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Cell and molecular biology for neurosurgeons (or everything you wanted to know about molecular biology but were afraid to download)

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We are fortunate to be physicians at a time when the molecular pathogenesis of disease is being unraveled. Beginning with the discovery of the structure of DNA to the Human Genome Project, molecular biology over the past 45 years has revolutionized medicine. Neurosurgery has a strong scientific tradition, but to remain active participants in this new era of medicine, we must understand the science of pathology at the molecular level, applying our unique perspective to its advancement. We will review most of the current techniques used today by cell and molecular biologists. Interwoven with these descriptions will be a brief discussion of pertinent molecular and cell biology concepts, a full review being beyond the scope of this article.

SOUTHERN BLOT ANALYSIS

The Southern blot (or DNA blot), named for its inventor, has revolutionized the way we analyze DNA. To understand this test, however, we must first review some basic science. Deoxyribonucleic acid is composed of four nitrogenous bases (adenine, guanine, cytosine, and thymine) arranged on a backbone of deoxyribose that is linked by 3'-OH to 5'-phosphate groups forming phosphodiester bonds. Two strands of DNA running in opposite directions can bind to each other through hydrogen bonds between the bases to form a double helix. Adenine and thymine form a pair, as do guanine and cytosine. Because DNA consists of a sense strand and complementary antisense strand, the entire genome can be replicated by unwinding the two strands and synthesizing a complementary strand to each half. This concept of using DNA or RNA as a template for synthesizing or matching a complementary strand of DNA or RNA is central to molecular biology.

The mammalian genome contains 3 X 10^9 base pair (bp) that code 40,000 to 120,000 genes. (This process of transcribing DNA into RNA will be covered in the next section.) Every cell in the body contains the exact same sequence and amount of DNA except for sperm and egg cells, which contain half the total. Thus, for example, DNA extracted from a white blood cell would qualitatively be the same as DNA from an astrocyte. For this reason many studies are performed with cultured human peripheral

white cells (lymphocytes), immortalized with a virus that makes available a continuous supply of DNA unique to an individual. Cells that have diverged from the norm, such as cancer cells, may be compared with the presumably normal lymphocytes. In the DNA extraction technique, enzymes called proteases are used to break down cell walls and then organic compounds are used to separate and dissolve the DNA, RNA, and protein. After the protein is precipitated and removed from this mixture, RNases are added to remove RNA, and the DNA is precipitated by high salt content. This DNA contains the complete sequence (including coding and noncoding regions) for the individual from whom the cells were taken and cultured: this is called genomic DNA.

To make this DNA useful for analysis, we must divide it into smaller pieces and package it so that interesting pieces can be saved and reproduced. To these ends, we use restriction endonucleases, first isolated from bacteria, to recognize short sequences (4-6 bp) of double-stranded DNA for cleavage. For example, the **Escherichia coli** restriction enzyme EcoRI recognizes and separates the following sequence:

The resulting DNA fragments have protruding, "sticky" ends that easily form complements with other EcoRI fragments. The process of DNA cloning relies on restriction enzymes to trick bacteria into reproducing the large amounts of foreign DNA necessary, for example, when a portion of a gene may need to be stored and used repeatedly as a probe to identify similar DNA sequences. This process begins as the bacterial plasmid (extrachromosomal circular DNA) and the DNA of interest are separated using EcoRI. The foreign DNA is then ligated into the plasmid by virtue of the sticky ends and the plasmid is introduced into the bacterial cell, which gives the plasmid a home in a process called transfection. A further refinement to this process involves an antibiotic resistance gene, usually ampicillin, that is placed into the plasmid so only bacterial cells that have taken up the new plasmid can grow in the antibiotic-spiked media. As these bacteria grow in culture they will actively produce the DNA strand of interest. The bacteria can also be frozen and thawed for later use.

Complete digestion of genomic DNA with a restriction enzyme should result in fragments of DNA of approximately the same size. However, if the conditions are altered so that the DNA is only partially digested, a wide range of fragment sizes can be produced. The partially digested DNA is placed on an agar slab and separated vertically by an electrical current applied to the gel; the largest fragments will migrate more slowly because of resistance provided by the agar proteins. A smear of DNA emerges with the faster-migrating DNA fragments at the bottom of the lane and the largest fragments at the top. Adding ethidium bromide to the gel allows the DNA smear to fluoresce under ultraviolet light (Fig. 1). Next, the DNA is blotted onto a nylon membrane that can be hybridized with any probe of interest. The probe is a single-stranded piece of DNA or RNA that contains phosphorus-32 in place of some phosphate groups and allows areas of hybridization to be visualized as bands on autoradiography film. The extent to which the probe is an exact complement of the target sequence can be controlled by factors such as temperature, salt concentration, and presence of blocking DNA during the hybridization and washing steps. A DNA "ladder" composed of known-size fragments is usually run on the gel to allow accurate sizing of the unknown bands. The relative intensity of the bands on autoradiography reflects the number of copies of that particular sequence in the genome.

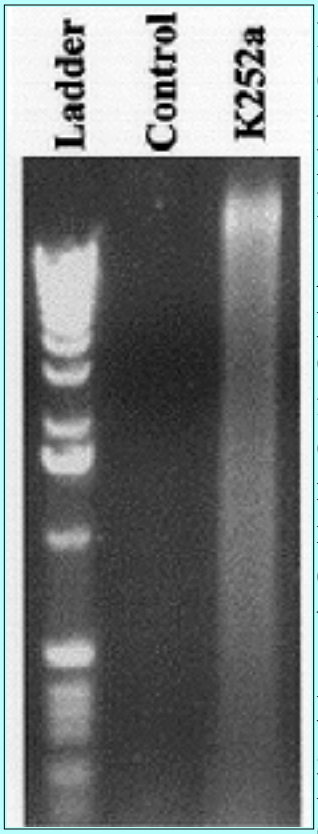

Fig. 1. Ethidium bromide-impregnated gel showing DNA. The DNA was extracted from untreated T98G glioma cells, and the cells were treated the tyrosine kinase inhibitor K252a. A 1-kb DNA ladder is seen in the far left lane. The smear of DNA in the K252a-treated cells suggests degradation of DNA indicative of apoptosis.

A classic use of the Southern blot is analysis of restriction fragment length polymorphisms (RFLPs). The total complement of DNA in an organism is composed of half paternal and half maternal genetic material; therefore, if alternate forms of a gene (alleles) exist, an individual may process two slightly different copies of the same gene (heterozygosity). These small sequence variations do not necessarily result in different forms of a protein because they can occur in the noncoding regions; however, if the variation occurs in a restriction enzyme site, different sized fragments may result following restriction enzyme digestion because the enzyme fails to recognize the altered cutting site. This variation is called a "restriction fragment polymorphism" and can be detected on a Southern blot by a probe complementary to the area of interest. Because RFLPs reflect changes in the genotype and not necessarily the phenotype, they can be used to determine potential linkage between the RFLP marker and a genetically transmitted disease. For example, if a specific restriction fragment is always found in members of a family who carry the disease but is absent in those who do not, then it is likely that the RFLP is close to the mutant gene responsible for the disease. Restriction fragment length polymorphisms can also be used to compare the DNA of normal cells and neoplastic cells. In contrast to normal cells, the loss of one RFLP band in cancerous cells indicates the loss of one allele (usually through deletion) and the potential presence of a gene that results in neoplastic transformation when inactivated (tumor suppressor gene). This strategy was used in the identification of **p53** as a tumor suppressor gene.

NORTHERN BLOT ANALYSIS

Ribonucleic acid can be analyzed in the same manner as DNA by using the Northern blot (or RNA blot). Approximately 2 X 10^9 bp of the total 3 X 10^9 bp in the human genome is nonrepetitive DNA that encodes genes. This DNA is transcribed into RNA by a complex of proteins that include RNA polymerase and transcription factors. Ribonucleic acid is single stranded, uses ribose as the base sugar, and uracil instead of thymine as a nitrogenous base. Three forms of RNA exist: ribosomal (rRNA), transfer (tRNA), and messenger (mRNA). The DNA that becomes mRNA is composed of coding regions called exons and noncoding regions called introns, both of which are spliced out of the final mRNA transcript. Messenger RNA encodes proteins by specifying its amino acid content through a code composed of three RNA nucleotides. The actual formation of protein involves ribosomes, encoded by rRNA and tRNA, which recognize the triplet code and bring the appropriate amino acid to the ribosome complex. This process will be described in the next section.

Northern blot analysis is performed in the same manner as Southern blot except that RNA is used as the template. Either single-stranded DNA or RNA can be used as a probe, and the autoradiographic techniques are similar. Due to the ubiquity of RNA degrading enzymes, all reagents and equipment must be treated to render them free of RNase. When total RNA is run on a gel, 80% of the mass is rRNA, whereas mRNA makes up only 5% of the total mass. The two species of rRNA that encode the 60S and

40S ribosomal subunits are approximately 1.6 and 4 kb in size and are seen prominantly on total RNA Northern blots. The intensity of the rRNA band in each lane can be used to normalize the amount of total RNA loaded in each lane, and the migration of the 1.6- and 4-kb bands can be used to provide approximate sizes for unknown bands. By running mRNA alone, the Northern blot can provide clearer and more sensitive blots because fewer of the repetitive RNAs are present. A larger amount of starting material is needed given the small amount of mRNA present in each cell. An RNA ladder needs to be run next to the mRNA to permit identification of band sizes and the amount of RNA in each lane needs to be normalized by hybridizing the blot with a commonly expressed gene like **ß-actin** or **GAPDH**.

An alternative technique of RNA detection is in situ hybridization, which analyzes intact cells or tissue sections. One advantage of this technique over the Northern blot is its provision of a spatial representation of RNA expression in the tissue or organ of interest. Briefly, an entire tissue section is hybridized with the probe of interest and then examined either by autoradiography, if a radioactive probe is used, or fluorescent microscopy in fluorescent in situ hybridization.

WESTERN BLOT ANALYSIS

Protein, the last major cellular component, is analyzed by the Western blot (or protein blot). Fundamental to this analysis is RNA translation, the basics of which warrant brief review. After the introns have been spliced out of mRNA, it is transported to the cytoplasm where translation occurs by ribosomes and tRNA molecules. Each ribosome complex contains binding sites for two tRNA molecules, and the entire apparatus translates the mRNA code in a 5' to 3' direction. The mRNA message is decoded by tRNA molecules that can recognize a sequence of three consecutive nucleotides called a codon. There are 64 possible combinations of the four nucleotides that are used to identify the 20 amino acids found in humans. The code is termed "degenerate" because each amino acid has more than one triplet (or codon) assigned to it, with three codes (UAA, UAG, and UGA) reserved to indicate termination of the signal. The start of the reading frame is signaled by the code AUG, which represents methionine. A polypeptide is produced as new aminoacyl-tRNAs are bound by the ribosome and donate their amino acid to the growing peptide chain. Proteins destined to stay within the cell are synthesized on free ribosomes in the cytoplasm, with the ultimate destination determined by the presence of short sequence motifs. Charged NH_2-terminal sequences of approximately 25 amino acids on the protein typically indicate a mitochondrial destination, whereas the nuclear localization signal is found internally. Posttranslational modifications such as peptide cleavage and glycosylation are also important for determining protein localization and function.

The principle of the Western blot is similar to the Southern and Northern blots except for the absence of nucleic acid hybridization (Fig. 2). Protein is separated on a polyacrylamide gel by electrical charge and blotted onto a membrane that is then incubated with antibodies raised against the protein of interest. Radioactivity can be conjugated with the antibody. It is much simpler and safer, however, to use chemiluminescent systems that conjugate an enzyme to a secondary antibody that recognizes the first antibody and produces a light signal that develops on an autoradiograph, which can be further quantified by optical densitometry. As with the other techniques, the main limitation of the Western blot is that the protein on the blot represents a pooled sample, and therefore it is not possible to distinguish differences in protein synthesis between individual cells.

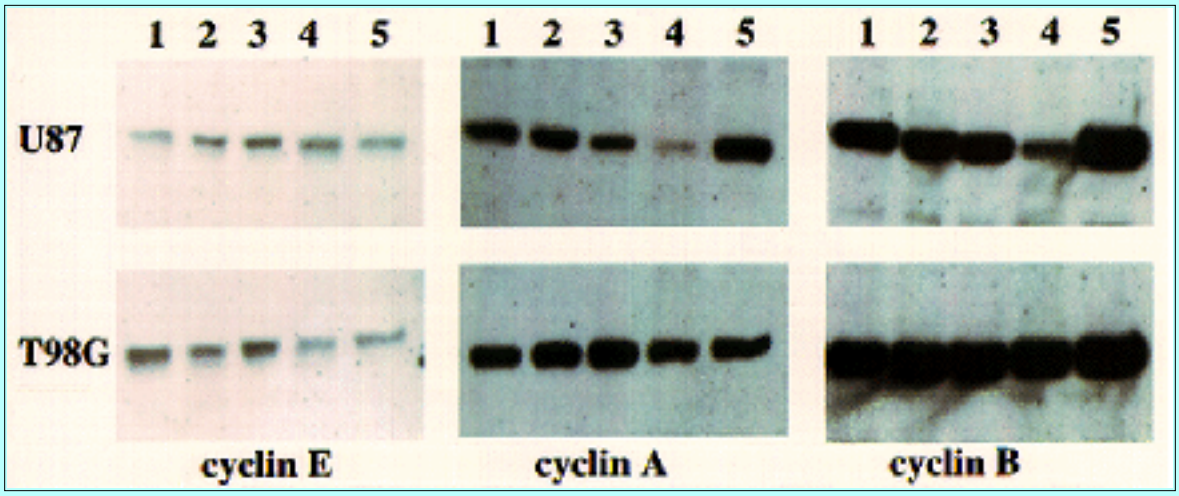

Fig. 2. Western blot analysis of cyclin proteins in U87 and T98G glioma cells under various conditions. Protein was extracted from cells and size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblotting was performed with anticyclin antibodies as shown. A nonradioactive detection system was used.

Immunohistochemistry is to the Western blot what in situ hybridization is to the Northern blot: it provides a spatial representation of protein expression. Whole-tissue sections are incubated with a primary antibody to the protein of interest, followed by the addition of a secondary antibody that is conjugated to the desired detection system, usually peroxidase or fluorescent labeling. These techniques are extremely helpful in clinical neuropathology. Immunohistochemical analysis, for example, can identify tumors of glial origin by detecting glial acidic fibrillary protein.

IMMUNOPRECIPITATION AND KINASE ASSAYS

A major limitation of protein detection assays is that they do not provide information about function or activity. One of the most important functions of intracellular proteins is the transduction of signals from external stimuli to the nuclei, where the appropriate cellular response can be manufactured. The fuel driving these signal transduction pathways is protein phosphorylation, generated by the activity of protein kinases. To study protein phosphorylation and kinase activity, immunoprecipitation techniques were developed that use polyclonal or monoclonal antibodies to isolate the protein. The protein-antibody complex is removed from the mixture by using agarose-bound protein A, a bacterial protein that binds to immunoglobulin regions. When protein A beads are centrifuged out of the mixture, they carry the protein-antibody complex. This protein can then be eluted from the agarose and used in a highly purified and concentrated form. The phosphorylation status of the protein can be determined by running a Western blot and using an antibody that detects phosphate groups like antiphosphotyrosine (Fig. 3).

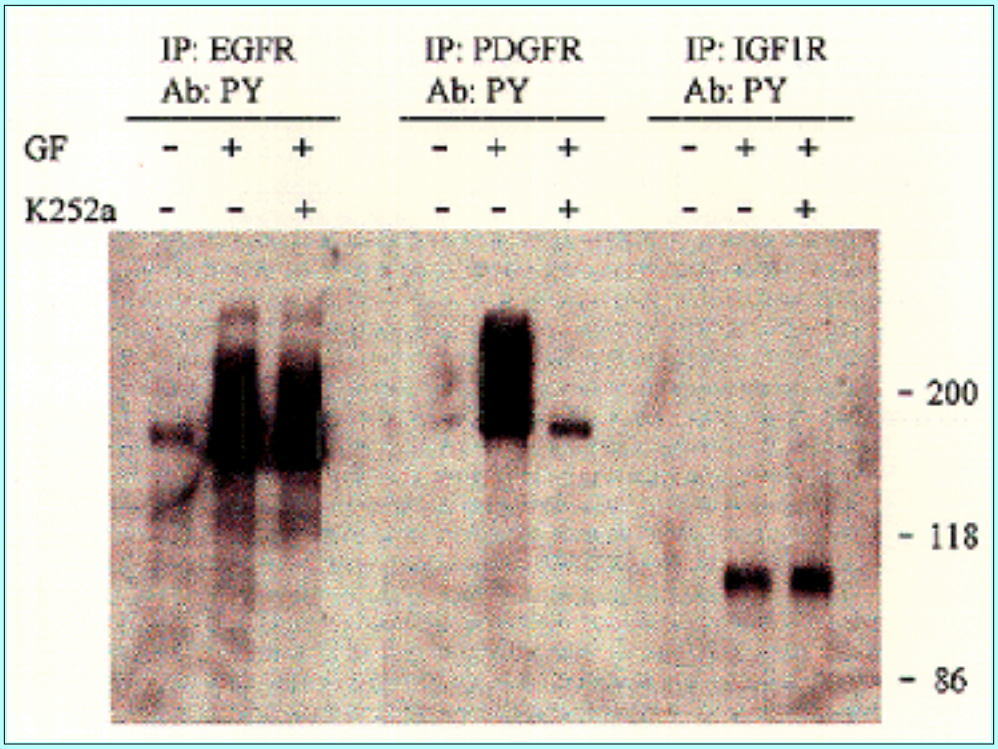

Fig. 3. Antiphosphotyrosine immunoprecipitation blot showing T98G cells that were exposed to growth factor (epidermal growth factor, platelet-derived growth factor, or insulin-like growth factor-1) in the presence of the tyrosine kinase inhibitor K252a as indicated. Protein was extracted and immunoprecipitated using an antibody to the indicated growth factor receptor. Immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting was performed with antiphosphotyrosine antibody. Receptor phosphorylation is inhibited by K252a in response to platelet-derived growth factor only (middle lanes).

If kinase activity is present the protein of interest can be assayed as long as the substrate is known. The kinase and its substrate are incubated together in the presence of gamma phophorus-32-adenosine triphosphate (ATP), which can then donate its most peripheral gamma-radiolabeled phosphate group to be incorporated into the newly phosphorylated substrate. The amount of radioactivity is proportional to the kinase activity and can be quantified by autoradiography or scintillation counting. An alternative to this technique is an in-gel kinase assay in which the immunoprecipitated protein is run on a gel impregnated with the substrate. The entire gel can be incubated with the radiolabeled ATP and then dried and exposed to autoradiography film. The advantage of this technique is that the size of the protein kinase can be determined.

DEOXYRIBONUCLEIC ACID SEQUENCING

The Human Genome Project has attracted much public attention and has galvanized efforts in the scientific community to reach a goal previously thought unattainable: to decipher all 3 X 10^9 bp that comprise the human genome. Although DNA sequencing is now automated for purposes of the genome project, it is still performed in many laboratories similarly to its original description by Sanger, et al.[2] The sequencing reaction requires a primer specific for the 5' end, DNA polymerase, and a mixture of excess nucleotides that contain a small amount of a dideoxynucleotide that lacks the 3'-OH group. The dideoxynucleotide blocks further elongation by preventing the 5'-P group of the next nucleotide from forming a phosphodiester bond with the 3'-OH group. Thus, dideoxyguanosine triphosphate causes all

synthesized DNA to end in guanine; similarly, dideoxy-ATP stops the sequences at adenine. By running four reactions in parallel, each with a different dideoxynucleotide, a series of fragments can be separated by polyacrylamide gel electrophoresis, a technique more sensitive than agarose electrophoresis and able to distinguish fragments that differ in size by only a single bp (Fig. 4). The DNA fragments are usually end labeled with sulfur-35 and detected by autoradiography, with the four reactions run next to each other. The sequence in the 5' to 3' direction is read from the bottom of the gel by noting in which dideoxy lane the band is found.

Fig. 4. Portion of a sequencing gel. A cDNA fragment has been cloned into a PCR-based vector system and sequenced using predetermined primers. The sequence is read from the bottom up and the four lanes represent GATC (left to right).

POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is a powerful technique that allows high-fidelity amplification of minute amounts of DNA or RNA for virtually any molecular biology application. Originally described by Mullis, et al.,[1] in 1986, it later earned him a Nobel prize. Two developments led to its widespread use: automation and a thermostable DNA polymerase. The reaction starts when a DNA molecule is heated and "melts" into two strands. The addition of two primers, each recognizing a short sequence at opposite ends of the same molecule, allows both strands to be replicated by DNA polymerase and a mixture of nucleotides. The reaction must be cooled, allowING annealing between the DNA template and the two primers, and then heated slightly for DNA elongation. Repeating this cycle allows the two new strands to be duplicated, thus forming four strands, all identical to the original. Normal DNA polymerase degrades at the temperature needed to melt the DNA strands (95šC), but the discovery of the bacteria **Thermus aquaticus** in deep-sea heat vents suggested that their DNA polymerase could withstand multiple cycles of heating and cooling. The use of Taq polymerase in PCRs obviates the need to add new DNA polymerase with each heating cycle. Automated thermal cyclers can complete a heat/cool cycle in 5 minutes, thus allowing a typical experiment of 25 to 30 cycles (4 X 10^6-fold DNA amplification) to be completed in several hours. Countless variations of the PCR technique have been devised, and they can be found in all facets of medical science.

REPORTER GENES

When studying eukaryotic gene transcription, it is essential to be able to quantitate and localize transcriptional activity. Eukaryotic gene transcription is largely regulated by a promoter sequence that begins 100 to 250 bp before the initiation codon. This promoter region binds a number of transcription factors that influence binding by RNA polymerase. Although this review focuses only on RNA polymerase II, which produces mRNA, two other RNA polymerases exist: RNA polymerase I, which

produces rRNA, and RNA polymerase III, which produces tRNA.

Transcription factors that interact with RNA polymerase II can be divided into three groups: 1) basal factors, which form a complex with RNA polymerase II near the startpoint and determine the site of initiation; 2) upstream factors, which are DNA-binding proteins that recognize short sequences called consensus elements, usually located within 100 bp upstream of the startpoint; and 3) inducible factors, which are also DNA-binding proteins that recognize DNA regions called "response" elements that contain consensus sequences, located within 250 bp upstream of the startpoint. The inducible factors are frequently specific to a tissue type and may be activated at different times, thus making them important for differential spatial and temporal gene expression. Constitutively expressed or "housekeeping" genes contain promoters that usually contain only the basal and upstream factors. The basal factors for RNA polymerase II are called TFIIX, where X is a letter that identifies the individual factor. The two important components contained in TFIID (a member of the TFIIX family) are the TATA-binding protein and associated factors. The TATA box is a ubiquitous 8-base promoter sequence found approximately 25 bp upstream from the startpoint and composed entirely of A/T bp and appears to be crucial for proper positioning of the transcription complex. There are several other common upstream consensus sequences and transciption factors: CAAT box, CTF/NF1; GC box, SP1; octamer, Oct-1,2; and kappa-B, NF-kappa-B. Well-described response elements and their DNA-binding protein include: heat shock response element, heat shock transcription factor; glucocorticoid response element, steroid receptor; and phorbol ester or TPA response element, AP-1 (jun/fos).

These transcription factors contain common motifs that allow them to bind DNA. The zinc finger is a conserved Cys/His sequence found in TFIIX and SP1 that binds zinc ions and maintains the protein in proper alignment for DNA binding. Helix-loop-helix proteins contain two amphipathic (containing two surfaces, one hydrophilic and one hydrophobic) alpha-helices separated by a link (the loop) that allows the two helices to interact independently and form dimers. Examples of helix-loop-helix factors are myoD and c-myc. The leucine zipper is a stretch of amino acids that are rich in leucine residues, which form an amphipathic alpha-helix that promotes dimerization. The transcription factor AP-1 is composed of jun/fos dimers that interact through leucine zippers.

The study of RNA transcription and promoters necessitated easy quantification of transcription and led to the development of reporter gene assays. The prototypical reporter gene assay is the chloramphenicol acetyl transferase (CAT) assay, which places **CAT** under the influence of a eukaryotic promoter in a plasmid construct. The **CAT** gene is bacterial in origin and has no eukaryotic counterpart, thus making it useful for characterizing eukaryotic promoters because the transfected gene is the only source of CAT in the cell. The amount of CAT produced under the experimental condition is measured by adding ^{14}C-chloramphenicol to cell extracts, separating acetylated and nonacetylated forms by chromatography, and then measuring their amounts by autoradiography or scintillation counting. The **lacZ** reporter gene encodes ß-galactosidase that can cleave substrates, resulting in a blue color, whereas the firefly luciferase, **luc**, gene produces a quantifiable light signal.

RETROVIRUS APPLICATIONS

Although eukaryotes and most prokaryotes store their genetic information as DNA, a specific subset of viruses called retroviruses store their genes as single-stranded RNA. In retroviruses the flow of information is reversed, that is from RNA to DNA. Retroviruses reproduce by infecting actively dividing cells, integrating their genome into the DNA of these cells whereby the process of host cell DNA

replication and transcription can create more retrovirus particles. Reverse transcriptase (RT) transcribes single-stranded RNA into double-stranded DNA, which then integrates into the host genome. Practical applications for RT have been found in molecular biology because RNA is unstable and frequently found in low concentrations, whereas DNA is much hardier; therefore, RT can be used to transcribe any RNA sequence into its complementary DNA (cDNA), which is then available for PCR amplification, cloning, or sequencing. The creation of a cDNA library also makes use of RT technology. The complete mRNA sequences of any tissue can be transcribed into cDNA by RT, cut into fragments by a restriction enzyme, and then placed into a bacterial or phage (a bacteria virus) vector. All the genes in that tissue are now represented in DNA form without the intervening noncoding and repetitive DNA sequences. The bacteria or phage can be grown, and using a variation of the Southern blot, colonies of bacteria or virus can be hybridized with the probe of interest. Positive colonies can be found in the library, extracted, and amplified for further analysis.

The recent explosion in gene therapy technology has been fueled in part by the application of retrovirus biology. The ability of retroviruses to integrate their genome into other cells is exploited by removing some retroviral genes and replacing them with the gene of interest. A tumor may be eradicated by delivering a "suicide" gene that kills any cell that takes up the retrovirus. To control the potentially unchecked nature of retroviral reproduction, the viruses can be made "replication defective" by removing critical retroviral genes that promote reassembly of the virus particle. Thus, only in a controlled environment, such as in a helper cell line that supplies the missing replicative viral sequences, can the retrovirus reproduce. When the helper cell line is transplanted into tumor, it releases retroviral particles that carry the suicide gene but lack the necessary genes to reproduce. One unique feature of retroviruses is that they only infect dividing cells, which, in the brain, would exclude all neurons. Therefore, to apply this technology to neurodegenerative diseases or stroke, other less selective virus vectors may be required. The adenovirus and herpes simplex virus can transform both astrocytes and neurons and may be used to transfer nerve growth factor, ciliary neurotrophic factor, or other neuroprotective factors into the brain.

CELL PROLIFERATION ASSAYS

Tumor cell growth and therapeutic interventions can be assessed only if there exist reliable, quantitative measures of proliferation. Tumor cells, like all cells in the body, can be characterized by their cell cycle, which determines the rate of proliferation and is responsible for coordinating the events needed for DNA replication and cell division. The eukaryotic cell cycle has five distinct phases: 1) G_0, the quiescent phase of the cycle that cells can enter from G_1 if they are no longer actively dividing (2N DNA); G_1, the first gap phase that occurs prior to DNA synthesis (2N); 3) S, the DNA synthesis phase (2-4N); 4) G_2, the second gap phase that occurs between S and the final phase (4N); and 5) M, the phase that culminates the cycle by permitting the cell to undergo mitosis (4N). The time to traverse one entire cell cycle would represent the theoretical doubling time of the tumor if all the cells in the tumor were in an active phase of the cell cycle (no G_0 cells) and if no cell loss occurred. Because both of these conditions are rarely met, the actual tumor-doubling time is longer than the cell cycle time.

Progression through the cell cycle is regulated by a protein complex composed of a regulatory cyclin subunit and a catalytic cyclin-dependent kinase (CDK). The CDKs behave as serine/threonine kinases and activate proteins that prepare the cell for DNA replication and cell division. Growth factors and other external stimuli increase expression of cyclin D during the G_1 phase, which leads to increased cyclin

D/CDK4,6 activity. One target of the CDKs is the product of the retinoblastoma tumor-suppressor gene **Rb**, which, after phosphorylation, becomes inactivated and releases the transcription factor E2F to stimulate expression of other proteins needed for DNA synthesis. Thus, inactivation of pRb by cyclin D/CDK4,6 allows progression through the G_1/S checkpoint. Expression of cyclin E rises dramatically at the G_1/S checkpoint and cooperates with CDK2 in permitting passage into S phase. Cyclin A/CDK2 and cyclin A/Cdc2 complexes increase during S and G_2 phases, whereas cyclin B/Cdc2 (maturation promoting factor) becomes most prominent during the G_2 and M phases and catalyzes the final events of the cell cycle that culminate in mitosis.

The eukaryotic cell cycle is most tightly regulated at two checkpoints: G_1/S and G_2/M. Processes that damage DNA such as ionizing radiation or chemotherapy can arrest cells at either checkpoint. Teleologically, cell cycle arrest allows the cell time to repair DNA damage before proceeding with DNA replication or cell division. Cells lacking this property can perpetuate DNA mutations and become hypersensitive to radiation or frankly neoplastic. Patients with ataxia telangiectasia, a disease that causes cancer susceptibility, lack a protein called ATM and do not demonstrate normal G_1/S arrest following exposure to ionizing radiation. The rare autosomal dominant disease Li-Fraumeni syndrome causes cancer susceptibility and is associated with a germline **p53** mutation. Among its many functions, wild-type p53 is a transcription factor that upregulates expression of $p21^{Waf1/Cip1}$, the prototypical small molecular weight CDK inhibitor (CKI). The p21 binds to CDK2,4,6 along with proliferating cell nuclear antigen and prevents progression through the G_1/S boundary. Other CKIs include $p27^{Kip1}$ (cyclin E/CDK2) and $p16^{INK4}$ (cyclin D/CDK4). These CKIs play an important role in cell cycle regulation and their dysfunction may play a role in neoplasia.

Cell proliferative potential can be measured directly by counting cells or indirectly by measuring a biochemical variable that correlates with cell turnover. The simplest techniques are to count actual cell numbers under the microscope or to measure absorbance after incubating cells with a dye that only forms in viable cells. An important variation, the colony-formation assay, uses the ability of tumor cells to form colonies from a single clone of cells under anchorage-dependent (tissue-culture plates) or anchorage-independent conditions (soft agar-covered plates). Indirect measurements of cell turnover can be made by quantifying cell cycle events. Cellular uptake of [^{3}H]thymidine is easily measured and provides a quantitative measurement of DNA synthesis. The thymidine analog, bromodeoxyuridine, allows nonradioactive measurement of the DNA synthesis rate and also the number of cells in the S phase when examined in tissue sections. The nuclear antigen Ki-67 or MIB1 is found in actively dividing cells during G_1, S, G_2, or M but is excluded in the G_0 phase. It provides a measure of the tumor growth fraction, which can provide an estimate of the doubling time if the cell cycle time is known.

The last technique we will discuss is flow cytometry. This procedure involves a machine that can measure DNA content in individual cells as they pass through a light beam. A DNA profile is then obtained that quantifies the percentage of cells in each of the cell cycle phases. Cells that contain less than 2N or more than 4N DNA can be identified (aneuploidy). A very versatile technique, flow cytometry can also be used to count cell numbers, identify antibody-labeled cells, and determine apoptotic cell death.

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Recommended Further Reading

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sense and antisense: the orientation of DNA is arbitrarily defined as "sense" in the 5' to 3' direction and "antisense" in the 3' to 5' direction.

bp or base pair: the individual bases in a DNA or RNA molecule that form a complement with each other through hydrogen bonds, A:T, G:C.

ethidium bromide: a compound that intercalates in DNA molecules and fluoresces under ultraviolet light.

blotted: the blotting process requires gravity, negative pressure, or electrical current to drive DNA, RNA, or protein from a gel onto a durable membrane that can be treated with antibodies or radiolabeled probes.

blocking: repetitive DNA sequences can obscure messages present in low-copy numbers because of excessive binding of probe. The quality of the autoradiograph can be improved by incubating the blot with blocking DNA-like-sheared salmon sperm DNA.

tumor suppressor gene: these genes normally function in the cell to limit cell growth or, alternatively, they may induce cell death. The prototypical tumor suppressor gene, **p53**, is frequently mutated in glioma cells.

ribosome: a large molecule, composed of two subunits, that synthesizes proteins by coordinating translation of an mRNA template with tRNA molecules that carry amino acids.

kb or kilo base: the size of genes are commonly described in units of 1000 bp.

aminoacyl-tRNA: a tRNA molecule that is complexed to an amino acid.

protein kinase: a protein that functions to phosphorylate another protein. Growth factor receptors have tyrosine kinase activity and therefore add phosphate groups to the tyrosine residues of their substrate.

antiphosphotyrosine: an antibody that recognizes phosphorylated tyrosine residues.

end labeled: DNA can be radiolabeled throughout the entire strand by using phosphorus-32 conjugated to a nucleotide, or it may be end labeled by adding a single radioactive group on the end of the strand.

NF-kappa-B: a transcription factor that is upregulated by stress signals such as ultraviolet light and free radicals. It is negatively regulated by I-kappa-B protein.

c-myc: a protooncogene responsible for proliferation and possibly apoptosis. It is closely related to the **myc** family of oncogenes.

cDNA library: a representation of mRNA transcripts in DNA form. A cDNA library can be screened with an unknown probe to determine if any genes exist that contain the unknown sequence.

2N DNA: The amount of DNA in a nondividing, diploid cell. During DNA synthesis the amount of DNA doubles and is denoted as 4N.