Lecture 11 - cDNA Cloning Lab Practicums (AMG text pp. 133-138)
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Using cDNA as a Reagent to Analyze Gene Expression

Research Objective
A researcher used differential cDNA cloning to isolate a cDNA transcripts representing growth factor induced genes. One of these cDNA clones corresponded to a previously uncharacterized open reading frame in the human genome which the exasperated researcher named "Another Moronic Gamble" (AMG). Since increased steady-state mRNA levels could be due to either transcriptional initiation events, or to a post-transcriptional process (e.g., regulation of RNA turnover rates), he decided to use the RNase Protection Assay and Nuclear Run-on Assay to determine if increased AMG mRNA levels was due to increased transcription at the AMG promoter. The long term is to identify which growth factor-regulated transcription factors are responsible for rapid changes in gene expression during in vitro differentiation of human stem cells.

Available Information and Reagents

1. AMG cDNA and genomic clones have been isolated and characterized.

2. A human stem cell line (HSCL) has been obtained from the National Institutes of Health and shown to be responsive to Growth Factor (GF). It is not known if GF regulates AMG transcript levels in HSCL cells.

3. A plasmid construct containing AMG cDNA sequences (pAMG) has been cloned into a cRNA expression vector which can be used to make large quantities of anti-sense RNA for the RNase Protection Assay. The same pAMG plasmid can be used for the Nuclear Run-on assay.

Basic Strategy and Comments
RNase protection assay (RPA) can provide a more accurate measurement of RNA expression due to the enhanced sensitivity and reproducibility of solution hybridization. In this procedure, an antisense RNA "riboprobe" is synthesized in vitro in the presence of 32P-rCTP using a cloned DNA as the template and purified bacteriophage RNA polymerase.

Riboprobes are hybridized in solution to total RNA to form specific RNA:RNA homoduplexes. Unhybridized probe, and single strand regions of non-complementary RNA, are digested using a mixture of RNases that cleave single-stranded but not double-stranded RNA molecules. The RNase Protection Assay can be used to monitor gene expression using cloned cDNA corresponding to exonic sequences, or to monitor splicing pathways (or splicing intermediates) using cDNA riboprobes corresponding to alternatively spliced transcripts.
In this example, cDNA sequences corresponding to exons 2 and 3 are used as the probe region and are converted to cRNA that contains 5’ and 3’ non-indentical sequences derived from the multiple cloning site (MCS) of the plasmid vector.

The AMG cRNA probe is synthesized in vitro using a segment of the AMG cDNA cloned into the EcoRI site of a plasmid vector containing the T7 bacteriophage promoter on the 3’ side of the gene. The resulting cRNA probe is larger than the internal segment that is complementary to the cellular AMG transcript due to vector sequences.

A time course experiment using RNA isolated from bronchial epithelial cells treated with Growth Factor for various amounts of time reveals that AMG transcript levels increase within two hours of treatment and are maximum at 12 hours post-treatment. The GAPDH (glyceraldehyde phosphate dehydrogenase) cRNA serves as an internal RNA loading control for genes that are not regulated by Growth Factor treatment.

The principle of nuclear run-on assays is that isolated nuclei contain transcription complexes that are stalled on the DNA template due to an acute loss of ribonucleotide substrates. By providing 32P-UTP to isolated nuclei in vitro, it is possible to reactivate the stalled RNA polymerase complexes on the DNA templates.

By providing of 32P-a-UTPs to isolated nuclei in vitro, it is possible to reactivate the stalled RNA polymerase complexes on the DNA templates. The amount of gene-specific radiolabeled RNA synthesized in one nuclei preparation, as compared to another, should reflect the number of transcriptional initiation events present at the time the nuclei were isolated.

In this example, nuclei were prepared from cultured bronchial epithelial cells that had been cultured in the absence (-) or presence (+) of a growth factor that had been shown to increase the steady state RNA levels of the hypothetical AMG gene. By hybridizing the in vitro synthesized pool of 32P-labeled RNA, with filter membranes that had been spotted with denatured AMG cDNA, and gene sequences from GAPDH as an internal control, it is possible to quantitate the amount of hybridized probe RNA and determine that growth factor treatment does increase the number of transcriptional initiation events at the AMG gene. Non-specific RNA-DNA hybridization can be monitored with a plasmid vector control.

Prospective
The data confirm that the AMG gene is regulated at the level of transcriptional initiation and should provide a starting point to identify transcription factors that transduce the growth factor signal to the transcriptional machinery.

**How could RPA be used to quantitate the steady-state level of a specific gene transcript in a cell in terms of molecules/cell rather than simply relative to a housekeeping gene transcript?**

**What makes the nuclear run-on assay so difficult to use, i.e., what are its limitations?**
What molecular genetic strategy using the firefly luciferase gene could be used to show that the AMG promoter is directly regulated by growth factor signaling? What might be the explanation if this type of assay gave a negative result, i.e., growth factor treatment had no effect on luciferase activity?

Assuming that the luciferase assay DID work, how would you isolate the putative transcription factor responsible for growth factor mediated induction of AMG transcription?

In Vitro Expression Cloning of Intracellular Signaling Proteins

Research Objective
A graduate student has isolated a mutant neuronal cell line that contains a genetic defect in a receptor-stimulated intracellular signaling pathway required for post-translational activation of a well-characterized cysteine protease. He plans to use in vitro expression cloning (IVEC) to identify cDNA clones that encode protease-activating functions in extracts prepared from receptor-stimulated mutant cells. The biochemical complementation assay he has developed utilizes a high-throughput screen based on detecting in vitro cleavage of a cysteine protease-specific fluorogenic substrate using a 96 well formatted plate fluorimeter.

Available Information and Reagents
1. Extracts from stimulated mutant cells contain unactivated protease and therefore lack substrate cleaving activity under normal assay conditions. It is possible to stimulate substrate cleavage in the mutant extracts by adding a small amount of extract prepared from unstimulated wild-type cells.

2. A directional cDNA library has been constructed using mRNA that was isolated from the wild-type cell line. This library is cloned into a plasmid vector containing a T7 bacteriophage promoter which can be used to direct the synthesis of “sense” RNA that is suitable for in vitro translation using a reticulocyte protein synthesis reaction mix.

3. The cDNA plasmid library has been normalized and subdivided into 384 pools (four 96 well plates) of independent E. coli strains, with each pool containing ~50 members. This normalized library is estimated to consist of approximately 20,000 different cDNA recombinants.

Basic Strategy
Marc Kirschner and colleagues developed a variation of expression cloning based on the use of coupled in vitro transcription and translation methods to synthesize proteins encoded by cDNA inserts in a plasmid library. The basic strategy is to use an in vitro biochemical assay to identify plasmids that encode proteins with the desired function. By exploiting sib selection using pools of cDNA clones, it is possible to perform a type of "reverse biochemistry" to go directly from biochemical function to cloned cDNA without knowing anything about the protein ahead of time.
Plasmid DNA will be isolated from each pool using a 96 well mini plasmid prep format utilizing nucleic acid binding resins to purify plasmid DNA from E. coli lysates. Sib selection will be used to subdivide plasmid pools into individual E. coli strains by plating the corresponding bacterial stock from a positive well onto agar plates. The plates will be incubated overnight and then each of 96 wells will be inoculated with bacteria from single colonies that have grown up on the agar plates. Based on the estimated complexity of the plasmid pools (~50 recombinants/pool), each 96 well plate should contain two positive wells that represent duplicates of the same cDNA recombinant. Once individual cDNA clones are identified that encode protease activating function, the cloned inserts will be sequenced and characterized.

Comments
A variation of in vitro expression cloning was used in the 1980s to identify a limited number of genes that could be assayed in Xenopus oocytes that had been microinjected with in vitro transcribed cDNA templates. By using sib selection strategies, researchers were able to identify genes encoding channel forming proteins and proteins required for early events in Xenopus development. The term "IVEC", and the idea of using this strategy to screen normalized cDNA libraries was formerly proposed by Marc Kirschner and colleagues in a series of proof-of-principle papers. Their initial application of IVEC was to identify substrates for cyclin-dependent protein kinases by screening for the appearance of electrophoretic mobility shifts of in vitro labeled proteins.

Prospective
Assuming that expression levels are physiological, this in vivo assay should provide good evidence that cloned cDNA encodes the defective protein, and that receptor stimulation is required for complementation. The next logical experiment would be to determine the nature of the genetic mutation that gave rise to the mutant cell phenotype. For example, a Northern blot could be performed to see if the gene is expressed at normal levels, and if it is, it would suggest that there is a mutation in the coding sequence which prevents the production of a functional protein. This would have to be tested by cloning and sequencing the corresponding cDNA.

What are two characteristics of a plasmid cDNA library that would be required to make IVEC feasible?

What are two characteristics of a biochemical assay that would be required to make IVEC feasible?

How could IVEC be used to identify cDNAs encoding protein substrates for a specific kinase that had been previously cloned? What would be the components of the assay?