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Messenger RNA differntial display strategies in birds and amphibians

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Abstract. As an inroad to the discovery of genes involved in important biological activities, various techniques have been developed for detecting genes based on their expression levels. Arbitrary amplification of different messenger RNA (mRNA) populations and their comparison on display autoradiograms made mRNA differential display one of the most straightforward approaches to identification of differentially regulated mRNAs. Over the past decade this strategy has been employed in many in vitro and in vivo systems. The use of the method in bird and amphibian model systems is reviewed here, emphasizing several unique combinations of model system and design of differential display screen.

Key words. Differential gene expression; song system; circadian rhythm; limb regeneration; limb identity; birds; amphibia.

Introduction

Differential gene expression is an essential biological mechanism that regulates development, differentiation, regeneration, plasticity and function of tissues and organs. However, the successful detection and isolation of differentially expressed genes has remained a challenge. Almost 10 years ago, Liang and Pardee [1] described messenger RNA (mRNA) differential display (DD) as an alternative methodology for the identification of different gene activities. The method is used to compare the expression of genes under two or more conditions. In short, mRNAs from experimental tissues are reverse transcribed into subsets of corresponding complementary DNAs (cDNAs). Their 3′ termini are arbitrarily amplified by polymerase chain reaction (PCR) and the resulting products are displayed as patterns of bands on sequencing gels. Bands with a differential appearance are excised from the gel, reamplified, and subsequently verified and analyzed. The advantage over conventional protocols lies in its straightforward approach to generating fingerprints of expressed mRNAs, without the need for cDNA libraries. The PCR based technology is very sensitive, utilizing only small amounts of input RNA, and allowing the detection of low-abundance messages. Since the initial publication, more than 1500 reports have described its application for the identification of differentially expressed genes in a wide range of biological systems.

Many improvements to the method have been proposed, in particular addressing issues such as reducing false-positive clones [2–4], enhancing long-range amplifications [5], and systematically surveying the repertoire of differentially expressed mRNAs [4, 6]. It would be beyond the scope of this report to describe the DD method in detail, and I will rather schematize an integrated strategy used in our laboratory which facilitates its use as a robust molecular screen for the detection of differentially regulated mRNAs, and their characterization as expressed sequence tags (ESTs). For a more in-depth description of current protocols, I refer to other reports in this issue and elsewhere [7–9]. Instead, I emphasize ways how mRNA DD has been applied to identify differentially expressed genes making use of unique features of bird and amphibian experimental model systems.

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mRNA DD in birds

The chicken, a versatile model for studying differential gene expression

Among birds, the chick has been shown to be a fast and powerful system for experimental manipulation and functional studies. Classical embryologists took advantage of the easy access to the embryo and, by performing transplantation experiments, identified important principles in developmental biology. With the advent of modern molecular tools, the chick remained an important model and has been utilized in a substantial number of mRNA DD screens. One important area of discovery has been the identification of hormone-responsive genes. Chick liver and particularly the oviduct, which is dependent on estrogen, have been used to identify genes that are direct targets of the estrogen receptor [10–12]. Chamberlain and Sanders [12] have isolated an estrogen-regulated transcription factor and its putative binding site in the 5′-flanking region of the ovalbumin gene, thus defining a novel regulatory cascade triggered by estrogen. In addition, mRNA DD was applied to normal and growth hormone (GH) receptor-deficient dwarf chickens and identified genes that play a role in mediating GH action [13, 14]. Chondrocyte differentiation and bone growth have been other productive research areas that made use of the DD technology [15–17]. Investigators have also used this strategy to find genes involved in the chick visual system; establishment of retinotectal topography during embryonic development [18], retina differentiation from precursor neuroectodermal cells [19], and image projection on the retina [20, 21]. DD has been employed in order to isolate genes that are involved in central nervous system (CNS) pattern formation [22], and in development of the inner ear [23]. In examining gene expression after noise trauma, several investigators identified differentially expressed genes in the chick basilar papilla [24, 25], and in the cochlea where new hair cells are produced to replace those that were lost [26]. These studies have paved the way for gain- or loss- of-function studies to directly test the role of these differentially regulated genes in repair of damaged epithelium or in hair cell regeneration. A selection of the various research areas and identified differentially expressed genes is summarized in table 1.

Zebra finches as a model to elucidate the song system of songbirds

Male zebra finches display two song behaviors, either when the bird is alone or in the presence of a female. The brain structures responsible for song learning and production are organized as an interconnected network of nuclei known collectively as the song system [27]. The high vocal center (HVC) integrates auditory and motor activities and constitutes a nodal nucleus in the song system. HVC projects to the nucleus robustus archistriatalis (RA), which in turn projects to hypoglossal motor neurons that innervate the vocal organ, the syrinx. This pathway is the motor backbone of the song system and is necessary for production of learned song. Another anterior pathway connects HVC and RA indirectly, and is necessary for acquisition but not production of learned song. The identification of genes preferentially expressed in HVC provides the potential to unravel molecular components involved in song system regulation (table 1). One of the most interesting aspects of the song system is that it is dependent on the production of new neurons. Adult vertebrate neurogenesis is a rare event, although it has recently been described in subregions of the mammalian brain. Therefore, genes that are found by DD to be regulated in the HVC may provide a window on the molecular mechanisms of adult vertebrate neurogenesis.

Screen design

Brain regions belonging to the HVC and those of the underlying neostriatal shelf differ markedly in cytoarchitectonics, connectivity and physiology [28]. Denisenko-Nehrbass and co-workers [28] made use of these features, particularly the straightforward anatomical recognition of regions of interest as visualized under dark-field illumination. In order to have carefully separated tissue preparations for the identification of genes specifically expressed in song nuclei, brains of male zebra finches were sliced sagitally and regions belonging to the HVC and those of the underlying shelf were punched out (fig. 1A). RNA was prepared and subjected to mRNA DD analysis.

Genes preferentially expressed in the HVC

The screen for molecular markers of HVC in the zebra finch brain identified several candidate DD genes with differential expression between HVC and the shelf. To confirm their differential expression, the authors employed in situ hybridization on brain sections comparable to those used for DD tissue extraction. Using the cloned DD fragments as in situ probes, one cDNA produced a very distinctive expression pattern (fig. 1B). It revealed high levels of mRNA in HVC, but no expression in the shelf or in other structures including caudal neostriatum, archistriatum, hippocampus, brain stem and cerebellum. The open reading frame of a full-length cDNA from an embryonic zebra finch library encoded a class 1 aldehyde dehydrogenase (ALDH), and was named zRalDH (for zebra finch retinaldehyde-specific ALDH). The presence of a retinoid-synthesizing enzyme in specific regions of the adult brain had not yet been demonstrated. Consequently, the authors investigated whether zRalDH would be involved in retinoic synthesis, and found that zRalDH had a very high affinity for retinaldehyde and that the brain ar-

Table 1. Screens for differential gene expression reported in birds.

Genes involved in	Experimental tissue	Expression verification	Identified genes	Reference
Chicken				
Estrogen targets	Liver	Northern	ADL CYP450 ovoinhibitor PGDS	10
Estrogen targets	Oviduct	Northern	MRP1	11
Estrogen targets	Oviduct	Northern	delta EF1	12
GH targets	Liver	Northern	sulfotransferase gene family member	13
GH targets	Liver	Northern	GHRG-1	14
Chondrocyte differentiation	Long bone growth Plate	RT-PCR	B-cadherin EF2 HT7 EX-FABP	15
Chondrocyte differentiation	Retinoic acid-treated Chondrocytes (in vitro)	RNase protection In situ hybridization	chondromodulin-I proliferation-specific 1.9-kb mRNA	16
Chondrocyte differentiation	Long bone growth Plate	RT-PCR	novel mRNA encoding transmemberane protein	17
Nasal-temporal axis specification	Eye, retinal neuro-Epithelium	RNA dot blot	nasal/temporal-specific mRNAs	18
Retina precursor cells	Eye, retina	Northern	cyclin D1	19
Vision deprivation	Eye, retina, choroids	Northern	cytochrome-c oxidase	20
Vision deprivation	Eye, photoreceptors	Northern	neuroendocrine-specific protein A and C	21
CNS patterning	Midbrain, hindbrain	In situ hybridization	receptor kinase antagonist sprouty 2	22
Inner ear development	Otocyst	Northern	novel 0.8kb mRNA	23
Noise trauma, Hair cell regeneration	Basilar papilla	RNA slot blot	parathyroid hormone-related protein, delta-subunit of neuronal-specific Ca^{2+}/cal-modulin-regulated protein kinase II, GTP-binding protein CDC42	24
Noise trauma	Basilar papillar	RNA slot blot	UBE 2B	25
Hair cell regeneration	Cochlea			26
Songbirds				
Song system regulation	Brain	In situ hybridization	zRalDH (class I aldehyde dehydrogenese	28

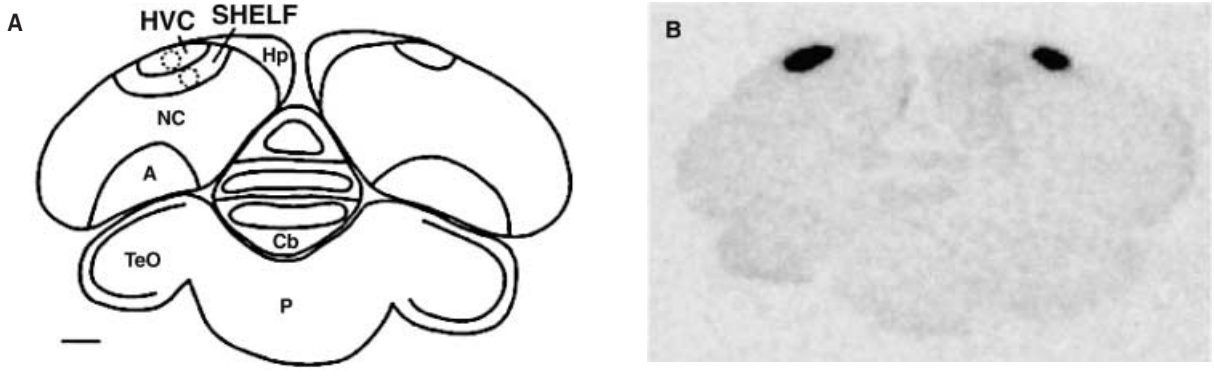

Figure 1. Isolation and expression analysis of zRalDH in zebra finches. (*A*) Diagram of frontal brain section through HVC indicating punch sites (dashed circles) where tissue has been removed for DD screen. (*B*) In situ autoradiogram of brain section probed with zRalDH [same level as in (*A*)] demonstrating restricted expression in HVC. Abbreviations: A, archistriatum; Cb, cerebellum; Hp, hippocampus; HVC, high vocal center; NC, caudal neostriatum; P, paleostriatum; TeO, optic tectum. Dorsal is up; scale bar, 0.5 mm. (By courtesy of Claudio Mello, New York, NY, USA, and Neuron, USA).

eas expressing the gene generate retinoic acid. In addition, they demonstrated that blocking zRalDH enzyme activity in the HVC of juveniles disrupted the normal process of song maturation. In sum, this study identified a retinoid-regulated process in the CNS, which plays a modulatory role in the maturation of the learned song behavior.

mRNA DD in amphibians

The African clawed frog, a model for studying embryonic development

For a long time the frog has also been a favorite model for experimental developmental biologists because its fairly large oocytes make it easy to manipulate. Not surprisingly, many investigators have adopted mRNA DD as a tool to identify genes in biological processes in the frog. The different research areas are briefly summarized with a focus on one biological problem, the circadian clock, to highlight the exceptional synergy of model system and DD strategy. As shown in table 2, employing the display technology led to the isolation of several novel genes. Maternally transcribed messages have been identified by their differential expression or localization to play a role in axis formation of the early embryo, patterning the nervous system and cell cycle control [29–31]. Also, *Xenopus* embryos or explants proved to be very useful for the identification of downstream target genes of known transcriptional inducers such as fibroblast growth factor (FGF) or activin [32–36].

Xenopus as a vertebrate model system to study circadian timekeeping

Circadian rhythms, generally detected as daily oscillations in behavior, physiological or biochemical processes, are widespread in organisms ranging in complexity from the blue-green algae to flowering plants and humans. Progress in understanding the underlying molecular principles of biological timekeeping came first by cloning three clock genes in *Drosophila* and *Neurospora*, respectively. However, although circadian clock mutants had been identified in mouse, the identification of clock genes in vertebrates remained elusive. Unlike other vertebrates, the clock in *Xenopus laevis* had been localized to the photoreceptor layer within the retina and made the frog an excellent system to uncover the molecular mechanisms of vertebrate circadian control [37].

Table 2. Screens for differential gene expression reported in amphibians.

Genes involved in	Experimental tissue	Expression verification	Identified genes	Reference
Frog				
A-P axis patterning	Oocyte, embryo	In situ hybridization	Enhancer of Zeste	29
A-P axis patterning	Oocyte	In situ hybridization	Mitotic checkpoint gene Xbu3	30
RNA localization Animal/vegetal cortex	Oocyte	Northern	An4a Beta-TrCP	31
Lithium response	Oocyte	In situ hybridization	Xoom	32
FGF targets	Embryo explants	Northern, quantitative PCR	Er1	33
Concanavalin A targets	Embryo animal cap explants	Northern	Neurula-specific ferredoxin reductase-like protein Nfrl	34
Activin targets	Embryo animal cap explants	In situ hybridization	Antipodean	35
Retinoic acid targets	Embryos	In situ hybridization	X17C	36
Circadian clock	Retina, photoreceptors	Northern	Nocturnin	38
Cricadian clock	Retina, photoreceptors	Northern, In situ hybridization	Four clock-controlled mRNAs	39
Newt				
Regeneration/ differentiation	Regeneration blastema	RNase protection, Northern	Skeletal myosin heavy chain (MHC)	4
Regeneration/ pattern formation	Regeneration blastema	RNase protection, In situ hybridization	Tbx5	43
Regeneration	Muscle	In situ hybridization	Rad (ras associated with diabetes)	44
Meiosis	Testis	Northern	Dynein intermediate Chain	45

Screen design

Green and Besharse [38, 39] built on the expectation that the general principles of biological clocks should be conserved, and devised a clever screen for clock-controlled genes in the *Xenopus* retina. In their experiments they made use of the fact that the *Xenopus* eye cup contains a circadian clock, and that the clock functions for extended periods in culture and can be synchronized to light-dark cycles (fig. 2A). They used cultured eye cups to eliminate any potential influence of an extraocular clock, and to ensure that rhythmicity was due to the clock and not to an acute effect of light or darkness, they cultured the eyecups in constant darkness throughout the experiment. In this setup, clock function could be verified by monitoring a known output, the rhythmic release of melatonin.

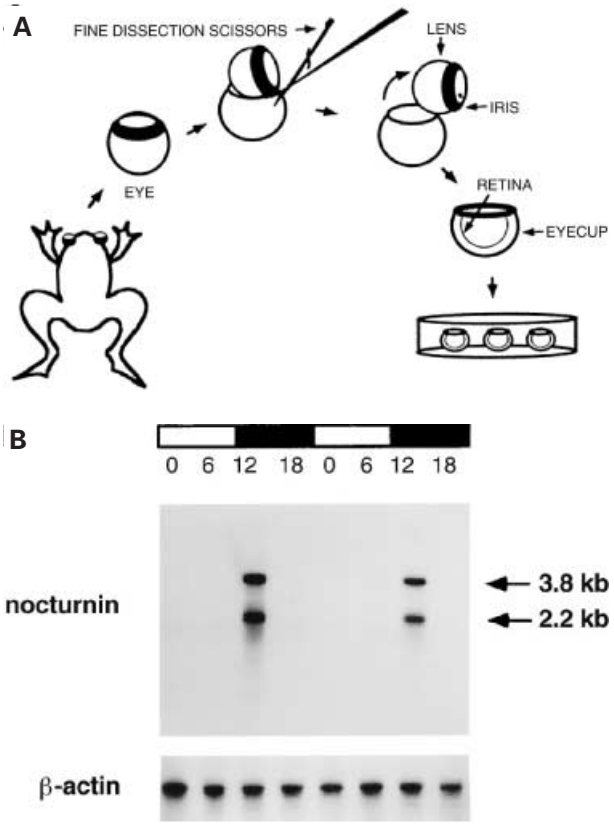

Figure 2. Experimental setup for isolation of clock-controlled mRNAs and expression analysis of nocturnin in *Xenopus laevis*. (*A*) *Xenopus* were entrained to a light/dark cycle before eyecups were prepared and cultured in constant darkness. At 6-h intervals over 2 days, retinas were harvested for DD analysis, and culture medium was collected for melatonin assay. (*B*) Expression patterns of nocturnin mRNA in comparison to β-actin from individual retinas by Northern blot. Bars above the blot indicate the lighting conditions: black bars indicate night (dark) and white bars indicate day (light). Numbers above the lanes denote Zeitgeber Time (ZT) in hours, in which ZT 0 is defined as the time of normal light onset and ZT 12 is defined as time of normal dark onset. (By courtesy of Joseph Besharse, Kansas City, KS, USA, Methods Mol. Biol., USA, and Proc. Natl. Acad. Sci. USA).

For two days at fixed intervals, retinas were removed from cultured eye cups, RNA was extracted and used to screen for transcripts displaying a daily pattern in expression.

Differential gene expression during light-dark cycles

In scanning more than 2000 bands on display gels, the authors identified four that revealed a consistent temporal pattern of expression over the experimental 2-day time interval. One of these candidate genes was present at the time of normal dark onset on both days and not detectable at the other times, and because of its nighttime expression was named 'nocturnin'. The 445-bp band was cloned and used as a probe to verify expression patterns, and to screen retina cDNA libraries for the isolation of full-length clones (fig. 2B). Employing in situ hybridization, the transcript proved to be specific to photoreceptors. Its rhythmic high-level expression at night persisted in eye cups kept in constant darkness, demonstrating that the rhythm was under the control of an endogenous clock. Nuclear run-on experiments demonstrated a robust circadian rhythm of transcriptional initiation in constant darkness, proving that clock control was transcriptional. The deduced nocturnin amino acid sequence revealed a novel protein that contained a leucine zipper-like protein dimerization motif. The sequence also showed significant homology to CCR4, a non-DNA-binding transcriptional coactivator in yeast, which would suggest transcriptional regulatory functions. Sequences similar to nocturnin have since been isolated from several other species, including chicken, cow, mouse and humans [37, 39].

Urodele amphibians as a model system to study limb development and limb regeneration

Regeneration can be viewed as the reiteration of embryogenesis in order to repair or replace damaged or lost tissues. In mammals, regeneration is facilitated by stem cells. These cells are maintained in an undifferentiated state, and have the capacity to self-renew and differentiate into a number of cell types [40, 41]. Urodele amphibians (newts and salamanders) can replace lost appendages through the formation of a regenerating blastema. The blastema cells are derived by local dedifferentiation of adult mesenchymal tissue in the stump. They reenter the cell cycle, proliferate and undergo differentiation and morphogenesis to completely replace the lost structure. Newts can regenerate limbs and tails throughout their lifetime [42], and thus make a unique model system for studying the control of cellular dedifferentiation, growth and differentiation. Such processes have been expected to rely on the differential expression of genes, and two research groups have employed mRNA DD as an inroad for their identification, [4, 43, 44], as summarized in table 2.

Screen design

In order to identify novel genes involved in the formation of a blastema (regeneration-specific) as well as in its morphogenesis (pattern-specific), we analyzed differences in gene expression during the course of forelimb, hindlimb and tail regeneration (fig. 3A). We hypothesized that regeneration-specific genes should display a set of mRNA/PCR products that are common to the different tissue types employed. These shared expression profiles may characterize mRNAs that play a role in wound healing, dedifferentiation, growth and differentiation. In addition, working on the assumption that the identity of either an arm, a leg or a tail might be controlled by different gene activities, we expected to display messages that were differently regulated in the different types of appendages. These tissue-specific mRNAs may be involved in controlling the formation of individual morphological patterns, typical of an arm or a leg.

Screen methodology

Regenerating blastemas of the various appendages were staged by morphology and time, and approximately 100 samples per differentiation stage were pooled. Reactions representing a time course of regeneration were analyzed in neighboring lanes on display gels (fig. 3B). To make mRNA DD amenable as a molecular screen, we have optimized the technology by (i) introducing two-step PCRs with greatly reduced amplification cycles, and (ii) directly sequencing the cDNA PCR products, thus eliminating conventional cloning steps and allowing efficient analysis and characterization of many DD isolates as ESTs [4]. Using this approach, we prepared a library of cDNA fragments corresponding to mRNAs with temporal and spatial differences in expression. To confirm differential expression of DD isolates in experimental tissues, we routinely used quantitative reverse-transcription (RT)-PCR with gene-specific primers to amplify fragments of similar size ranging from 200 to 250 bp in length. PCRs were performed in parallel for three or more different cycle numbers to assure that amplification was in a linear range, and gel band intensities were quantitated on a phosphorimager and normalized to elongation factor 1α (EF-1α), a gene found to be expressed at a constant rate [43]. Although many of the DD products showed homology to known genes, the vast majority of cDNAs were within the 3′ untranslated region and did not reveal the nature of the gene product. To gain structural information on the putative proteins, we used 5′ rapid amplification of cDNA ends (5′ RACE) for efficient isolation of full-length cDNAs. As an inroad towards the function of an individual cDNA isolate, we performed RNase protection to appreciate subtle differences in expression levels, and whole-mount in situ hybridization to visualize the three-dimensional distribution of mRNA [43].

Differential gene expression during limb regeneration

Approximately 600 mRNA/PCR fragments displayed differential expression patterns during limb regeneration. Roughly half of the isolates appeared to be downregulated in regenerating blastemas, and half of them were upregulated. All cDNA fragments were processed for sequencing and redundant isolates were eliminated, yielding a total of 177 down- and 183 upregulated RNA/PCR fragments. GenBank searches of these differentially expressed sequence tags revealed homologies to previously recorded sequences for about 20% of the isolates. Among the genes downregulated during regeneration, we identified predominantly muscle-related mRNAs such as myosin light and heavy chains, actin, titin and creatine kinase. This was an expected finding since a regenerating blastema does not contain muscle, as compared with a normal limb. Among the upregulated genes we found the majority to code for EF-1γ, transfer RNA (tRNA) synthetases, and so on; i.e. genes that are directly linked to the physiologic state of the proliferating blastema cells. In total, the screen identified approximately 120 mRNAs that appeared to be differentially expressed in the forelimb, hindlimb or tail territory of the animal, and these may represent potential pattern control genes. Approximately 43 differentially regulated mRNAs were identified as common to all four experimental tissues. These have been considered candidates for genes that may play a role in wound healing, tissue dedifferentiation and differentiation.

Genes involved in limb-specific patterning

As candidate genes that may control limb identity, we have analyzed mRNAs that were exclusively expressed in regenerating forelimbs or regenerating hindlimbs. One of the forelimb-specific cDNA isolates contained a T-box motif and was later identified to be the newt homolog of the mouse and human T-box gene *Tbx5* [43]. Using the evolutionarily conserved T-domain as a molecular tag, we isolated the closely related *Tbx2*, *3*, *4* and *5* from both newt and chick, and investigated their temporal and spatial expression patterns during limb development and limb regeneration, respectively [43, 46]. In these studies we demonstrated that the forelimb-specific *Tbx5* and the hindlimb-specific *Tbx4* revealed properties expected of a gene having a role in controlling limb morphology. Comparable to developing limbs, gene expression in regenerating newt limbs was limb type specific (fig. 3C). However, *Tbx4* and *Tbx5* were constitutively expressed at low levels in adult newt limbs. This may suggest that the residual gene activity is needed for regeneration to occur. Our studies in regeneration also indicated that *Tbx4* and *Tbx5* might provide proximal/distal axial positional information [43, 47].

These presumptive transcription factors revealed a remarkable evolutionary conservation among vertebrates

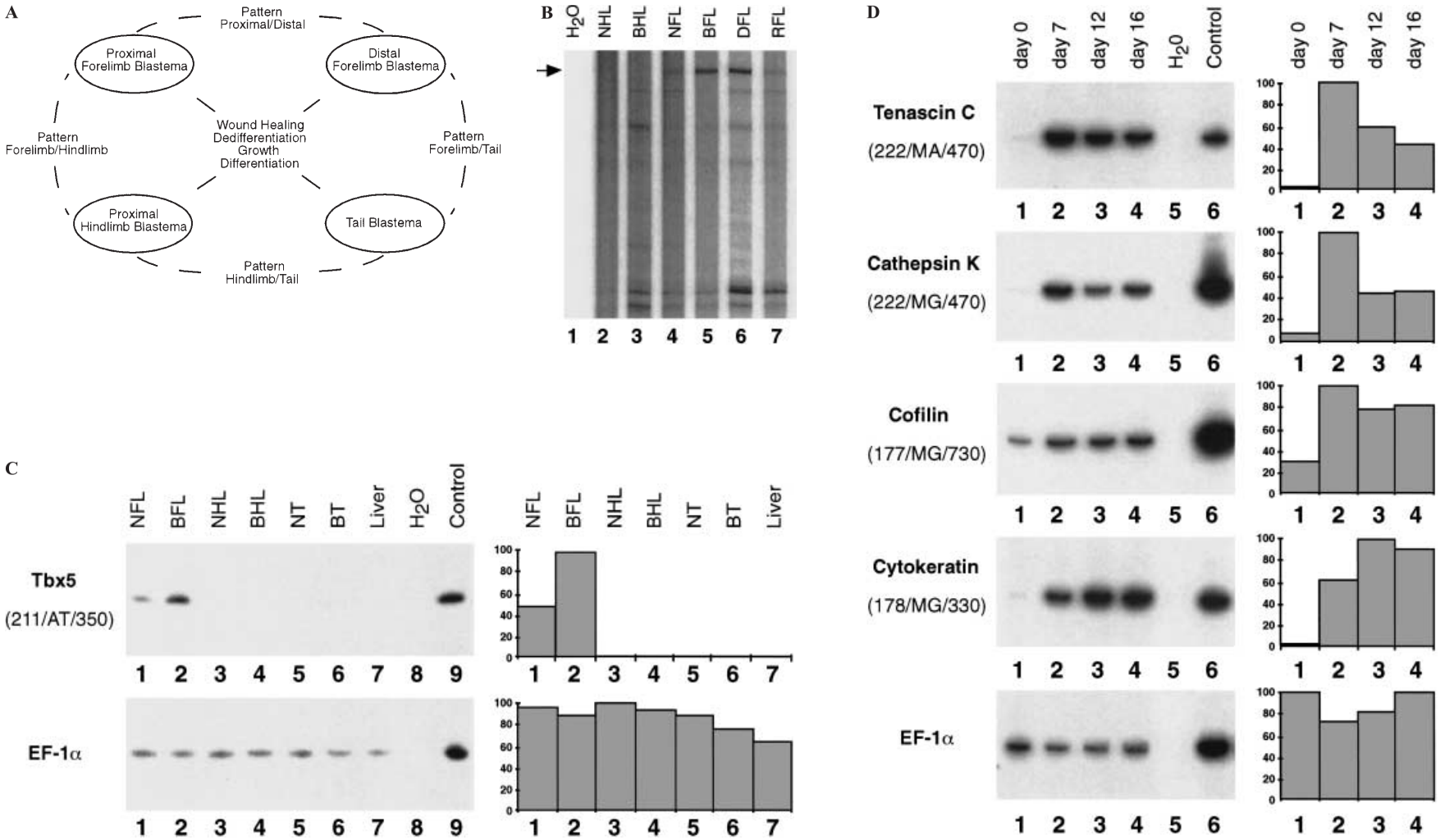

Figure 3. Isolation and expression analysis of pattern-specific genes and regeneration-specific genes in *Notophthalmus viridescens*. (*A*) Experimental design of screen. Newts were amputated at either proximal or distal positions of forelimbs, proximal hindlimbs and tails. Regenerating tissues were collected for DD screen at day 0, 7, 12, 16 and 42 (complete regeneration). (*B*) mRNA DD showing expression patterns of Tbx5 with RNA from normal and regenerating forelimbs, and normal and regenerating hindlimbs. Lanes: (1) H_2O control; (2) normal hindlimb, d0 [NHL]; (3) blastema hindlimb, d12 [BHL]; (4) normal forelimb, d0 [NFL]; (5) blastema forelimb, d12 [BFL]; (6) digit-stage forelimb, d16 [DFL]; (7) regenerated forelimb [RFL]. The arrow indicates the position of the differentially expressed 350 bp PCR product [211/AT/350]. (*C*) RT-PCR analysis demonstrating the spatial expression patterns of newt Tbx5 mRNA in various tissues, and quantitation of relative levels of expression after phosphoimager scanning. Lanes: (1) normal forelimb, d0 [NFL]; (2) blastema forelimb, d12 [BFL]; (3) normal hindlimb, d0 [NHL]; (4) blastema hindlimb, d12 [BHL]; (5) normal tail [NT]; (6) blastema tail [BT]; (7) liver; (8) H_2O; (9) cDNA control. (*D*) RT-PCR analysis demonstrating temporal expression of forelimb regeneration with primers specific for newt DD isolate 222/MA/470, 222/MG/470, 177/MG/730, 178/MG/330 and the normalizing EF-1α. Lanes: (1) normal forelimb, d0; (2) blastema forelimb, d7; (3) blastema forelimb, d12; (4) blastema forelimb, d16; (5) H_2O; (6) cDNA control. In (*C*) and (*D*) expression levels of the RT-PCR products have been quantitated by phosphoimager scanning, and each data point in the bar diagram represents the ratio of the PCR products amplified to the EF-1α normalizing gene and is given as percentage of the respective peak values. (Courtesy of Hans-Georg Simon, Chicago, IL, USA, and Development, England)

from urodele amphibia to humans. A mutated allele of the homologous human gene *TBX5* has been reported to cause Holt-Oram Syndrome (HOS), a disease characterized by hand, arm and shoulder malformations, as well as septation defects of the heart [48, 49]. The dominant phenotype of the mutation demonstrates an essential regulatory role of this transcription factor for proper development and shaping of the upper limbs.

Genes involved in cell dedifferentiation and differentiation

Recent studies suggest that maintenance of the differentiated state of a cell or tissue requires continuous regulation. In understanding the mechanisms that underlie the stability of differentiation, the reversal of the process, termed dedifferentiation, is of particular interest. In concentrating on cDNAs whose expression is upregulated (i) at the correct time window for dedifferentiation, and (ii) in all regenerating appendages irrespective of the amputation level, we have identified candidate genes that might play a role in the control of the differentiated or dedifferentiated state of a cell. The data indicate different classes of molecules, extracellular matrix components, and secreted proteases and inhibitors, as well as putative transcription factors [H.-G. Simon, unpublished observations]. Many of the genes have been previously cloned in other species, but not all have known functions, particularly not in the context of regeneration and blastema formation. Overall, we observed two typical expression profiles after amputation, an immediate peaklike response and a more gradual response. Four examples represent these observations (fig. 3D).

Tenascin C (222/MA/470) is a component of the extracellular matrix which mediates cell adhesion and has been previously shown to be expressed both during mammalian wound healing and newt limb regeneration [50, 51]. Cathepsin K (222/MG/470), a cysteine protease which degrades bone collagen in mammalian osteoclasts is involved in bone remodeling and bone resorption [52]. In regeneration it is likely that cathepsin K activity is important for replacement of lost bone structures, in a continuous manner from old to new. The early upregulation of these two genes in the newt suggests mechanistic similarities between wound healing and the dedifferentiation stages of regeneration. Cofilins (177/MG/730) are members of a superfamily of actin-binding proteins and mediate the stability of actin filaments [53]. With the transition from a differentiated state to a less differentiated state, and from quiescence to proliferation, a large supply of cofilin would be required to regulate a dynamic cytoskeleton. Cofilins were expressed in normal limb tissue, and a week after amputation their expression level increased to a high steady-state level while blastema cells were proliferating and building cell mass. A new newt cytokeratin (178/MG/330) displayed a more gradual upregulation after amputation. Cytokeratins are intermediate filament proteins found in epithelial and epidermal cells, and are thought to play a role in giving shape and structural integrity to a cell. This type I keratin may be the heterodimerizing partner of a previously identified newt type II keratin [54] in basal keratinocytes of the skin and the wound epidermis of the blastema.

The regeneration model system has clearly provided access to different classes of genes controlling cell function, including those that are involved in controlling the differentiated state of a cell and those that play a role specifying regional identity and pattern.

Perspectives

Many biologically relevant questions have to be addressed experimentally in vivo, employing appropriate model organisms instead of using cell lines. As has been reviewed here, display protocols have been useful in revealing differentially expressed mRNAs in different animal systems. However, complex systems have inherent problems, and some technical notes should be considered for a successful outcome of a DD screen. As starting material for producing the RNA for the DD, tissues should be taken from a number of different animals. This reduces risks of animal-to-animal variability, which may produce different bands on the display gels superimposed on experimental results. In addition, to minimize false positives due to genomic variability and low-stringency PCR, it is important to use duplicate or triplicate samples and a stringent criterion for band selection. For instance, we compared the bands in neighboring lanes and selected those that showed the same expression pattern over a time-course, from early to late regenerates (fig. 3B). This allowed us to obtain a trend of individual gene activities over time and proved to minimize the isolation of false positives. To confirm differential expression, various methods have been used. However, protocols with similar sensitivities as DD such as RT-PCR, RNase protection or in situ hybridization produced the most reliable results and are therefore recommended.

Over the past 20 years, certain animal systems have been selected as models based on their accessibility to genetics, and more recently, sequencing projects have been established to unravel their genomes. Many questions, though, cannot be answered by genetic manipulation alone, and it is essential to make use of the impressive diversity of species to probe various aspects of biological function. For several important model systems such as the chick, there is no systematic attempt to identify and map all expressed genes. Thus, researchers cannot rely on ESTs deposited in databases covering the genome of their chosen animal model. For this reason, mRNA DD will remain a method of choice to detect differential gene ex-

pression. While an integrated DD technology including a direct sequencing approach [4] facilitates its use as a systematic molecular screen, the confirmation of hundreds of bands is very labor intensive. To overcome the limitations of analyzing individual genes or at best small groups of genes, the profiling of gene expression with cDNA arrays may be a practical alternative [55]. The gene array technique enables the monitoring of expression patterns and expression levels at the same time for hundreds or thousands of genes. DNA arrays typically contain cDNAs from both novel genes and known control genes spotted on a solid support, e.g. glass slide. The novel genes could be, for example, RT-PCR products coming from a DD screen. Hybridization of the arrays with fluorescent probes derived from RNA isolated from two or more different experimental conditions would allow for a simultaneous analysis of large numbers of candidate genes, and a verification of their differential expression patterns.

In sum, combining mRNA DD and gene array technologies holds great promise for an efficient identification and isolation of differentially expressed genes in a wide range of model organisms in order to address many biological problems.

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