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A multi-well version of *in situ* hybridization on whole mount embryos of *Caenorhabditis elegans*

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ABSTRACT

We report an efficient procedure for *in situ* hybridization with a multi-well format on *Caenorhabditis elegans* embryos for large scale screening of gene expression patterns in this organism. Each hybridization well contains embryos at various stages throughout embryogenesis. The validity of the method was confirmed through results with control genes whose expression patterns have been reported; *glp-1* in very early embryos, *myo-2* in pharyngeal muscle and *unc-54* in body wall muscle. Several collagen genes and a pepsinogen gene were also examined to establish a set of lineage-specific markers. As a pilot project, we examined ~100 unique cDNA species classified by our cDNA project, finding that ~10% of the cDNA groups were expressed in specific cells and at specific stages.

INTRODUCTION

The nematode *Caenorhabditis elegans* is one of the best organisms for studying the molecular mechanisms of development, since an enormous amount of information has been accumulated with respect to anatomy, development, genetics and the genome. The entire cell lineage has been traced from zygote to adult, which consists of 959 somatic cells (1). A fertilized egg asymmetrically divides to produce the somatic founder cell AB and the germline founder cell P1. The P1 blastomere divides three times in stem cell fashion, producing three somatic founder cells EMS, C and D and the germline precursor cell P4. EMS further divides to produce two blastomeres E and MS. These blastomeres generate a variety of tissues; hypodermis (derived from the AB and C blastomeres), body nervous system (from AB), body wall muscle (from AB, MS, C and D), pharynx (from AB and MS), intestine (from E), somatic gonad (from MS) and germline (from P4). The fates of the blastomeres are determined in the early phase of embryogenesis. The mechanisms of fate determination by maternal genes have been under extensive investigation. However, the mechanisms of the subsequent execution of fate are largely unknown, since a large number of downstream genes have not yet been identified. Thus, identifying genes that are expressed in specific cell lineages will provide important clues to these mechanisms.

The most straightforward way to this end is to look at the expression patterns of genes in this organism one by one. Several methodologies for *in situ* hybridization and promoter trapping have been reported (2–6). However, a much more efficient strategy for systematic analysis of patterns of gene expression is needed, since a large number of genes identified in the *C.elegans* genome projects are awaiting analysis. The consortium of the Sanger Centre and Washington University has sequenced >25% of the genome, from which >4000 genes have been predicted (7,8). In our laboratory, we are carrying out a cDNA project from which ~4500 cDNA species, corresponding to 35% of the total number of genes, have been identified (Y. Kohara *et al.*, manuscript in preparation). Current progress suggests that all genes of this organism, estimated at ~13 000, will be identified within a couple of years.

In this paper, we present an efficient procedure for *in situ* hybridization suitable for this end and the result of a pilot project for large scale screening of gene expression patterns.

MATERIALS AND METHODS

Clones

Plasmid pJC124 of *col-3* DNA (9) was supplied by J. Kramer, cDNA clone cm01b7 (10) was supplied by R. H. Waterston and other cDNA clones were from our stock (Y. Kohara *et al.*, manuscript in preparation; their sequences can be seen in DDBJ/GenBank or ACEDB).

Preparation of hybridization probes

cDNAs in λZAPII vectors were PCR amplified using vector primers BS619 (TGAATTGTAATACGACTCAC) and BS711 (TGCAGGAATTCGGCACGA). *clb-2* DNA was PCR amplified from genomic DNA using primers clb-2 03 (ACAACCTGGACTTCGTGGAG) and clb-2 02 (GCCAGAATCCGTGATTGGTG). The amplified cDNA were purified by Sephacryl S-400 spun column chromatography. Digoxigenin (DIG)-labeled antisense DNA was made by linear PCR as described (11), in reaction mixtures (10 µl) using DIG–dUTP, amplified cDNA (~50–200 ng) and anchored oligo(dT) primers. In the case of *clb-2*, a gene-specific primer was used. Unincorporated substrates were removed by Sephadex

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G50-spun column chromatography. The eluates were subjected to partial digestion by DNase I in the reaction mixture (25 µl), containing 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 400 µg/ml phenol-extracted salmon testis DNA and 1 µl 14 ng/ml DNase I (freshly diluted in 0.1 M NaCl) at 37°C for 30 min. The reaction mixtures were heated at 75°C for 5 min and then stored at –20°C. We used 5 and 2.5 µl (and sometimes 1 and 0.5 µl) of the mixtures as probes for duplicate hybridizations.

Preparation of embryos

Standard techniques for cultivation and handling of worms have been described (12). Worms of the wild-type N2 strain were harvested from a mixed stage population and digested with alkaline hypochlorite. The resulting embryos were allowed to hatch to L1 larvae by incubating overnight in S-basal buffer (0.1 M NaCl, 50 mM KPO_4, pH 6, 5 mg/l cholesterol). The L1 population was fed to young adults in liquid culture. The worms were collected, digested with alkaline hypochlorite for ~10 min and then forced out through a 23 gauge needle onto nylon mesh (50 µm). The embryos in the filtrate were washed four times with M9 buffer (0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.5% NaCl, 1 mM $MgSO_4$) and finally resuspended in 100 µl M9 buffer in a siliconized microcentrifuge tube.

Removal of eggshell and fixation

The suspension of embryos was added to an equal volume (100 µl) of 15 mg/ml yatalase solution (an enzyme complex containing chitinase, chitobiase and β-1,3-glucanase activities; TAKARA Shuzo Co., Japan) in 0.3 M mannitol, 50 mM HEPES, pH 7.2, 10 mM NaCl, 10 mM $MgCl_2$ and 2 mM DTT and was immediately vortexed for 70 s at room temperature. Chitinase (Sigma C-6137) at 1 mg protein/ml also works, but we found that yatalase had better reproducibility than chitinase. The embryos were washed three times with embryo handling buffer (0.3 M mannitol, 50 mM HEPES, pH 7.2, 10 mM NaCl, 10 mM $MgCl_2$, 0.04% EGTA, 2 mM NH_4NO_3, 0.1% gelatin and 2 mM DTT), once with basal EH buffer (embryo handling buffer without EGTA, NH_4NO_3, gelatin and DTT) and then resuspended in basal EH buffer at a ratio of 100 µl packed embryos/ml buffer at 4°C. It is recommended that the extent of devitellinization be monitored at this point by observing the elongation of embryos of the 2-fold stage due to breakage of the vitelline membrane. To achieve devitellinization in 95% of embryos after subsequent methanol treatment, it is necessary for 20–30% embyros of the 2-fold stage to show elongation at this point.

Drops of basal EH buffer were placed in the wells of 8-well microscope slides (Flow Laboratories) that had been coated with poly-L-lysine. Embryo suspension (5 µl) was delivered to each well and the embryos were left to settle to the bottom for 8–10 min at 4°C.

Excess buffer was removed and the slides were immediately immersed in methanol at –20°C for 5 min. The embryos were rehydrated by immersing the slides in a series of mixtures at 4°C; in methanol for 5 min, in 70% methanol + 30% fixative [3.7% formaldehyde in 0.08 M HEPES, pH 6.9, 1.6 mM $MgSO_4$, 0.8 mM EGTA and 1× PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4)] for 2 min, in 50% methanol + 50% fixative for 2 min, in 30% methanol + 70% fixative for 2 min and finally in the fixative for 20 min. The slides can be stored at –20°C in ethanol for at least 2 months after the following dehydration treatment at room temperature; in 30% ethanol + 70% PBS for 5 min, in 50% ethanol + 50% PBS for 5 min, in 70% ethanol + 30% PBS for 5 min and finally twice in ethanol for 5 min each.

Proteinase K treatment

The slides were rehydrated at room temperature in the following series of solutions; in 70% ethanol + 30% PBS containing 0.03% H_2O_2 for 2 min, in 50% ethanol + 50% PBS for 5 min and finally in 30% ethanol + 70% PBS for 5 min. The slides were immersed in PBT (PBS with 0.1% Tween-20) for 5 min. To cut the glycosidic bonds of the proteoglycans that appear in late embryos, the slides were immersed in 0.2 N HCl for 20 min at room temperature. After washing twice in PBT for 5 min each, the slides were incubated in proteinase K solution (10 µg/ml in PBT) at room temperature for 11 min. The digestion was stopped by immersing the slides in 2 mg/ml glycine in PBT for 2 min. After washing twice in PBT for 2 min each, the specimens were refixed by immersing the slides in fixative at room temperature for 20 min. After washing twice for 5 min each in PBT, the slides were immersed in 2 mg glycine/ml PBT at room temperature for 5 min and then washed once in PBT for 5 min.

Hybridization on a 96-well dot blotting apparatus

The slides were immersed in the following series of buffers; in 50% basal hybridization solution (hybridization solution without salmon testis DNA and yeast tRNA) + 50% PBT for 10 min and then in basal hybridization solution for 10 min. Pre-hybridization was performed as follows. The slides were wiped off and waterproof lines surrounding the sample wells were drawn with a PAP pen (Cosmo Bio Co., Japan). The sample well region surrounded by the waterproof line was covered with 150 µl heat-denatured hybridization solution (50% deionized formamide, 5× SSC, pH 7.0, 100 µg/ml heparin, 0.1% Tween-20, 100 µg/ml sonicated salmon testis DNA and 100 µg/ml yeast tRNA) and incubated at 48°C for 1 h in a moist chamber.

After pre-hybridization, the slides were placed in the proper positions on a sheet of silicone rubber that was placed on the lower block of a 96-well dot blotting apparatus (SRC96D; Schleicher & Shuell). The upper block, which has 96 holes individually equipped with O rings, was placed on top of the slides and quickly assembled in such a way that the holes and the wells matched perfectly. To each sample well was added 50 µl hybridization solution containing 5 or 2.5 µl heat-denatured probe DNA, followed by layering with 100 µl mineral oil to prevent evaporation. The top of the block was sealed with sealing tape and hybridization was performed at 48°C overnight in a moist chamber. A similar method of multi-well hybridization was reported for FISH mapping on human chromosomes (13).

After hybridization, 0.4 ml 50% basal hybridization solution + 50% PBT was delivered into each hole of the apparatus to dilute the probes to minimize cross contamination in subsequent handling. The mixtures in the holes were discarded by inverting the apparatus. The apparatus was quickly disassembled and the slides were washed twice in 50% basal hybridization solution + 50% PBT for 10 min each at 48°C, four times in 0.8× PBS, 0.1% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; Sigma C3023) for 20 min each at 48°C and then in PBT twice at room temperature.

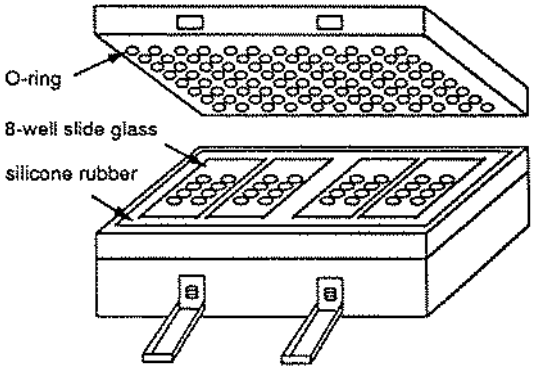

Figure 1. Apparatus for multi-well hybridization. Pre-hybridized slides on which embryos are fixed are placed in the proper position on a sheet of silicone rubber that is placed on the lower block of a 96-well dot blotting apparatus (SRC96D; Schleicher & Shuell). The upper block, which has 96 holes individually equipped with O rings, is placed on top of the slides and quickly assembled in such a way that the holes and the wells match perfectly. Hybridization solution is added to each sample well using a multi-channel pipette and then mineral oil is added to prevent evaporation. The top of the block is sealed with sealing tape and hybridization is performed at 48°C overnight in a moist chamber.

Detection

The slides were incubated twice in PBtr (PBS, 0.1% Triton-X 100, 0.1% BSA and 0.01% NaN_3) at room temperature for 10 min each and then subjected to an alkaline phosphatase-mediated color reaction as previously described (3). The color reactions were stopped by washing twice in PBS + 20 mM EDTA. The embryo specimens were mounted in Mount-Quick Aqueous (Cosmo Bio) and observed on a Zeiss Axioplan microscope with Nomarski optics. In cases in which staining with DAPI was also done, the specimens were mounted in 90% glycerol, 1× TBS, 1% *n*-propylgallate.

RESULTS

In situ hybridization with the multi-well format on whole mount embryos of *C.elegans*

During the first 30 min after fertilization, a *C.elegans* embryo forms a very tough eggshell that makes the embryo impermeable to fixatives and hybridization probes. The eggshell can be broken physically or enzymatically, but once the eggshell is broken, the embryo becomes very fragile due to a rapid change in osmotic pressure. This is particularly serious in early embryos, since the cells are large. Therefore, the main point of the method is removing the eggshell while maintaining good morphology in the embryo. After testing various procedures, we have established a protocol in which the eggshell is removed enzymatically by treating the embryos with chitinase in isotonic buffer. The processed embryos are stuck to poly-L-lysine-coated multi-well slides and then subjected to fixation procedures. Currently, we use 8-well microscope slides whose intervals between the wells match the standard 96-well format perfectly. The slides are assembled with a 96-well dot blot apparatus and are subjected to hybridization as depicted in Figure 1. The O rings, with which the holes of the dot blot apparatus are individually equipped, prevent leakage of the hybridization solution, enabling us to perform multiple hybridization reactions on a single slide. One apparatus accommodates four slides, meaning that 32 different probes can be analyzed on the apparatus. Furthermore, one great advantage with *C.elegans* is that each well of the slides contains a population of embryos at various stages throughout embryogenesis.

Another point of the method is the nature of the hybridization probes. We use digoxigenin (DIG)-labeled single-stranded DNA

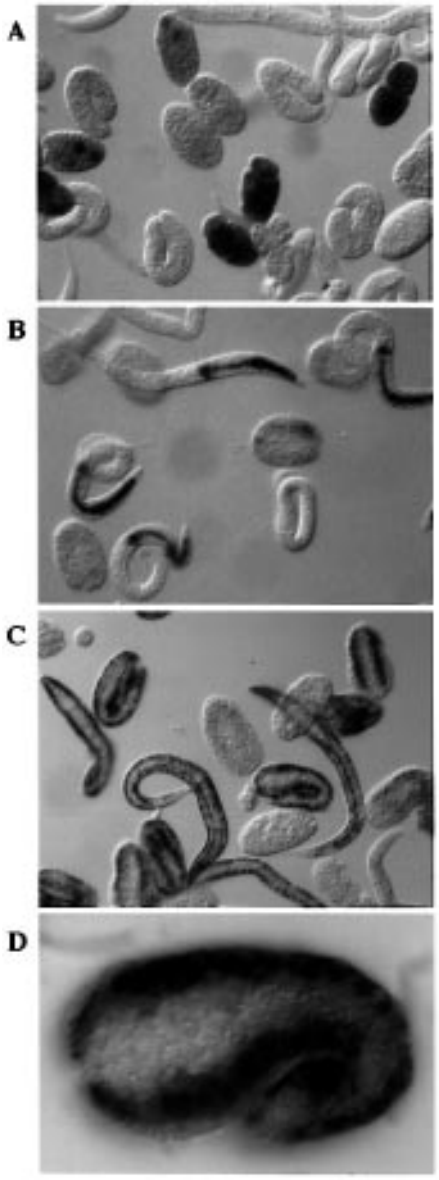

Figure 2. *In situ* hybridization on whole mount embryos with control gene probes. The dark signals mean positive hybridization signals visualized by an alkaline phosphatase-mediated color reaction. The specimens are observed on a microscope with Nomarski optics. Each hybridization well contains embryos at various stages throughout embryogenesis. (**A**) *glp-1* probe. Signals are seen in very early embryos (two to four cell embryos). In the embryos at late gastrulation, two dots are seen; the cells are unidentified. (**B**) *myo-2* probe. Only the pharynx is stained by the probe. (**C**) *unc-54* probe. No staining is seen in early embryos. Only body muscle cells are stained. (**D**) From the same experiment as (C), but of an embryo at a higher magnification to identify individual body wall cells expressing *unc-54* mRNA. The size of the embryo is ~50 μm.

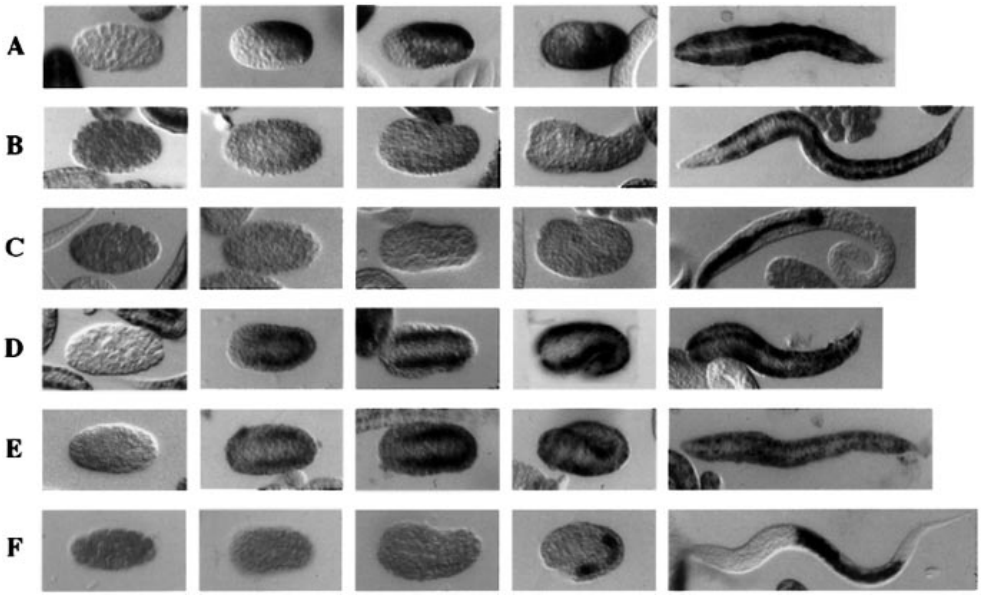

Figure 3. A collection of cell lineage-specific probes. Typical results at various developmental stages are rearranged along the time line for embryogenesis. The embryos in each column are roughly at the same stage; (from left to right) early gastrulation, late gastrulation, comma, 1.5-fold, 3-fold. Anterior is to the left and dorsal is up, except in (A) column 4, (B) columns 3 and 4, (C) column 4, (E) column 4 and (F) column 3, where dorsal is down. (**A**) CELK01595 (cDNA clone yk51e4, accession no. D73478), encoding cuticle collagen; (**B**) *col-3*, encoding cuticle collagen; (**C**) *myo-2*, encoding pharyngeal myosin; (**D**) *unc-54*, encoding body muscle myosin; (**E**) *clb-2*, encoding α(IV) collagen, a main component of the basement membrane; (**F**) cm01b7, encoding a pepsinogen homolog. For *myo-2* and *unc-54*, the data were rearranged from the experiments in Figure 2.

probes, which are made by linear PCR on cDNA inserts, using anchored oligo(dT) primers to minimize the effect of a long poly(A) stretch. We found that the method was sensitive to the size of the DNA probes; the presence of probes >500 bases frequently produced a high background, particularly on early embryos that are rich in yolk protein. Thus, we established an efficient protocol which facilitates shortening of a large number of probes to ~100–300 bases through partial digestion with DNase I. The concentrations of probes in the hybridization reactions are also important parameters for the signal-to-noise ratio of the results. We determined the optimal concentrations for several test probes, but the concentrations must be optimized for individual probes. This task would be quite cumbersome if we apply the method to a large number of cDNA clones. To bypass the problem, we adopted a duplication strategy in which hybridization was performed in two (or sometimes four) wells with serially diluted concentrations of the probes, expecting that one of the concentrations would give the best results.

Verification of the method

To test the accuracy of the method, we applied it to the control genes, *glp-1*, *myo-2* and *unc-54*, whose expression patterns have been reported. The maternal gene *glp-1* plays an important role in fate determination of the anterior blastomere AB and its mRNA is detected from oocyte to very early embryo (2). The *myo-2* gene encodes pharyngeal muscle-specific myosin and is expressed in pharyngeal cells (14). The *unc-54* gene encodes body wall muscle-specific myosin and is expressed in body muscle cells (14).

Figure 2 shows typical images of the results of *in situ* hybridizations with these probes. Background signals are sufficiently low. With the *glp-1* probe, signals are seen in very early embryos (two to four cell embryos) (Fig. 2A). The results coincide with those previously reported (2,3). The *myo-2* probe stained only the pharynx (Fig. 2B) and the *unc-54* probe stained only body muscle cells (Fig. 2C and D), which agrees with the results from immunostainings (14). The combined results show the validity of the method. Since the specimen contains embryos at various stages throughout embryogenesis, we can learn about the stage in which transcription of a zygotic gene starts. Transcription of *myo-2* seems to start in 2-fold embryos, but not in 1.5-fold embryos (Figs 2B and 3C). Transcription of *unc-54* starts in the posterior region in late gastrulation (Figs 2C and 3D).

Collection of tissue-specific markers

Although the identification of cells is easy in embryos earlier than mid-gastrulation, it becomes harder in embryos later than gastrulation. More tissue-specific markers, such as the *myo-2* and *unc-54* probes, are desirable, which will make it easy to interpret the results produced by a large scale screening of gene expression patterns. Thus, we are collecting such probes through *in situ* analysis of genes whose expression is expected to be tissue specific. These markers will also be useful as differentiation markers.

Cuticle collagen genes were chosen as markers for hypodermis. *Caenorhabditis elegans* has ~100 cuticle collagen-related genes (9), of which some are unique in the genome. The *col-3* gene is one of the unique cuticle collagens (9). Figure 3A shows that *col-3* is expressed in the main body syncytium of the hypodermis from late-stage embryos. Another cuticle collagen gene (cDNA CELK01595) showed a different pattern of expression, starting from the posterio-dorsal region of embryos at late gastrulation

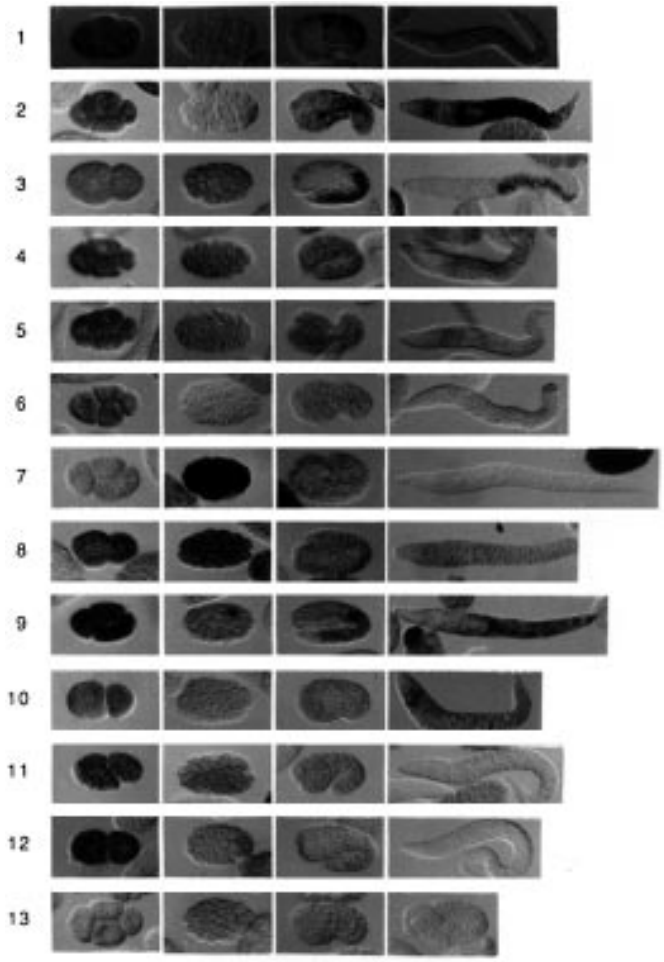

Figure 4. A pilot project of *in situ* screening on 100 cDNA species classified by our cDNA project. The 12 cDNA species that gave specific patterns of expression are listed. No. 13 is an example that shows no signal. The developmental stages of the columns are (left to right) very early stage (two to four cell stage), gastrulation, comma to 1.5-fold, 3-fold. (1) CELK00125 (clone yk11f3, accession no. D32354); (2) CELK00207 (clone yk2g10, accession no. D27178, similar to vacuolar ATP synthetase); (3) CELK00231 (clone yk3a10, accession no. D27723); (4) CELK00285 (clone yk5h5, accession no. D27911); (5) CELK00323 (clone yk5c2, accession no. D27861); (6) CELK00425 (clone yk23d3, accession no. D32914); (7) CELK00484 (clone yk19g11, accession no. D32629, similar to nucleolin); (8) CELK00807 (clone yk16h8, accession no. D27662, corresponding to R09A8.3 predicted protein, similar to ring canal protein); (9) CELK01035 (clone yk29c5, accession no. D33324, similar to uridine diphosphoglucose pyrophosphorylase); (10) CELK01121 (clone yk32g9, accession no. D33567); (11) CELK01149 (clone yk34e2, accession no. D33679, corresponding to F31E3.4 protein); (12) CELK01166 (clone yk35c7, accession no. D33737); (13) CELK01413 (clone yk6b12, accession no. D27921). When not indicated, the cDNA clones have no significant similarity in the public databases.

and finally detected all over the main body syncytium except for seam cells in late embryos (Fig. 3B).

The *clb-2* gene encodes one of the α(IV) collagens associated with the basement membrane that separates the hypodermis from muscle (15). It has been speculated that either gut cells or muscle cells secrete the product of *clb-2* (16). Figure 3D and E clearly indicates that *clb-2* is expressed in body wall muscle cells, because the expression pattern is essentially the same as that of *unc-54*.

A homolog to pepsinogen, cDNA clone cm01b7 (10), showed specific expression in gut cells from the 1.5-fold stage (Fig. 3F) as expected, because pepsinogen is one of the most abundant products in the stomach in vertebrates.

A pilot experiment for large scale screening

We applied the *in situ* method to ~100 cDNA species that had been classified in our cDNA project (Y. Kohara *et al*., manuscript in preparation). As shown in Figure 4(1–12), 12 of the clones show specific patterns of expression. The patterns are roughly categorized into several groups: (lanes 4–6, 8, 11 and 12) maternal expression; (lanes 2 and 9) both maternal and zygotic expression; (lane 7) zygotic expression from early stage; (lanes 1, 3 and 10) zygotic expression from mid-stage. Other cDNAs showed ubiquitous distribution throughout embryogenesis (data not shown) or no expression (like lane 13). This set of cDNAs is derived from a cDNA library of a mixed stage population including larvae and adults as well as embryos. Therefore, the cDNA groups that gave no signals may be expressed post-embryonically.

DISCUSSION

The main points of our method are the removal of the eggshell by an enzymatic procedure before fixation and the usage of a multi-well apparatus for hybridization. These make the method so efficient that currently one person can perform 192 hybridizations (96 different probes) at one time using six dot blot apparatuses. It takes a week to do the task, including preparation of the probes. The quality of the results is high enough to identify positive cells. Background signals are a common problem with *in situ* methods, but our duplication strategy, in which hybridization for a probe is duplicated with different probe concentrations, makes the results very reliable with minimum reduction of efficiency. It is also a great advantage to have each hybridization provide information on mRNA distribution at all stages of embryogenesis. Thus, with our plan it is quite feasible that within a year we will survey the expression patterns of the set of 4500 cDNA species that we have classified.

Accumulation of data on expression patterns will enable us to more finely classify patterns with respect to cell lineage and developmental stage, which will lead to identification of sets of genes that show the same expression patterns. For example, the cDNA group CELK00231 showed a very similar pattern to that of a pepsinogen homolog [Figures 3F and 4(3)]. The *clb-2* gene showed essentially the same pattern of expression as that of *unc-54* (Fig. 3D and E), indicating that body muscle cells secrete α(IV) collagen, the main component of basement membrane, which is encoded by the gene.

The *in situ* screening currently being performed in this laboratory is finding many more such examples (our unpublished results). Some of these genes might be under the same regulation mechanisms. In *C.elegans*, the genomic sequences surrounding these genes are, in many cases, available from the genomic sequencing project. These sequences will become immediate targets for analysis of the regulatory regions by experimental means and/or through informatics methods. The factors that regulate these genes will be expressed in the same cell lineage but earlier than the expression of target genes. Our screening is also revealing examples of sets of genes which are expressed in a specific cell lineage but at different stages (our unpublished

results). If one of these genes has a similarity to a transcription factor, it will be of interest to disrupt the gene to examine the transcription of other genes that are normally expressed later in the cell lineage. For this end, a system for transposon-mediated gene disruption is available in *C.elegans* (17).

For closer examination of the time and place of the start of expression, multiple labeling detection using a confocal microscope should be considered. We use the color reaction to detect hybridization signals for permanent storage of the specimens, but the *in situ* method can be applied to a fluorescent detection system simply by changing the mounting reagent.

In situ analysis gives information only on the distribution of mRNA and other mechanisms, including translation regulation, protein localization and protein modification, also play important roles in development. However, since this work revealed that >10% of the probes showed specific patterns of mRNA distribution, *in situ* screening will provide a large amount of invaluable material for studying the molecular mechanisms of development. Currently we are focusing on embryogenesis, but we are planning to extend the screening to post-embryonic stages with several modifications, ultimately aiming at understanding the entire life of the worm.

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