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## In situ analysis of gene expression in *Xenopus* embryos

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### Abstract

The molecular anatomy of the vertebrate embryo was systematically analysed through gene expression during early development of the *Xenopus* frog using whole-mount in situ hybridization. Expression patterns are documented and assembled into the database Axelldb (<http://www.dkfz-heidelberg.de/abt0135/axelldb.htm>). Synexpression groups representing genes with shared, complex expression pattern that predict molecular pathways involved in patterning and differentiation have been identified. These sets of co-regulated genes show a striking similarity with operons, and may be a key determinant facilitating evolutionary change leading to animal diversity. **To cite this article:** N. Pollet et al., *C. R. Biologies 326* (2003).

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### Résumé

**Analyse in situ de l'expression des gènes dans les embryons de Xénope.** L'anatomie moléculaire de l'embryon de vertébré a été étudiée de manière systématique à travers les patrons d'expression géniques au cours du développement précoce du Xénope par hybridation in situ sur embryons entiers. Les descriptions de ces profils d'expression géniques sont documentés dans la base de données Axelldb (<http://www.dkfz-heidelberg.de/abt0135/axelldb.htm>). Nous avons identifié des groupes de synexpression, représentant des gènes dont l'expression complexe quasi identique prédit des voies moléculaires utilisées dans le développement et la morphogenèse. Ces groupes de gènes co-régulés sont très similaires aux opérons et pourraient être des éléments clés des changements évolutifs conduisant à la diversité du monde animal. **Pour citer cet article :** N. Pollet et al., *C. R. Biologies 326* (2003).

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**Mots-clés :** base de données ; développement ; expression génique ; génomique fonctionnelle ; groupes de synexpression ; hybridation in situ ; transcriptome ; *Xenopus laevis*

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## 1. Introduction

Today, the nuclear genomic DNA of different eukaryotes, unicellular and pluricellular, has been sequenced. Of the recently completed *Drosophila melanogaster* genome sequence, 17% of the 13,600 genes identified did not show any similarity to anything previously described [1]. Even if a molecular function can be tentatively assigned for the majority of the remaining genes, their biological role is often unknown.

The study of all these genomic sequences using informatics tools will not be sufficient to make the connections between sequence and biological role [2]. To take the benefit of this intimate knowledge of genomes will require systematic detailed functional studies using experimental set-up that take advantage of various model organisms, what can be called experimental genomics.

Geneticists and molecular biologists have been interested in quantifying gene transcripts for many years and for various reasons [3]. The characterization of the transcripts is the first step to make connections between genomic sequences and the many biological roles of the encoded genes. The description and analysis of gene expression patterns is crucial to elucidate the physiological functions of genes and to understand the network of genetic interactions that underlies the process of normal development. Knowing the patterns of expression of large numbers of genes allows the identification of specific promoters, marker genes useful to monitor cells in a specific state and genes that are tightly co-regulated.

In functional genomics, the generation of the expression data for large numbers of genes should be a means of placing newly characterized sequences into context with respect to their sites of expression, to study the correlation between gene expression and function, and to correlate the expression profiles with regulatory sequences. In particular, developmental expression patterns of related genes in different vertebrate species have proven predictable power for other vertebrate species, including man, where such analyses are not easily feasible.

### 1.1. Bioinformatics and gene expression

While the study of the expression pattern of a gene is a prerequisite to understand its biological role,

the characterization of the expression of most known genes is incomplete. As a consequence it is almost impossible to compare gene expression patterns, and there is few specialized public databases available storing the data.

Databases of gene expression are needed as a resource for the emerging field of functional genomics. Currently, there are gene expression databases available for the worm *Caenorhabditis elegans* [4], the fly *Drosophila melanogaster* [5] and the mouse [6], but one is lacking for *Xenopus* although it is a major model system in embryology since decades.

Some laboratories are engaged in large-scale in situ hybridization screening [7]. Due to these and other non-systematic efforts we expect a dramatic increase in the number of characterized gene expression patterns in the future. The development of specialized databases will therefore be imperative to manage and interpret this particular data.

### 1.2. Functional genomics and the embryo

The embryos are particular well suited to experimentation, as decades of experimental embryology have shown, and have the potential to reveal *in vivo* the biological role of genes, what some authors have called the natural history of genes [8].

It is generally accepted that a major mechanism in cellular differentiation and development is based on differential gene expression. Therefore, description of gene expression is now considered an essential part of the characterization of novel genes. The expression pattern often gives important clues about the function of a gene and makes readily testable predictions. Moreover, mechanisms of embryonic patterning as well as evolutionary relationships between seemingly unrelated structures in different animals could be derived by such analysis. Despite this utility, the number of genes whose embryonic expression pattern has been documented by in situ hybridization is relatively small, on the order of a few hundreds in mouse, *Xenopus* and zebrafish with many of them being orthologs.

To identify all classes of developmentally important genes, expression-based and other molecular screens are needed to supplement classical genetic screens. In *Drosophila*, the most productive of these screens to date have used P element-based en-

hancer traps, but P element insertion is not random and enhancer trap screens are biased toward identifying genes that are favored for insertion by P elements [9, 10]. In a screen based on in situ hybridization, 80% of the genes found were not previously described, underscoring the potential of this approach [11].

We are working on the systematic analysis of gene expression patterns during early development of *X. laevis*. This gives us the tools to study the molecular anatomy of the embryo while isolating new genes involved in early development. Besides, we developed a knowledge base focusing on gene expression.

Contrarily to most of the current work on gene expression profiling that uses microarrays in yeast or human, we developed a large-scale approach coupling whole mount in situ hybridization and partial cDNA sequencing [7,11–16].

## 2. Results

### 2.1. Strategy

Spatial and temporal embryonic expression profiles of the genes represented in a neurula stage cDNA library were determined by RNA in situ hybridisation to whole-mount *Xenopus* embryos [13,17]. This developmental stage was selected because most of the genes expressed during gastrulation are still transcribed, and genes involved in neurogenesis are already active.

RNA probes were prepared from individual, randomly picked cDNA clones and screened on albinos embryos at stages gastrula, neurula and tailbud. This enabled to characterize gene expression at the critical phases of mesoderm regionalization, neurogenesis and organogenesis.

When a restricted expression pattern was observed, it was described in a semi-quantitative way and pictures of stained embryos taken. The corresponding cDNAs were partially sequenced.

### 2.2. Categorisation and analysis of expression patterns

We defined main categories of gene expression patterns as follows. Expression of a gene at all examined embryonic stages and in most cells of the embryos was scored ubiquitous. Distinctive patterns of expression,

with clearly visible different domains, were scored patterned and grouped accordingly to the developmental stage at which the patterning was observed. Finally, absence of staining at any of the examined stages was scored as undetected. Additionally, we defined sub-categories for the patterned main category. These followed either anatomical aspects (e.g., gastrula mesoderm), cellular compartmentalization (e.g., nuclear localization) and temporal differences (e.g., gastrula only).

In a pilot study, 1765 cDNAs taken at random in a cDNA library made from neurula stage *X. laevis* embryos have been characterized by whole mount in situ hybridization on gastrula, neurula and tailbud stage embryo [13]. The results can be summarized as follows: 26% of the cDNA clones corresponds to genes with a patterned expression, 51% of the cDNAs corresponds to ubiquitously expressed genes and for 23% of the cDNAs, no signal can be detected after hybridization. The analysis of partial cDNA sequences from the 449 cDNA clones coming from the differentially expressed genes shows that they identify 273 unique genes (less than 1% of the fraction of the genome expressed during embryogenesis), from which 207 are newly described in *Xenopus* and 24% without known homologs.

We attempted a functional classification of the 208 cDNAs with attributable function: strikingly, 27% of all unique cDNAs with attributable function represent genes with a potential regulatory role in development (growth factors and receptors, signal transduction components and transcription factors). The most prominent figure is the increase in the complexity of gene expression patterns as development proceeds, and notably in the central nervous system (82% of genes at stage 30) and in the tailbud region. In *Xenopus* embryos, the expression in endoderm can not be reliably assessed due to the limitations of the whole-mount procedure, where penetration of tissues rich in yolk is a problem [13,18].

We concluded from this pilot study that this systematic approach is a powerful mean to identify new regulators of development.

### 2.3. The molecular anatomy of the embryo

Among the genes identified, 65 can be used as differentiation markers in the tailbud stage embryo be-

cause their expression is so specific. These marker genes can be used to identify the progressive regionalization during early development, revealing the molecular anatomy of the embryo (the description of development from the point of view of gene expression). Using novel genes, we have thus described the regionalization of the gastrula embryo, of the epidermis and of the tailbud region of tailbud stage embryo.

#### 2.4. Synexpression groups

The study of the whole set of transcribed genes, the transcriptome, can provide new information on the function of genes for which sequence similarity searches does not give any clues. We compared the gene expression profiles and could group genes into sets according to their highly similar expression pattern. This is what we called synexpression groups.

In our pilot study we found four such synexpression groups: Delta1, Bmp4, endoplasmic reticulum and chromatin. For example, genes of the Bmp4 group are all found expressed dorsally in the eye, heart, tailbud and lateral plate mesoderm of tailbud-stage embryos. This group consists of seven members which all encode components of the BMP signalling pathway, as studied in early dorso-ventral patterning of mesoderm.

A literature survey shows that there is many more (glycolysis, cholesterol biosynthesis, GABA signaling...) uncovered by gene expression profiling using microarrays or RT-PCR experiments in yeast, rat or human [19].

In a sense, we can see synexpression groups in eukaryotes as a form of regulation that reminds us of the bacterial operon. It is probable that this organization, like a subroutine in the genome program to make an analogy with informatics, has been an ingredient in evolution that facilitated the phenotypic changes observed in eukaryotes [19].

Within a synexpression group, there is at least one gene whose function and biological role are known. For the Bmp4 group, it is the *bmp4* gene encoding a member of transforming growth factor beta family. For the Delta1 group, it is *Xdelta1*, a gene encoding a ligand of the Notch receptor. For the endoplasmic reticulum group, it is genes encoding proteins involved in protein import into the endoplasmic reticulum. For the chromatin group, most of the gene members

are structural components of chromatin (histones and HMG-box proteins) or transcription factors.

This categorization of genes is informative in many aspects. Genes are grouped because they are involved in the same cellular process, metabolic or signaling pathway. It is therefore possible to predict the biological role of those genes qualified as orphans today when their expression is characteristic of the one of a well-described gene. Hypothesis can thus be formulated, and tested experimentally afterwards (this is what has been done for *Xvent2* and *Bmp4*, members of the Bmp4 synexpression group [20,21]). Recent findings are confirming the notion of synexpression group [22,23], and indicates that synexpression groups are conserved throughout evolution [24,25].

#### 2.5. Data availability

A *Xenopus laevis* database (Axelddb) was developed with the aim to compile the expression patterns, the DNA sequences and associated informations coming from this study [26,27]. We used ACEDB (A *Caenorhabditis elegans* database) as our database management system [28]. ACEDB is publicly available and widely used in many genomic centers, its basic data model is easy to tailor, and it comes with powerful data visualization capabilities. We modified the basic ACEDB data model by adding objects with information specific for expression patterns, synexpression groups and expression domains. ACEDB provides a convenient framework for browsing and manipulating the integrated results, as well as a scriptable access and a web interface [29]. Access to Axelddb can be made in two ways. First, a web interface is available at the URL: <http://www.dkfz-heidelberg.de/abt0135/axelddb.htm>. Second, data (including pictures) and models for the UNIX version of ACEDB are available at the ftp server <ftp.dkfz-heidelberg.de> in `outgoing/abt0135/axelddb`. Users can query the database through class objects: clone, expression pattern, expression domain, tissue and through sequence similarity searches (Fig. 1).

### 3. Conclusion

We used a whole-mount in situ hybridisation based screen in *Xenopus* embryos to identify differentially

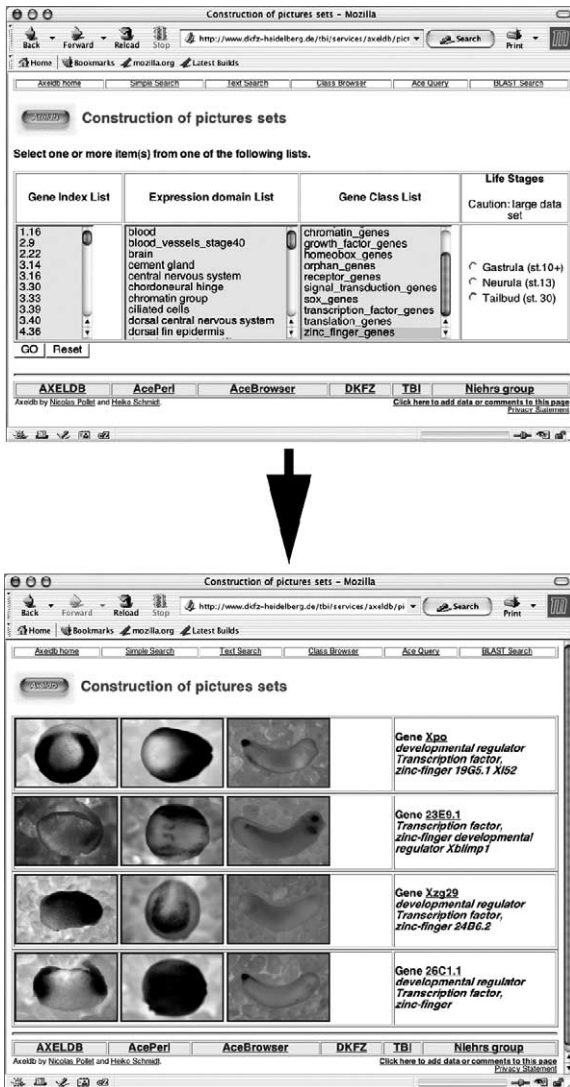


Fig. 1. Browsing pictures in Axeldb. Example of a script enabling the query of Axeldb based on gene names, expression domains, gene class or life stage. The output of a search for zinc-finger genes is shown. Both pictures and gene names are hyperlinks.

expressed genes during early development. The expression profiles of 273 genes and their associated sequence information is available on a public database, Axeldb.

By comparing expression profiles, we identified groups of genes with shared, complex expression pattern which also share function. These synexpression groups predict molecular pathways involved in pat-

terned and differentiation. Within groups, strong predictions can be made about the function of genes without sequence similarity. These results indicate that large scale expression screening is an alternative to identify molecular pathways and elucidate gene function of unknown genes.

A great advantage of the in situ screen is the immediate availability of the cloned cDNA, which readily allows a gain-of function test by microinjection of synthetic mRNA in *Xenopus*. By this approach two novel homeobox genes discovered in this screen could be implicated in dorso ventral mesoderm patterning [20, 30].

A number of methods have been developed to study gene expression systematically, from RT-PCR to DNA arrays. While these approaches enable the reading of transcript levels in vitro, they do not combine this quantitation in vivo with the spatio-temporal dimension of gene expression. As of today, information on the spatio-temporal distribution of mRNA in vivo is most often obtained using reporter gene systems (green fluorescent protein fusion from gene trap by example) or in situ hybridization. To project the spatio-temporal dimension of gene expression in the genomics era will need to apply systematically such methods and develop new ones.

Using filter-arrayed cDNA libraries, robotic processing of DNA and RNA probes and automated whole-mount in situ hybridization, gene expression screening can be largely automated [17,31]. Hence, there is the perspective of carrying out a saturating analysis of embryonic gene expression.

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