Under these circumstances we could determine that anti-PP-1 injection was preventing one of the daughter cells from completing its mitotic exit—respreading and chromatin decondensation. Staining for the injected IgGs after incubation and fixing not only allowed us to unambiguously identify the injected cells, but also to show the location of injection, since the IgGs remain accumulated at their site of injection.

Although these two examples reveal some of the possibilities of the antibody microinjection technique, the new user should always consider the effective controls that are required for antibody microinjection. A simple set of rules should include the following: the inhibitory activity should be present in the antisera from the outset (i.e., in the serum). Antibodies that show good binding by blotting may be of no use for microinjection since the first case involves recognition of denatured epitope sequences and the second requires binding to sites present in the native conformation.

Acknowledgments

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[7] Small Pool Expression Screening: Identification of Genes Involved in Cell Cycle Control, Apoptosis, and Early Development

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Introduction

Traditional genetic and biochemical methods have been quite successful in identifying genes that are essential for cell cycle progression and early embryonic development, among other diverse biological processes. Nevertheless, only a small fraction of the genes in the vertebrate genome has been functionally characterized. In this chapter, we describe a systematic and broadly applicable approach to cloning genes based solely on the biological activities or biochemical properties of the gene products. This approach does not depend on knowledge of the DNA sequence of the

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gene or purification of the gene product. We describe several potential applications of this expression cloning approach, and also discuss its use in related types of screening procedures.

Expression Cloning Strategy

The sib selection approach to expression cloning was developed in the mid-1980s for the identification of genes encoding plasma membrane receptor proteins[1] and secreted growth factors.[2] To clone membrane receptors, the basic strategy was to inject tissue RNA into oocytes of the frog *Xenopus laevis,* and then to assay the oocytes electrophysiologically for receptor activity. Active RNA was then used to construct a cDNA library, which was subdivided into pools of cDNA. These cDNA pools were transcribed *in vitro* to make RNA pools, which were again screened for receptor activity by oocyte injection. Positive pools were progressively subdivided and reassayed until a single clone was identified. Genes encoding a large number of G-protein-coupled receptors and membrane channels have been cloned using this approach. A similar cloning strategy has been applied to other biological assays that involve injection of RNAs into *Xenopus* oocytes[3] or embryos[4–6a] or transfection of cDNAs into mammalian cell cultures (reviewed in Ref. 7).

Although the oocyte and embryo-based cloning approaches have been used very successfully, they have generally been limited to identifying highly active or abundant genes, or genes for which a sensitive assay is available. To circumvent this problem, we modified the approach in one simple way. Rather than subdivide the cDNA library into pools containing more than 10,000 cDNA clones, as has been traditionally done, we subdivided the cDNA library into smaller pools containing only 100 clones (Fig. 1). This reduction in pool size has a number of important consequences. First, it substantially increases the amount of each clone in the pool. This increases the probability of detecting even weakly active proteins using existing assays and also broadens the range of assays that can be used to screen cDNA or RNA pools. Second, it makes it possible to construct small protein pools,

[1] Y. Masu, K. Nakayama, H. Tamaki, Y. Harada, M. Kuuno, and S. Nakanishi, *Nature (London)* **329,** 836 (1987).

[2] G. C. Wong, J. S. Witek, P. A. Temple, *et al., Science* **228,** 810 (1985).

[3] K. D. Lustig, K. Kroll, E. Sun, R. Ramos, H. Elmendorf, and M. W. Kirschner, *Development* **122,** 3275 (1996).

[4] W. C. Smith and R. M. Harland, *Cell* **70,** 829 (1992).

[5] P. Lemaire, N. Garrett, and J. B. Gurdon, *Cell* **81,** 85 (1995).

[6] K. D. Lustig, K. Kroll, E. Sun, and M. W. Kirschner, *Development* **122,** 4001 (1996).

[6a] P. E. Mead, I. H. Brivanlou, C. M. Kelley, and L. I. Zon, *Nature* **382,** 357 (1996).

[7] H. Simonsen and H. H. Lodish, *Trends Pharmacol. Sci.* **15,** 437 (1994).

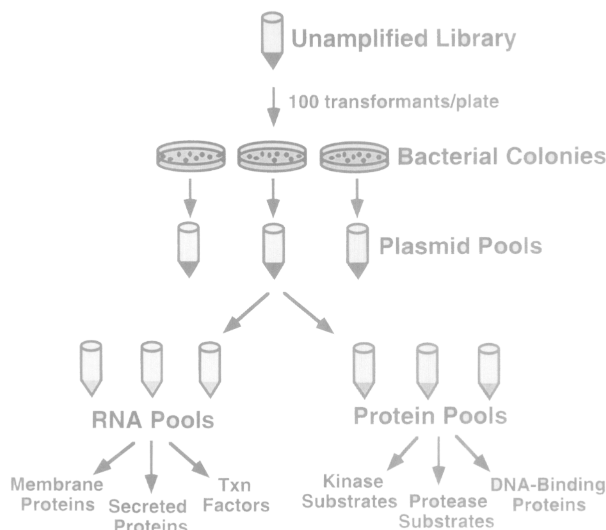

FIG. 1. Overview of expression screening strategy.

which can be used in substrate, interaction, and activity screens *in vitro*. Finally, it makes it easier to rapidly isolate a single clone once an activity is identified, since small pools are less likely than large pools to contain multiple active cDNAs and they can be subdivided in only one to two steps to a single active cDNA.

We first describe general methods used to prepare library pools of cDNA, RNA, and protein, and the sib selection techniques used to subdivide a pool once it is found to contain a candidate activity. We then describe how protein pools can be screened *in vitro* for substrates of kinases and proteases and for DNA-binding proteins. Finally, we describe how this approach can be applied to a functional screen for signaling proteins involved in early embryogenesis.

General Methods

Preparation of cDNA Pools

In most expression screens that we carry out, we use an oligo(dT)-primed cDNA library that is constructed in the high copy number plasmid pCS2^{+} [8] from poly(A)$^{+}$ RNA isolated from gastrula stage *Xenopus* embryos.[9] In principle, it should be possible to carry out the procedures de-

[8] D. L. Turner and H. Weintraub, *Genes Dev.* **8,** 1434 (1994).
[9] K. D. Lustig and M. W. Kirschner, *Proc. Natl. Acad. Sci. U.S.A.* **92,** 6234 (1995).

scribed as follows with plasmid or phage libraries constructed in other appropriate T7, T3, or SP6 expression vectors as well. Only cDNAs larger than 0.5 kb are ligated directionally into pCS2$^+$. Electromax DH10B *Escherichia coli* (BRL/GIBCO, Gaithersburg, MD) are transformed with the ligation mix by electroporation, incubated for 1 hr at 37° and then stored as an unamplified 20% (v/v) glycerol stock at −80°.

To prepare 100 pools of library cDNA, each containing 100 cDNA clones, an aliquot of the frozen unamplified library containing 10,000 independent transformants is thawed, diluted, and plated on 100 LB-agar plates containing antibiotic. After an overnight incubation at 37°, or when bacterial colonies are approximately 1 mm in diameter, the colonies on each plate are collected with a rubber policeman and pooled. A small aliquot of the pooled bacteria is stored as a 20% (v/v) glycerol stock at −80°. Plasmid DNA (between 5 and 20 μg) is then isolated from the 100 bacterial cultures and stored at −20°. Commercially available minipreparation kits from Promega (Wizard, Madison, WI) give a higher yield of plasmid DNA than kits from Qiagen (QIAprep Spin, Chatsworth, CA) or Bio101 (Rapid-Pure, Vista, CA) but generally lead to more variable results in *in vitro* translation reactions. With the use of any of these kits, one person can easily perform 100 minipreparations in a single day.

Preparation of Protein Pools

Protein pools are prepared directly from cDNA pools using a coupled transcription/translation system. For our screens, we prepare protein pools using the TNT coupled reticulocyte lysate system from Promega, according to the manufacturer's instructions. We typically scale the reaction volume down to 5 or 10 μl and add 0.1 to 0.5 μg of pooled cDNA per reaction. Radioactively labeled protein pools are prepared by carrying out a 10-μl translation reaction in the presence of 8 μCi [^{35}S]methionine (1000 Ci/mmol) and omitting unlabeled methionine from the amino acid mix. In practice, 100 protein pools can be prepared in about 3 hr. Protein pools are stored on ice or at −80° until used. There is considerable variability in the size range and total number of proteins that are synthesized from different plasmid pools. Although more than 85% of the plasmids in the library have cDNA inserts, we typically observe only between 15 and 30 ^{35}S-labeled protein bands after translation of a cDNA pool containing ~100 transformants. This may reflect the presence in the library of a high proportion of noncoding sequences or genes lacking efficient initiation codons, perhaps because some of the cDNAs are not full length. The protein bands are frequently of differing intensities, suggesting that certain genes are preferentially recruited by the RNA and protein synthesis machinery in the *in vitro* transcription/translation reaction.

Preparation of RNA Pools

Commercial transcription kits are available, although it is much more cost effective to purchase the transcription reaction components separately. Pooled plasmid DNAs are first linearized by an overnight incubation at 37° with 20 to 40 units of the appropriate restriction enzyme. The linearized DNA is extracted with phenol : chloroform (1 : 1, v/v), ethanol precipitated, and resuspended in water. Pools of capped RNAs are prepared by *in vitro* transcription of pools of cDNAs (~5 μg), exactly as described.[10] RNase-free DNase is used to degrade template DNA after the transcription reaction is complete. The DNA-free RNA pools are extracted with phenol : chloroform (1 : 1, v/v), ethanol precipitated, and resuspended in water. RNA is stored undiluted at −80° until used. It is possible for one person to linearize and transcribe 100 cDNA pools in 2 days.

Sib Selection of Positive Pools

Once a cDNA pool containing an activity is identified, it is progressively subdivided until a single active cDNA is isolated. To subdivide the pooled cDNAs, we generally plate a small aliquot of the corresponding glycerol bacterial stock on LB-agar plates. Alternatively, we transform fresh bacteria with the positive cDNA pool and plate the transformants on LB-agar plates with antibiotic. After an overnight incubation at 37°, a small sample of each discrete colony is transferred with a toothpick to a single well of a 96-well flat bottom plate containing 150 μl LB and the appropriate antibiotic. An adhesive plastic film (SealPlate, EXCEL Scientific, Wrightwood, CA) is placed on top of the plate to reduce cross-contamination between wells. After an overnight incubation at 37°, the cultures are mixed by repipetting, and 100-μl samples of bacterial culture from each of the eight wells in a column are pooled. Plasmid DNA is isolated from each of the 12 column pools and, depending on the screen, protein or RNA pools are synthesized and assayed. If no positives are detected, 96 more colonies are picked and the process is repeated. Positive column pools are subdivided by individually preparing and testing the cDNA in each of the eight wells. Alternatively, a two-dimensional matrix (e.g., 12 column pools by 8 row pools to make 20 pools) can be used to identify a single active cDNA in one step. Because cross-contamination between wells can occur, it is necessary to verify that the final observed activity is a consequence of the action of a single cDNA. This is accomplished by plating out the bacteria in the positive well, picking single colonies and retesting as above.

[10] M. Wormington, *in* "*Xenopus laevis:* Practical Uses in Cell and Molecular Biology" (B. K. Kay and H. B. Peng, eds.), pp. 167–182. Academic Press, San Diego, 1991.

Screening of Protein Pools for Enzyme Substrates and DNA-Binding Proteins

Identification of Proteins Phosphorylated or Degraded during Mitosis

Progression through the eukaryotic cell cycle is regulated by the sequential activation of cyclin-dependent kinases (CDKs), which are believed to initiate DNA synthesis and entrance into mitosis by phosphorylating key target proteins. Although several substrates of CDKs have been identified through traditional biochemical approaches, no general methodologies, including genetics, have been developed to identify protein kinase substrates directly. Similarly, the onset of anaphase and exit from mitosis are mediated by the proteolysis of anaphase inhibitors and mitotic cyclins, respectively; however, additional substrates of the mitotic protease are likely to exist.[11] Identifying protease substrates using standard biochemical techniques is problematic, because such proteins may either be unstable or present in small quantities. Here we describe a simple procedure for simultaneously screening library pools for substrates of mitotically regulated kinases and proteases. Pools of radiolabeled library proteins are incubated in mitotic or interphase extracts prepared from *Xenopus* eggs, which accurately reproduce the biochemical and cytological events of the cell cycle *in vitro*. Library proteins that are phosphorylated or degraded are identified by scoring for an alteration in protein mobility on SDS–polyacrylamide gels.

1. *Preparation of interphase and mitotic Xenopus extracts.* Extracts are prepared from unfertilized eggs obtained from the clawed frog *Xenopus laevis*,[12] with the following modifications. Meiotically arrested eggs incubated in 1× Marc's modified Ringer's solution (MMR; 5 m*M* HEPES, pH 7.4, 100 m*M* sodium chloride, 2 m*M* potassium chloride, 2 m*M* calcium chloride, 1 m*M* magnesium chloride) are driven into interphase by the addition of 0.4 μg/ml A23187, a calcium ionophore, and then crushed 50 min later. Extracts are maintained in the interphase state by the addition of 100 μg/ml cycloheximide, which prevents mitotic cyclin synthesis. Sucrose is added to a final concentration of 300 m*M* and the extracts are frozen in liquid N_2 and stored at −80° until used.

2. *Phosphorylation and degradation reactions.* The interphase extract is thawed on ice and divided into two aliquots. One aliquot is induced to enter the mitotic state by a 45-min incubation at room temperature with 30–50 μg/ml cyclin BΔ90, a nondegradable mutant of sea urchin cyclin

[11] A. W. Murray, *Cell* **81,** 149 (1995).
[12] A. W. Murray, *Methods Cell Biol.* **36,** 581 (1991).

B1 that keeps the mitotic degradation system constitutively active.[13] The amount of recombinant cyclin B1 needed to drive the extract into mitosis varies among different extract preparations and is determined experimentally. The other aliquot is maintained in interphase by addition of cyclin B1 storage buffer only (10 m*M* HEPES, pH 7.7, 1 m*M* magnesium chloride, 100 m*M* potassium chloride, 1 m*M* dithiothreitol, and 50 m*M* sucrose). To start the reactions, a 1.5-μl aliquot from a [^{35}S]methionine-labeled protein pool is added to 4.5 μl (~200 μg protein) of interphase extract or mitotic extract. The reactions are incubated at room temperature for 60 min and then terminated by adding 22.5 μl of 2× protein lysis buffer (125 m*M* Tris-Cl, pH 6.8, 2% (w/v) sodium dodecyl sulfate, 20% (v/v) glycerol, and 10% (v/v) 2-mercaptoethanol). *Xenopus* cdc25, a well-characterized protein phosphatase that is phosphorylated during mitosis but not during interphase, is used as a positive control for the phosphorylation reactions. Full-length *Xenopus* cyclin B1, which is degraded by the ubiquitin-dependent degradation pathway at the end of mitosis, is used as a positive control for the degradation reactions. [^{35}S]Methionine-labeled cdc25 and cyclin B1 proteins are prepared using the TnT kit and incubated with extracts as above.

3. *Separation of protein products.* The denatured interphase and mitotic reactions are incubated at 95° for 5 min and then 10-μl samples are loaded side by side on a 5–15% SDS–polyacrylamide gradient gel and run for approximately 2 hr at 30 mA. Gels are fixed in a methanol/acetic acid solution (5 : 7.5, v/v) for 15 min and vacuum dried onto Whatman (Clifton, NJ) 3-mm paper. Labeled proteins are detected by overnight exposure using a phosphoimager cassette (Molecular Dynamics, Sunnyvale, CA), or by a 5- to 6-day exposure on Kodak (Rochester, NY) XAR film.

4. *Identification of novel kinase substrates and degraded proteins.* Almost all pooled proteins exhibit a constant mobility and intensity in SDS–PAGE regardless of whether they are incubated in mitotic or interphase extracts (Fig. 2A). Potential mitotic kinase substrates are therefore identified by scoring for proteins that exhibit a lower relative mobility after incubation in the mitotic extract than after incubation in the interphase extract (Fig. 2B), reflecting their phosphorylation by one or more kinases. Mitotic protease substrates are identified by scoring the same samples for proteins that are present when incubated in the interphase extract but absent when incubated in the mitotic extract (Fig. 2C). The mitotic kinase substrate cdc25 (70 kDa) is phosphorylated by kinases in the mitotic extract that are not active in the interphase extract; phosphorylation leads to a visible (~20–50 kDa) increase in its apparent molecular mass (data not shown).

[13] M. Glotzer, A. W. Murray, and M. W. Kirschner, *Nature (London)* **349**, 132 (1991).

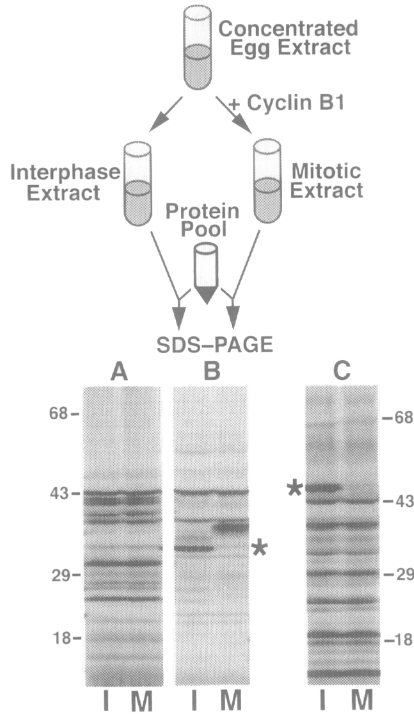

FIG. 2. Isolation of mitotically phosphorylated and degraded proteins. Three different [^{35}S]methionine-labeled protein pools (A, B, and C) were incubated in interphase extract (I) or mitotic extract (M) for 60 min and then resolved by SDS–PAGE. Asterisks indicate proteins that have been modified in a mitotic-dependent manner.

Cyclin B (55 kDa) is almost completely degraded by the mitotic extract but is not affected by the interphase extract (data not shown). Single cDNAs are isolated from positive pools by sib selection as described above.

A secondary screen is generally carried out to verify potential positives. When screening for putative kinase substrates, alkaline phosphatase treatment is used to show that the change in protein mobility is indeed caused by phosphorylation and not by another posttranslational modification. For degradation reactions, we determine whether degradation is ubiquitin dependent by using inhibitors of the ubiquitin-mediated degradation system, such as methyl ubiquitin or an N-terminal fragment of cyclin B. Finally, we test whether candidate proteins are similarly modified in a cell cycle-dependent manner *in vivo*.

About 25 pools can be easily prepared and screened by one person in a single day, and thus this approach can be used to screen at least 500 proteins each day. After screening through about 500 pools (about 10,000

detectable library proteins), Stukenberg *et al.* (1997) identified 20 candidate substrates for mitotic kinases, including novel proteins and proteins previously implicated in cell cycle control.[13a] In the same fashion, T. McGarry and M. W. Kirschner (unpublished results) have identified three proteins degraded during mitosis. The effect of these proteins on cell cycle progression is currently being investigated.

Identification of Apoptotic Substrates of the Caspase (ICE/CED-3) Family of Proteases

Cells of multicellular organisms respond to a variety of stimuli by activating an endogenous suicide mechanism known as programmed cell death or apoptosis; this tightly regulated process plays a fundamental role in development and in maintaining homeostasis.[14] Proteases of the caspase (interleukin-1β-converting enzyme (ICE)/CED-3) family, homologs of the *Caenorhabditis elegans* cell death gene product *Ced-3*, are critical components of the apoptotic cell death pathway.[15] A few substrates of these proteases have recently been identified but, given the growing size of the protease family, it seems likely that most substrates are yet unknown. Systematic and broadly applicable methods to identify apoptotic substrates of caspases have not yet been described. In this section, we describe two complementary small pool expression methods for identifying potential substrates of the caspase family. In one assay, pools of library protein are incubated in apoptotic extracts that contain multiple activated caspase family members. In the other, protein pools are incubated with purified caspases.

1. *Preparation of apoptotic and control Jurkat lymphoma cell extracts.* Human Jurkat cells are maintained in RPMI 1640 media (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS). Prior to making extracts, Jurkat cells are concentrated to 2×10^7 cells/ml in fresh RPMI with 10% FBS and divided into two aliquots, one of which is treated for 1 hr with 250 ng/ml anti-Fas monoclonal antibody CH-11 (Kamiya Biomedical Company, Thousand Oaks, CA). Treatment of sensitive cells with anti-Fas monoclonal antibody induces apoptosis by activating one or more caspases.[15] Cytosolic extracts from anti-Fas-treated cells (apoptotic extract) and untreated cells (nonapoptotic extract) are prepared by a modification of existing methods.[16,17] Cells are washed twice with ice-cold RPMI and

[13a] P. T. Stukenberg, K. D. Lustig, T. J. McGarry, R. W. King, J. Kuang, and M. W. Kirschner, *Current Biology* **7,** 338 (1997).

[14] H. Steller, *Science* **267,** 1445 (1995).

[15] A. Fraser and G. Evan, *Cell* **85,** 781 (1996).

[16] Y. A. Lazebnik, S. Cole, C. A. Cooke, W. G. Nelson, and W. C. Earnshaw, *J. Cell Biol.* **123,** 7 (1993).

[17] B. Zhivotovsky, A. Gahm, M. Ankarcrona, P. Nicotera, and S. Orrenius, *Exp. Cell Res.* **221,** 404 (1995).

resuspended in an extraction buffer containing 10 mM HEPES (pH 7.0), 2 mM magnesium chloride, 50 mM sodium chloride, 5 mM EGTA (pH 7.6), 40 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 μg/ml antipain; 1 μl of extraction buffer is added per 4 $\times$ 10^5 cells. Cells are then lysed by four cycles of freeze–thawing in an ethanol/dry ice bath, and the cell lysates are centrifuged for 15 min at 14,000 g (4°). The clear supernatant ("cytosolic" extract) is removed and stored in aliquots at −80° until used.

2. *Proteolytic cleavage reactions.* [^{35}S]Methionine-labeled protein pools are prepared as described above in a total volume of 5 μl. A 1.5-μl aliquot from each protein pool is incubated for 1 hr at 37° with 5 μl (approximately 38.5 μg protein) of (1) nonapoptotic extract, (2) apoptotic extract, or (3) apoptotic extract preincubated for 15 min with 10 μM acetyl-Tyr-Val-Ala-Asp chloromethyl ketone (YVAD-CMK) (BACHEM Bioscience, Inc., King of Prussia, PA), a specific peptide inhibitor of caspases.[18] A well-characterized caspase substrate, poly(ADP-ribose) polymerase (PARP),[19] is used as a control to verify extract activity. ^{35}S-Labeled human PARP protein is synthesized using the TNT system and incubated with extract as above. After 1 hr, the cleavage reactions are terminated by adding an equal volume of 2× protein lysis buffer and boiling for 5 min. The denatured cleavage reactions (three conditions per protein pool) are loaded side by side and proteins are separated by SDS–PAGE as above.

3. *Identification of putative apoptotic caspase substrates.* cDNA pools containing putative apoptotic caspase substrates are identified by comparing the protein pattern of the labeled pool under all three cleavage conditions. In practice, putative substrates have been identified by scoring for proteins that are present in both the nonapoptotic extract and the apoptotic extract containing YVAD-CMK, but not in the apoptotic extract (Fig. 3A). Alternatively, candidate substrates have been identified by scoring for novel protein fragments that are formed only after incubation in the apoptotic extract (Fig. 3B), reflecting partial cleavage or incomplete separation of the full-length substrate. Full-length PARP (115 kDa) is specifically cleaved by the apoptotic extract into a signature 85-kDa fragment; cleavage of PARP is inhibited by YVAD-CMK (data not shown). Single cDNAs encoding the putative substrates are isolated by sib selection as described above.

The same basic approach can be used to identify substrates for specific members of the caspase family. In practice, these two approaches (apop-

[18] N. A. Thornberry, H. G. Bull, J. R. Calaycay, K. T. Chapman, *et al., Nature* (*London*) **356,** 768 (1992).

[19] Y. A. Lazebnik, S. H. Kaufmann, S. Desnoyers, G. G. Poirier, and W. C. Earnshaw, *Nature* (*London*) **371,** 346 (1994).

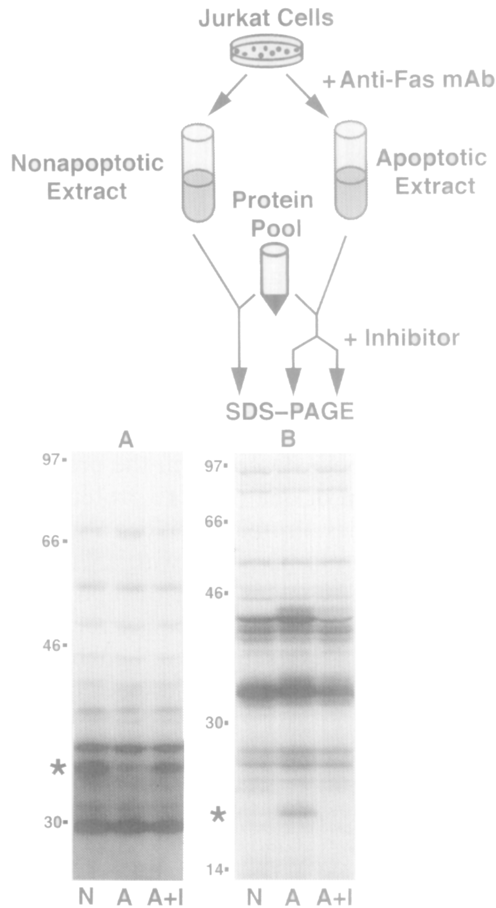

FIG. 3. Identification of caspase substrates. Two different [^{35}S]methionine-labeled protein pools (A and B) were incubated in nonapoptotic extract (N), apoptotic extract (A), or apoptotic extract containing a specific ICE/CED-3 protease inhibitor YVAD-CMK (A+I) for 60 min and then resolved by SDS–PAGE. Asterisks indicate proteins that have been modified in an apoptotic-dependent manner.

totic extract vs purified protease) are complementary and can be pursued simultaneously. The screening procedure is similar to that described above except that the protein pools are incubated with a purified protease instead of with protease-containing extracts. A typical reaction is incubated at 30° for 40 min and contains 1.5 μl of the [^{35}S]methionine-labeled protein pool, 1 μl (10 ng) of an affinity-purified caspase,[20] and 1.5 μl of CED-3 buffer (50 m*M* Tris-HCl, pH 8.0, 0.5 m*M* EDTA, 0.5 m*M* sucrose, 5% glycerol,

[20] D. Xue, S. Shaham, and H. R. Horvitz, *Genes Dev.* **10,** 1073 (1996).

5 mM dithiothreitol). As above, reactions are carried out in the presence or absence of selective caspase inhibitors.

About 40 protein pools can be prepared and screened by one person in a single day, and thus at least 1000 proteins can be screened in 1 day. Using extracts that contain activated caspases, V. Cryns and J. Yuan (unpublished results) have so far identified seven candidate protease substrates (out of 80 pools screened). Using purified *Ced-3* protease, D. Xue and H. R. Horvitz (personal communication) have so far identified four candidate substrates. Current studies are directed toward determining whether these proteins are cleaved in an apoptosis-dependent manner *in vivo* and, if so, whether their cleavage is necessary for programmed cell death.

Identification of DNA-Binding Proteins

Substrate screens, like those described in the previous section, involve assaying for the modification of one or more proteins in the library pools by extracts or purified enzymes. Protein pools can also be used in expression screens for proteins capable of interacting with a wide variety of different "bait" molecules including antibodies, individual proteins (or protein complexes), and nucleic acids. Here we describe a screen for proteins capable of binding to specific DNA target sequences. Unlabeled protein pools are incubated with a labeled DNA fragment, and specific binding is determined by using a gel mobility shift assay.

1. *Preparation of labeled probe.* Labeled DNA probes are prepared by end-labeling one of two complementary oligonucleotides with polynucleotide kinase or by filling in 5′-overhangs with the Klenow fragment of DNA polymerase in the presence of ^{32}P-labeled nucleotide. Unlabeled protein pools are prepared as described above in a total volume of 25 μl.

2. *Gel-shift assay.* Gel-shift assays are performed essentially as described.[21] A 2- to 5-μl aliquot of an unlabeled protein pool is mixed with a gel-shift cocktail containing 10 mM HEPES, pH 7.8, 40 mM potassium glutamate, 5 mM magnesium chloride, 1 mM EDTA, 5% glycerol, 0.5 mM dithiothreitol, 50 μg/ml poly(dI)-(dC) (final concentrations in a 20-μl reaction), and approximately 5×10^4 cpm ^{32}P-labeled double-stranded oligonucleotide probe. After a 20 min incubation on ice, the entire reaction mix is resolved on a nondenaturing 5% (w/v) polyacrylamide gel prepared in 0.5× TBE and run at 235 V for approximately 2 hr at 4°. Gels are transferred to Whatman 3M paper, vacuum-dried, and exposed overnight to Kodak XAR film at room temperature.

[21] M. Crossley, M. Merika, and S. H. Orkin, *Mol. Cell. Biol.* **15,** 2448 (1995).

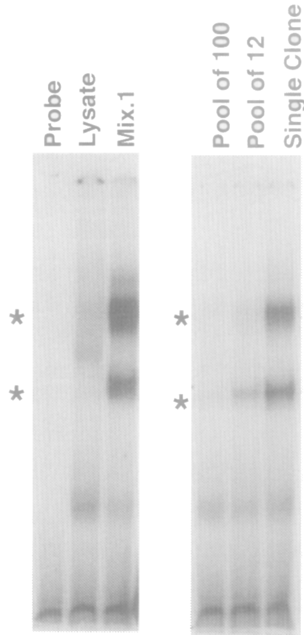

FIG. 4. Identification of DNA-binding proteins. Protein pools were incubated with a ^{32}P-labeled Mix.1 DNA-binding oligomer and then resolved on a nondenaturing polyacryamide gel. The three lanes on the left-hand side are control reactions, carried out as described in the text. The Mix.1 gene product is used as a positive control. The three lanes on the right-hand side show the enrichment of DNA-binding activity as a positive protein pool is progressively subdivided and retested for gel-shift activity. The single active clone in this pool turned out to encode Mix.2, which had been previously identified.[22]

3. *Identification of DNA-binding proteins.* Protein pools containing candidate DNA-binding proteins are identified by visually scoring for a reduction in the mobility of the labeled DNA fragment (Fig. 4). Two negative control reactions are generally carried out. One control contains probe and no protein; the other contains probe and an aliquot of the *in vitro* translation lysate (prepared without the plasmid pool template) to control for possible DNA-binding activities in the reticulocyte lysate. A positive control reaction containing a protein known to bind to the DNA target, if available, is also carried out. Single cDNAs encoding the putative DNA-binding proteins are isolated by sib selection as described above.

About 50 protein pools can be prepared and screened by one person in a single day, and thus at least 1000 proteins can be screened in 1 day. Using this approach to screen for proteins that could bind to the Mix.1

[22] P. D. Vize, *Dev. Biol.* **177,** 226 (1996).

DNA-binding site (P3),[23] P. Mead and L. Zon (unpublished results) have so far identified three Mix.1-related proteins (out of 100 pools screened).

Screening of RNA Pools for Functional Activity

Identification of Signaling Proteins by Localized Overexpression of RNA Pools

The increase in sensitivity obtained by small pool expression also substantially expands the usefulness of cloning strategies that involve RNA expression in oocytes or embryos. Such approaches have generally been restricted to identifying highly active signaling proteins, or proteins for which a highly sensitive assay is available. In this section we describe how an axis duplication assay first used to clone the *Xenopus* homeobox gene siamois[5] can be adapted to screen for signaling proteins with a range of functional activities.

1. *Preparation of Xenopus embryos. Xenopus laevis* embryos are generated by *in vitro* fertilization. Thirty to 60 minutes after fertilization, the jelly coat is removed from the embryos by a 4- to 6-min treatment with 2% (w/v) cysteine hydrochloride (pH 7.9). Dejellied embryos are washed five times with 1× MMR and then cultured in 0.1× MMR at 17–19°. Embryos are staged according to Nieuwkoop and Faber.[24]

2. *Microinjection of RNAs into embryos.* GpppG-capped RNA pools are prepared as described above. For primary screens, we generally inject a single blastomere of a four- to eight-cell embryo with ~10 nl of each RNA pool (typically 1–3 ng). To target the gene products to a variety of tissues, RNA pools are injected into the vegetal pole, marginal zone, or animal pole of the embryo. We have generally injected tissue on the ventral side of the embryo. Ventral blastomeres are distinguished from dorsal blastomeres based on their larger size and darker pigmentation. Embryos are injected with a Picospritzer automatic air injection system (Medical Systems Corp., Greenvale, NY) set to an injection pressure between 3 and 6 psi and an injection time of 0.3–0.5 sec. Injection needles are made from borosilicate glass capillary tubing (1.2 mm o.d. × 0.9 mm i.d.); the end of the needle is typically ~15 μm in diameter. About 10 min prior to RNA injection, the embryos are transferred to 0.2× MMR containing 4% Ficoll 400 and 50 μg/ml gentamicin. Each RNA pool is injected into about 25 embryos, which are then cultured at 18°. About 40 RNA pools (~1000 embryos) can be injected by a skilled person in a single day.

[23] D. Wilson, G. Sheng, T. Lecuit, N. Dostatni, and C. Desplan, *Genes Dev.* **7,** 2120 (1993).
[24] P. D. Nieuwkoop and J. Faber, "Normal Table of *Xenopus laevis* (Daudin)." North Holland, Amsterdam, 1967.

3. *Identification of candidate signaling proteins.* Injected embryos are visually scored at multiple times during gastrula, neurula, and tailbud stages for any alteration in morphology. This may include the formation of ectopic tissues that resemble specific body structures such as the notochord, neural tube, or head, or the abnormal development (e.g., increase or reduction in size, ablation, or transformation) of a particular tissue or embryonic region (Fig. 5). Primary positives are retested prior to being sib selected as above.

Twelve different biologically active genes have so far been cloned using this approach out of ~1000 pools screened[6] (K. Kroll, A. Salic, and M. Kirschner, unpublished results). These include previously isolated proteins, novel proteins, and new members of known families of signaling proteins, including homeodomain and T-box transcription factors, members of the Wnt signaling pathway, members of the transforming growth factor beta (TGFβ) signaling pathway, and proteins that induce or pattern neural tissue. About one-half of the cloned genes appear to act noncell autonomously. Embryonic injection of the original RNA pools encoding these genes induced a variety of effects including (1) complete duplication of the body axis, (2) formation of a partial secondary body axis containing notochord but no head structures, (3) formation of enlarged dorsoanterior structures, (4) formation of ectopic morphogenetic structures such as blastopore lips, or (5) abnormal development of posterior and ventral structures. Although it is more laborious to prepare and screen small pools than large pools, the increased labor seems offset by the high detection rate of positive pools.

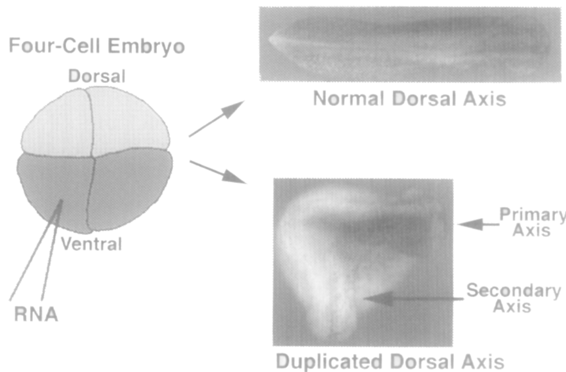

FIG. 5. Identification of signaling proteins involved in embryogenesis. A single ventral blastomere of a four-cell embryo (schematized here in animal pole view) is injected with RNA and then visually scored for any morphological change at multiple stages of development. Shown here are photographs of a normal tailbud-stage embryo and a sibling embryo that had been injected with RNAs encoding signaling proteins capable of inducing a secondary body axis. Embryos are shown in dorsal view with anterior to the right.

Such a small pool approach may allow this type of expression screen in *Xenopus* to reach saturation.

Comments

The small pool expression screening approach can be applied to a wide range of existing *in vitro* and *in vivo* assays. Using small pools of library proteins, three types of screen—substrate, interaction, and activity—can be carried out. The methods we describe to identify kinase and protease substrates should be broadly applicable to other substrate screens. The interaction screen we describe to identify DNA-binding proteins should also be easily applied to screens involving the binding of expressed library proteins to RNA, purified proteins such as antibodies, or other bioactive molecules. This approach is in some ways comparable to two-hybrid interaction screens, which enable the identification of cDNAs based on protein–protein interaction. In two-hybrid screening, however, it is not possible to use complex protein assemblies as the "bait." In the interaction screen we describe above, the "bait" can be any isolated complex of unlimited complexity. We have not yet attempted to screen protein pools for enzymatic or other biological activities, or for the ability to complement fractionated or purified systems, although this should be feasible. All of the screens we describe are limited to identifying activities that are the product of a single gene product acting alone or in complexes with other exogenously added proteins (e.g., found within an extract).

Many receptor proteins and secreted signaling molecules require post-translational processing or association with membranes or other cellular factors for activity. Screens for these types of proteins are therefore generally carried out by injecting RNA pools into oocytes or embryos or by transfecting cDNA pools into mammalian cells. Unbiased functional screens of RNA or cDNA pools in living cells, like the one described above, may make it possible to uncover novel biological functions of known proteins or, alternatively, to identify novel proteins with known biological activities. Substrate and interaction screens can also be carried out using whole cell translation systems, but this would generally require the reisolation of the *in vivo* translated proteins.

The abundance of each cDNA in an expression library generally correlates with the abundance of its mRNA in the tissue originally used to construct the library. Thus, some genes are common to different library pools and are assayed multiple times during an expression screen. Normalized or subtracted libraries that reduce or eliminate this skewed abundance would likely make for more efficient and rapid screening. Large-scale sequencing projects will have identified, within about 5 years, almost all of the genes in the genomes of several invertebrate and vertebrate organisms.

Ultimately, it will be possible to use genomic sequence information to construct an expression library containing only one full-length copy of each gene in the genome. Such a "completely normalized" library could then be divided into small pools and functionally screened.

[8] Localization of Cell Cycle Regulators by Immunofluorescence

By JONATHON PINES

Introduction

The cell cycle is regulated in both time and space. At mitosis, the cellular architecture undergoes dramatic changes in almost all its components—including the nucleus, the cytoskeleton, and the membrane compartment—that must be coordinated throughout the cell. Similarly, in replicating its genome the cell ensures that each origin fires only once per cell cycle, and there appears to be a set order in which particular origins initiate DNA synthesis. As might have been predicted, a spatial element to the regulators of the cell cycle machinery is now emerging. A number of regulators, such as the cyclins, their partner kinases the cyclin-dependent kinases (CDKs), the activatory Cdc25 phosphatases and the inhibitory wee1/myt1 kinases, form multigene families, and individual family members appear to be in specific locations within the cell. Furthermore, some of these regulators, such as cyclin B1, alter their location depending on the stage of the cell cycle. This has consequences not only for which substrates are available, but also for which components of the cell cycle machinery are able to associate at any point in the cell cycle. Thus to gain a complete understanding of how the regulators of the cell cycle machinery interact with each other and with the cellular architecture, we need to determine where they are in the cell, and at which points in the cell cycle they colocalize.

The optimal method is to visualize proteins in living cells through time. The techniques to achieve this are improving in resolution, sensitivity, and affordability.[1,2] Proteins can be introduced into cells after conjugation to fluorescent adducts, such as fluorescein isothiocyanate (FITC) or tetra-

[1] Fluorescence microscopy of living cells in culture, Parts A and B. (D. L. Taylor and Y.-L. Wang, eds.). "Methods in Cell Biology," Vol. 30. Academic Press, San Diego, 1989.

[2] D. Shotton, *in* "Electronic Light Microscopy. Techniques in Modern Biomedical Microscopy" (D. Shotton, ed.). Wiley-Liss, New York, 1993.

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