprinting experiments are often plagued with a high rate of false positives, or gene fragments that seem to be differentially expressed as an artifact of PCR. Finally, investigation of all potentially differentially expressed genes requires high-throughput PCR and extensive investments of time and labor to follow up and confirm differential expression on an individual gene basis.

Several products have been developed specifically for the differential display technique, including precast acrylamide gels (Invitrogen, Carlsbad, CA), primer and enzyme kits (Clontech, Palo Alto, CA; GenHunter, Nashville, TN), specialized fluorescent scanning and reagent systems (Beckman-Coulter, Fullerton, CA), and complete differential display services (GenHunter).

Expressed Sequence Tag Sequencing

Technological advances facilitating high-throughput sequencing led to the concept of expressed sequence tag (EST) libraries in the early 1990s (Adams et al., 1991; Okubo et al., 1992). The EST sequences are generated by randomly picking clones from a cDNA library and performing a single sequencing reaction to produce 300 to 500 bp of sequence per clone. Differences in gene expression may be identified by counting the number of times a particular sequence appears in EST libraries of genes from different sources. However, EST sequences are often generated from cDNA libraries that have been normalized to equalize the abundance of clones representing different transcripts (Soares et al., 1994). The EST sequenced from normalized cDNA libraries may be compared to identify transcripts that are expressed in one library and absent in another, but accurate quantitative data describing the relative abundance of genes within a library can only be obtained from EST generated from non-normalized cDNA libraries. Despite this restriction, the power of discovering differences in gene expression by comparing the abundance of sequences in EST databases increases with growth of the databases. Several Web-based resources have been developed to evaluate public databases of human EST for the purpose of identifying differences in gene expression among libraries (see review by Bortoluzzi and Danieli, 1999). Bortoluzzi et al. (2000)

demonstrated that this type of evaluation of gene expression using EST sequences from non-normalized libraries produces transcript profiles similar to those generated by more specialized methods.

Several EST sequencing projects have been initiated from cDNA libraries constructed from various livestock tissues (Abdrakhmanov et al., 2000; Grosse et al., 2000; Tirunagaru et al., 2000; Fahrenkrug, 2001; Tuggle et al., 2001). Although these libraries will generate valuable resources needed for other methods of gene expression analysis (see the following sections), searching these databases following the strategy of Bortoluzzi et al. (2000) is not appropriate because most of the sequences were generated from normalized or subtracted libraries.

Serial Analysis of Gene Expression

Serial analysis of gene expression (SAGE; Velculescu et al., 1995) is a technique for the analysis of gene expression that is essentially an accelerated version of EST sequencing. The SAGE technique is based on the concept that short sequence tags are sufficient to identify a gene transcript, provided the tags represent a known location within the gene (Velculescu et al., 1995). A SAGE tag is generated from an mRNA transcript by extracting a short sequence from a defined location. Typically, a SAGE tag includes nine bases of sequence downstream from the last endonuclease recognition site of a transcript. Multiple SAGE tags are ligated together in a cloning vector so that a typical sequencing reaction of 300 to 500 bp generates the sequences of 20 to 30 SAGE tags (see Figure 3 for details). Because each unique SAGE tag theoretically represents a unique mRNA transcript, an overview of all genes expressed in the original RNA sample is generated by SAGE tag sequences. Differences in gene expression among experimental samples can then be identified by comparing the relative abundance of specific SAGE tags in different libraries. Genes or EST sequences represented by the SAGE tags are identified by searching sequence databases to identify gene or EST sequences that contain the SAGE tag in the appropriate location. A thorough description of the bioinformatics involved in the analysis of SAGE data is provided by Lash et al. (2000). Since the original description of SAGE, modified protocols have been proposed to reduce the amount of mRNA required (Datson et al., 1999; Peters et al., 1999) and improve the efficiency of cloning tags (Powell, 1998; Kenzelmann and Muhlemann, 1999).

Important advantages of SAGE over subtractive hybridization and differential display are that SAGE data are quantitative and cumulative. Accurate, quantitative transcript profiles describing the abundance of all genes expressed in a cell or tissue are generated by SAGE, provided sufficient sequencing is completed. The resulting data may then be compared to all existing and future SAGE databases constructed in a similar manner. For example, a public gene expression data

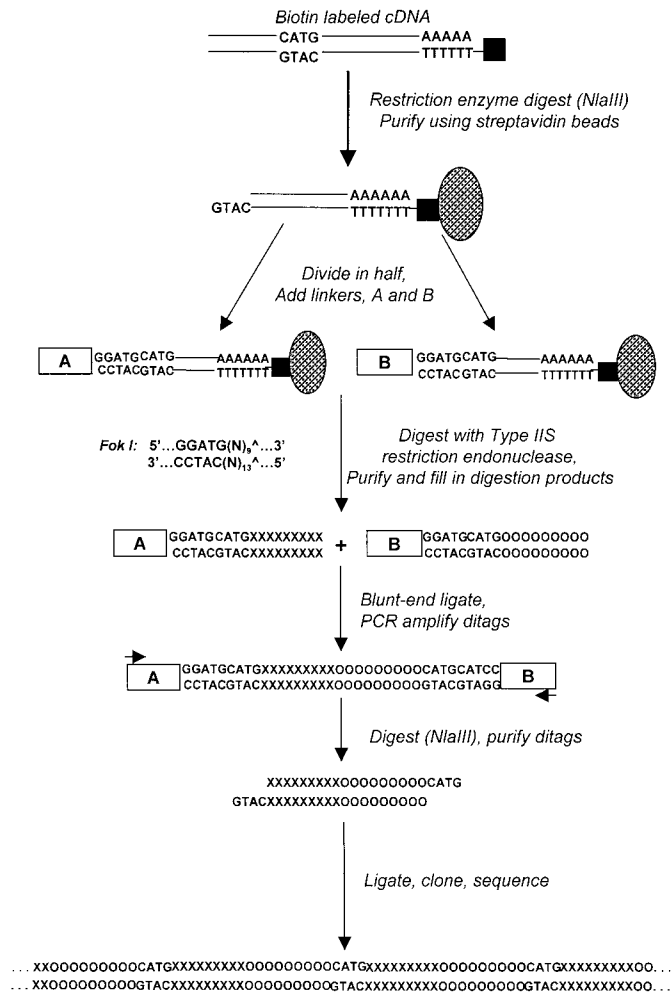


Figure 3. Serial analysis of gene expression (SAGE). Messenger RNA of one experimental sample is reverse-transcribed to cDNA using a biotin-tagged primer. The cDNA is digested with a restriction endonuclease (*NlaIII*) that recognizes a 4-bp sequence (CATG), and products from the 3' ends of the transcripts are purified. Two linkers, A and B, are added to the purified digestion products in separate reactions. The linkers contain sequence complementary to PCR primers and the recognition sequence of a Type IIS restriction endonuclease (*FokI*). The cDNA molecules are digested away from the primer by *FokI*. This enzyme recognizes its specific recognition sequence at the end of the linker and cleaves the cDNA 9 bases downstream. The resulting digestion products are purified, ligated together, and amplified by PCR. A final digestion with the original endonuclease (*NlaIII*) cleaves away the linker sequence, producing ditags that contain 9 bp of sequence from two different transcripts. The ditags are flanked by the *NlaIII* recognition site, which is used by analysis programs to delineate the tags. Multiple ditags are ligated together, cloned, and sequenced to produce the SAGE sequence data.

repository has been created for the storage and analysis of SAGE data (Lash et al., 2000). The power of this repository will continue to grow as more SAGE data are contributed. Another feature of SAGE data is that they can complement EST data generated from normalized or subtracted cDNA libraries. The EST sequences contribute sequence and annotation information, whereas SAGE contributes quantitative data describing the abundance of those sequences. Finally, although SAGE requires high-throughput sequencing capabilities, the amount of gene expression data provided by sequencing a SAGE library may be as much as 20 times greater than that provided by the same amount of EST sequencing.

One limitation of SAGE is identification of the genes represented by SAGE sequence tags. This process is first restricted to sequences found in accessible databases. However, this limitation will become less problematic as more EST sequences are generated and existing sequences are annotated. Another potential limitation is the misidentification of tags. This can result from sequencing errors and from the misidentification of the region in the database sequence that corresponds to the SAGE tag (see Zhang et al., 1997). In addition, certain transcripts may fail to be represented by SAGE tags depending on the specific enzymes used to generate the SAGE library (see Welle et al., 1999), and other transcripts may be represented by multiple SAGE tags because of single nucleotide polymorphisms or alternative splicing of transcripts. Many of these problems are expected to affect a relatively small portion of genes represented by SAGE tags, but they should not be overlooked when interpreting SAGE data.

To date, the application of SAGE in livestock species has not been reported. Currently, SAGE data could accurately describe the number and abundance of genes expressed in samples derived from livestock species, and comparisons of expression profiles could be made among samples. Unfortunately, the identification of genes represented by livestock SAGE tags may be difficult because of the relatively limited number of livestock EST sequences. However, livestock EST databases are growing rapidly, and techniques have been described to efficiently extend SAGE tags to obtain longer sequences (Chen et al., 2000; van den Berg et al., 1999). Therefore, SAGE may prove to be an effective and efficient way to identify novel, differentially expressed genes in livestock in the future.

Microarray Hybridization

The evaluation of gene expression using microarray technology that was originally described by Schena et al. (1995) is proving to be an effective approach for a variety of applications. Although this technology has been largely inaccessible to animal scientists in the past, the resources needed to create livestock microarrays are being generated, and specialized equipment for making and reading arrays is becoming more acces-

sible. Three general types of microarrays include oligonucleotide chips made by the synthesis of short oligos directly on a glass wafer (see www.affymetrix.com/products/tech_probe.html for more information), oligonucleotide arrays made by spotting presynthesized oligos onto glass slides or nylon membranes, and cDNA arrays, made by spotting PCR amplified inserts of cDNA library clones onto glass slides or nylon membranes. Although the technology to make oligonucleotide chips remains highly specialized and largely inaccessible for research in livestock species, steps toward making cDNA or oligonucleotide microarrays containing livestock genes are underway. The basic steps of analyzing gene expression using a cDNA microarray are outlined in Figure 4, and a thorough review of many of the technical aspects related to the production of

microarrays has been published (http://www.nature.com/ng/chips_interstitial.html).

One of the most important aspects of microarray experimentation is the selection of gene fragments, or probes, to be spotted on the array. Even though the number of genes represented on a microarray may be large (3,000 to 10,000), all of the genes important to a particular experimental question may not be represented. A cDNA library may be produced or obtained for the purpose of constructing microarrays. However, selection of the most appropriate library for a particular application and selection of clones within a library remain critical to the success of the microarray experiment. In some situations, it may be most effective to focus experiments on a smaller set of genes selected by specific criteria, such as tissue distribution profiles, functional classification, or known expression changes.

After probes are identified, clones with the desired inserts, as well as appropriate controls, must be assembled and organized for the production of cDNA microarrays. Depending on the situation, this may involve re-arranging a set of clones from a library, acquiring clones from commercial or other sources, and/or cloning sequences using standard molecular biology techniques. Regardless of the source of the clones, it is essential to know with confidence the sequence of each probe on the array to ensure accurate interpretation of the resulting microarray data. Thus, resequencing all or a portion of the organized clones may be necessary.

The DNA representing each selected clone is prepared by PCR amplification of the clone insert. Typically, the length of these clones will be greater than 500 bases, but it depends on the specific clones selected. The PCR products are purified and then spotted, or printed, to precise locations on a glass slide or nylon membrane. Instruments used for the production of microarrays have improved dramatically in recent years. Options range from following directions for building an arrayer from scratch (<http://cmgm.stanford.edu/pbrown/mguide/index.html>) to purchasing complete microarray systems (see www.gene-chips.com for links to suppliers, and www.cartesiantech.com/review.html for a recent review of the technology by Rose, 2000).

The mRNA representing experimental samples is prepared for hybridization with cDNA microarrays by reverse transcription and labeling with fluorescent (Cye3 and Cye5-dUTP) or radioactive (^{32}P dCTP) nucleotides. Fluorescent labels are generally used with glass slide microarrays, whereas radioactive labeling is used with nylon membranes. The Cye3 and Cye5 fluorophores are detected at different emission spectra, so two experimental samples may be labeled with the different fluorophores, hybridized together in a single competitive hybridization reaction, and then detected separately by a fluorescence scanner. In contrast, radiolabeled samples must be hybridized individually in serial hybridization reactions and can be detected using a phosphor imaging system. Regardless of the experimental design, microarray analysis is based on the premise that the inten-

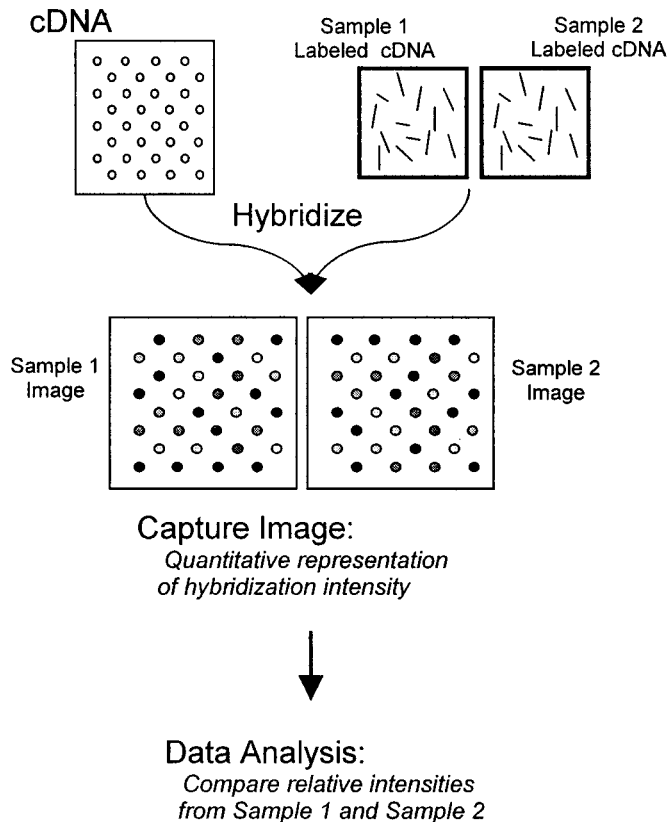


Figure 4. Overview of microarray hybridization. Messenger RNA representing two experimental samples, 1 and 2, is reverse-transcribed to cDNA and labeled. The resulting cDNA is hybridized to a microarray containing sequence fragments representing thousands of genes. Images are captured for each experimental sample such that the hybridization signal generated from each sequence fragment on the microarray is proportional to the abundance of that mRNA transcript in the original RNA sample. Comparisons of individual gene hybridization signals from different experimental RNA samples are then made to identify differences in gene expression between samples.

sity of the resulting hybridization signal for any sequence on the microarray is proportional to the amount of mRNA corresponding to that sequence in the original mRNA sample. Relative expression of each sequence represented on the microarray is evaluated by comparing hybridization intensity signals generated by different experimental samples.

Large quantities of data are generated with each microarray experiment, and appropriate analyses of these data are critical. A list of publications on this topic is found at <http://linkage.rockefeller.edu/wli/microarray/>. In general, the analysis of array data may be divided into three components: 1) identification and quantification of hybridization intensities, 2) visualization of data, and 3) clustering techniques. The first component includes the accurate identification of spots from the hybridization image, normalization for background, quantification of the hybridization intensities, and output of the data in a usable form. Data visualization includes sorting and presenting the data in a logical manner. Because of the vast amount of data created by a single hybridization experiment, it is important to be able to visualize the data in different formats to address multiple questions. Illustrations of different data presentation formats are provided by Brown and Botstein (1999). Finally, clustering algorithms allow groups of genes with similar expression patterns across multiple experimental samples to be identified (Eisen et al., 1998). Based on the assumption that genes with related functions are coregulated, clustering of microarray data becomes a powerful method to assign putative functional classifications to novel genes. Software programs that specialize in all areas of microarray analysis are available. A listing and description of many commercially available programs is presented at http://www.epd.unil.ch/biocomputing/array/software/MicroArray_Software.html, and software developed at Stanford University is freely available at <http://genome-www4.stanford.edu/MicroArray/SMD/restech.html>.

Microarray technology is becoming increasingly accessible to animal scientists, primarily through efforts to develop cDNA microarrays. Several research efforts are currently underway to develop the cDNA and EST library resources needed to identify and obtain clones for preparing DNA to spot on cDNA microarrays (e.g., Abdrakhmanov et al., 2000; Grosse et al., 2000; Tirunagaru et al., 2000; Fahrenkrug, 2001; Tuggle et al., 2001). Furthermore, initial data describing the production and testing of 768- and 3,800-element arrays containing sequences derived from bovine spleen and placental cDNA libraries have been reported (Band et al., 2001), and other livestock microarrays are in development. Another option currently available to animal science researchers is to use commercially available human cDNA microarrays (e.g., GeneFilters, Research Genetics, Huntsville, AL; Atlas arrays, Clontech, Palo Alto, CA; UniGem microarrays, Incyte Pharmaceuticals, St. Louis, MO) for cross-species hybridizations with live-

stock cDNA (Zou and Moody, 2001). However, some researchers have reported inconsistent results using this approach (Fitzsimmons et al., 2001). Based on current research efforts and interests in this field, it is anticipated that microarrays containing genes from cDNA libraries derived from a variety of livestock tissues will soon become available to the research community. This accomplishment will facilitate novel research efforts that begin to define livestock genomes at a more detailed level.

Summary

The methods reviewed in this paper are powerful technologies that expand the study of gene expression from single genes to the genomic level. No single technique is optimal for all applications, and the optimal strategy for specific situations may prove to be creative combinations of several techniques (for example, see Wang and Rowley, 1998). The continued refinement of genomics techniques and their related bioinformatics will provide researchers with efficient tools to investigate gene expression and gain a better understanding of the genetic regulation of physiological processes important to the improvement of livestock production.

Implications

The evaluation of gene expression is an effective way to identify genes important in the regulation of traits of economic importance in livestock production. Several techniques have been developed for this purpose, and important resources are currently in development that will facilitate continued research and discovery in this area. The application of these research methods in animal sciences will contribute to a greater understanding of the genetic regulation of traits important to livestock production.

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