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Chapter 1

DNA and its Relationship to Cancer

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Abbreviations

Deoxyribonucleic acid (DNA) ,
N1: nitrogen in 1st position,
H2A: histone 2A,
O6: oxygen in 6th position,
Å : Angstrom,
5': 5th position of pentose,
3': 3rd position of pentose,
T: Thymine,
A: Adenine,
G: Guanine,
C: Cytosine,
YY1: Ying-Yang-1,
10q24: 24th position of long arm in chromosome 10,
JNK: c-Jun N-terminal kinase 1 (alias for MAPK8:mitogen-activated protein kinase 8),
NF-kB: Nuclear Factor kappa B,
PCR: polymerase chain reaction,
c-jun: cellular homologue of jun oncogene,
c-fos: cellular homologue of fos oncogene,

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mRNA: messenger ribonucleic acid,
5S: sedimentation coefficient 5,
SRF: serum response factor,
ATM: ataxia telangiectasia mutated,
AP-1: activating protein-1,
BPDE-dG: 7r,8t – dihydroxy - 9t,
10t – epoxy - 7,8,9,10 –tetrahydrobenzo [alpha] pyrene N2 - deoxyguanosine,
AAF-dC: 2-acetylaminofluorene deoxycytosine,
XP: xeroderma pigmentosum,
XPA: xeroderma pigmentosum A,
ERCC1: excision repair cross-complementing rodent repair deficiency complementation group 1,
ROS: reactive oxygen species,
FEN: Flap endonuclease-1,
hOgg1: human 8-oxoguanine DNA N-glycosylase,
UDG: uracil DNA glycosylase,
MMR: DNA mismatch repair,
ARF: alternate reading frame,
MDM2: mouse double minute 2,
p53-binding protein;
oncoprotein MDM2,
ATR: ataxia telangiectasia and Rad3-related (FRAP-related protein-1),
DNA-PK: alias for PRKDC (protein kinase, DNA-activated, catalytic polypeptide; hyper-radiosensitivity of murine scid mutation, complementing 1),
CHK2: alias for CHEK2 (CHK2 [checkpoint, S.pombe] homolog; protein kinase Chk2)

DNA

Cells have two chemically similar information-carrying molecules: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Both DNA and RNA in their primary structures are linear polymers composed of monomers (single chemical units) called nucleotides. DNA is usually double-stranded, due to formation of hydrogen bonds between units of two complementary strands (1).

Each fragment of double stranded DNA that encodes for several genes and is individually inherited as a whole, is called a chromosome. A mammalian genome contains several chromosomes. Genetic information is passed on to descendant cells and organisms through DNA replication; DNA double strands unfold locally, to permit synthesis of complementary sequences by an elaborate enzymatic apparatus. During mammalian cell cycle, DNA is replicated in the S phase. Each daughter cell receives double stranded DNA that consists of one parent strand and one newly synthesized complementary strand. Each chromosome is represented twice in a somatic cell. This means that almost all genes are represented by two alleles in a mammal. Exception are the sex chromosomes x and y. Chromosomes that are not associated with sex determination are the autosomes.

Nucleic acid sequences that are not in a double stranded form may fold into hairpin structures when parts of one linear nucleic acid strand hybridize via hydrogen bonds to one another. Complex patterns that involve hairpins are the chromosomal telomeres of eukaryotic organisms in the case of DNA (1). Human chromosomes have guanine-rich sequences at the ends, which adopt a hairpin-folded conformation and bind specific proteins, to inhibit degradation. Telomerase is an enzyme which maintains chromosome length. In complete absence of telomerase activity chromosomes progressively shorten. This is one of the mechanisms which contribute to cell senescence. Cancer cells usually maintain high telomerase activity.

DNA is Condensed by Complex Formation with Basic Proteins, the Histones

Eukaryotic DNA is wrapped around small basic proteins called histones, in a way that a long stretch of DNA takes up very little space. Histones thereby constitute half the mass of a eukaryotic chromosome. The entire complex of a cell's DNA and associated protein is called chromatin. The elementary unit of DNA and histones, is called nucleosome core particle. The DNA connecting core particles is termed linker DNA. Five major histones are present in chromatin: four histones, called H2A, H2B, H3, and H4, associate with one another; the other histone is called H1. A quarter of the amino acid residues in each histone are either arginine or lysine, giving to histones strikingly basic properties. Nucleosomes will form on almost all DNA sites, although some sequences are preferred because the dinucleotide steps are properly spaced to favor bending around the histone core. The stacked layers of nucleosomes form loops. This results in a very high condensation of DNA.

The left-handed helical manner in which writhing of DNA around the histone core occurs, results in negative supercoils. If the nucleosomal DNA is straightened out, it will be underwound. This underwinding that follows nucleosome displacement, is exactly what is needed to separate the two DNA strands during replication, the process aimed to maintain the genome across cell generations and transcription, the process that initiates gene expression.

Dynamics of Chromatin

A typical feature of any gene is its promoter. This is a combination of specifically arranged DNA sequences, most often in the 5' direction from the coding sequence, that determine the conditions under which the gene is expressed. Catabolic genes that enable bacteria to survive in lactose are induced when a protein, that otherwise binds to promoter sequences of those genes, binds lactose and is thereby allosterically disabled from DNA binding. This enables expression of the catabolic genes. Eukaryotic gene regulation has similar features. Due to the higher complexity of eukaryotic organisms, their regulatory DNA sequences are more complex. Each one of the proteins that regulate gene expression recognizes short stretches of DNA, about 5 to 12 basepairs. Activity of such regulatory proteins is induced by changes in conditions, which result in alterations in the function of

intracellular signal transducers. Such alterations may cause covalent or structural modification in regulatory proteins. Modified regulatory proteins may enter the nucleus. While eukaryotic genome size is usually large, certain parts of it are more accessible than others, due to the different degree of DNA condensation. A cell type that needs a particular gene product more, maintains the DNA region of the corresponding gene, in a less chromatin-condensed state. Corresponding proteins that are necessary for the induction of the expression of that gene have therefore easier access to its regulatory sequences, without being diluted into billions of basepairs of potential target DNA. This gene-specific differential degree of DNA condensation is a feature of eukaryotic cell differentiation.

Regulation of Gene Expression at the Level of DNA Transcription is Strongly Dependent on DNA Sequence and is Mediated by Proteins

A major mechanism of the control of gene expression is regulation of DNA transcription into RNA. It is regulated by the control of chromatin accessibility, transcription initiation, mRNA elongation.

The process of DNA transcription into RNA consists of two steps: initiation, which requires recruitment of the template-directed enzyme RNA polymerase to the promoter of a given gene, and elongation, which involves the continuous addition of nucleotides to a growing RNA chain. In eukaryotes, RNA polymerase is recruited to a promoter by multiprotein complexes, many of which are common to large groups of genes. These complexes consist of basal transcription factors, which themselves are attracted to a promoter by protein-protein interactions with DNA-binding, inducible transcription factors that have been activated by intracellular signal cascades. Only few basal transcription factors recognize DNA. TATA-box binding protein recognizes the sequence TATAA or variants of it, located few bases upstream of transcription start sites of a very large proportion of eukaryotic genes.

A major role in the regulation of the expression of genes is played by inducible transcription factors. Most inducible transcription factors recognize specific DNA sequences on the promoter of their target genes, and bind to them via noncovalent protein-DNA bonds. These transcription factors are proteins, themselves controlled in variable degrees by regulation of their own synthesis, posttranslational modifications, and availability of interaction partners (2-8). These partners modify function of transcription factors and may themselves access DNA indirectly, via noncovalent interaction with DNA bound transcription factors. Inducible transcription factors may also repress gene transcription, depending on the specific arrangement of target sites on a given promoter, and the species of factors available. The term transactivators has been used for inducible transcription factors, because the DNA sequences that encode the factors themselves, are most often in different DNA regions than the genes they regulate. They activate therefore in trans, meaning they activate DNA sequences neither genetically nor physically linked to the DNA sequences that encode the transactivators themselves.

Inducible transcription factors bind to one or more sequences on a gene promoter, but their position relative to the transcription start site is not uniform for all genes that they regulate. An inducible transcription factor may have more than one target site on a gene

promoter. A given promoter has target sites for a variety of factors (1,3). The numbers and relative position of those sites determines the response of the promoter to changes in the activity of inducible factors (8).

Regulation of gene transcription is affected by a variety of mechanisms. Chromatin context and position of the gene in the nucleus affect the accessibility of the gene to the transcriptional apparatus (1). Availability of inducible transcription factors and signals that activate them determines which groups of promoters may be turned on at a given condition. Availability of cofactors, which do not themselves bind DNA, but modulate the activity of transcription factors through formation of active or inactive multiprotein complexes is also important. Antagonistic transcription factors may bind directly to each other preventing access to DNA targets or to RNA polymerase, or have overlapping DNA recognition sequences on the promoter of a target gene which they regulate. An example is the displacement of negative regulatory protein YY1 by SRF, upon SRF activation by myogenic signals (9).

Further mechanisms of control of gene expression at the level of DNA transcription into RNA include, but are not limited to, acetylation of histones and phosphorylation of RNA polymerase.

A great number of different events regulate DNA transcription into RNA (1). Events are integrated on the promoter of accessible genes, where a number of diverse transcription factors and enzymes are recruited by DNA-bound proteins. The exact combination of transcription factors that regulate expression of a particular gene is usually characteristic of the individual gene. On the other hand, genes that share certain conditions and whose expression is activated together, usually also share some sequence elements of their promoters (10). This facilitates their induction by sets and combinations of transcription factors that are active at the given conditions that the particular genes will be expressed (11,12). Proinflammatory genes, for example, share sequence elements that include recognition targets for factors such as AP-1 and NF-kB (2,3,13,14).

Enzymes that activate transcription factors are themselves controlled by an immense variety of different signals (15). An example is the phosphorylase JNK, which is controlled by changes in the intracellular oxidative environment (16), hormones (17), heat shock (18), upstream kinases that phosphorylate and thereby acitvate JNK itself (19), and phosphatases, that remove phosphate groups from JNK, deactivating it (2). JNK itself controls the activity of a number of diverse proteins, mostly by phosphorylation, but also by association with them. JNK substrates include a variety of transcription factors and other proteins that control gene expression (4-7).

The most prominent substrate of JNK is the protein c-jun, a protooncogene (4). First identified as a cellular homologue of the 17th gene of avian myeloblastosis virus, c-jun is phosphorylated by JNK and activated. It forms dimers with other proteins such as c-fos, forming the transcription factor AP-1. AP-1 binds to DNA sequences such as the pseudopalindrome ATGACTCAT or variants of it. AP-1 binds to promoter DNA sequences of various genes such as cyclin D1, many of which control the progression of the cell cycle (2,7).

To conclude, genes are DNA sequences. Expression of genes is controlled by a variety of conditions that affect accessibility of DNA in the context of chromatin, DNA transcription

into RNA (20), splicing of the transcribed RNA, translation of the spliced RNA in the cytoplasm. Posttranslational modifications and stability of the gene product as well as interactions with other macromolecules regulate the activity of the gene product and thereby control the effects of the gene itself.

Control of Gene Expression by Chromatin Remodeling

The transcriptional regulatory mechanisms utilized by prokaryotes and eukaryotes have some significant differences, many of which are related to the vast difference in genome sizes between these classes of organisms. However, much of the DNA in a eukaryotic cell is stably assembled into chromatin. The packaging of DNA with chromatin renders many potential binding sites for transcription factors inaccessible, in effect, reducing the size of the genome (8). Thus, rather than scanning through the entire genome, a eukaryotic DNA-binding protein scans a set of accessible binding sites that is close in size to the genome of a prokaryote. The cell type is determined by the genes that are accessible.

If cell nuclei are treated with the nonspecific DNA-cleaving enzyme DNase I, cleavage is not uniform. DNA regions that are adjacent to actively transcribed genes are cleaved more efficiently than other parts of the genome. This increased accessibility of transcribed DNA to DNase I reflects the fact that transcribed parts of the genome are less protected (and hence condensed) by histones and other proteins than the rest of the DNA in a nucleus. In addition to the above observation, it has been noted that DNA regions of approximately 1 thousand base pairs (1KB) from a given transcribed (expressed) gene have an increased sensitivity to cleavage by nucleases such as DNase I. It has been found that nucleosomes of those regions have an altered conformation or are absent altogether. Such hypersensitive sites (to DNase I) appear when and where a given gene is actively transcribed (8), meaning in those cells that will activate expression of the gene, and at those specific developmental stages that will require the gene product to be expressed. In those tissues that the gene product will not be expressed, chromatin of that gene will remain condensed throughout development.

When, during a study of gene regulation, a particular transcription factor is crosslinked to DNA in the extract of the nucleus of a eukaryotic cell and DNA is fragmented, antibodies specific for the transcription factor are used to precipitate the chromatin fragments that are bound by the transcription factor. After breaking of the crosslinks and identification of the DNA fragments, usually it is observed that only a very small percentage of the factor's binding sites in a given genome are occupied. This technique that characterizes a factor's binding sites via specific antibodies that pull down the factor and its associated chromatin is called chromatin immunoprecipitation. Other techniques exist for the identification of a factor's binding sites in the nucleus of a living cell, based on cleavage of DNA within the nucleus, and PCR amplification of the bound fragments. The fact that only few binding sites are occupied by a transcription activator probably reflects the fact that with the exception of the genomic regions that are essential for cellular function the rest of DNA is highly condensed by histones in chromatin. To make this part of gene regulation clearer, it has been recently found that several transcription factors recruit histone-modifying enzymes to genes

they regulate, and other transcription factors even possess histone-modifying properties themselves.

As a matter of fact, this exact modification of chromatin is a function of enhancers. Gene expression enhancer sequences, which may be located several thousand basepairs upstream or downstream from a given gene, or even within the gene itself, often serve as binding sites for proteins that modify chromatin regions (and sometimes interact with elements of gene promoters) or recruit chromatin modifiers. This property to influence chromatin condensation, and thereby control gene expression, makes enhancers effective at a distance and in a direction-independent manner.

Swapping (exchanging) enhancers between different genes can often make the genes inducible under the conditions that the proteins which bind to the given enhancer are expressed or activated by posttranslational modification. Enhancer binding proteins are typical for a given cell type or a given state of cell physiology (8). Control of enhancer status is therefore another step whereby cell phenotype (activity of certain proteins) controls gene expression.

Control of Gene Expression by DNA Methylation

Another level of control for gene expression is provided by DNA methylation (1,8). The carbon number 5 of cytosine can be methylated by some mammalian enzymes (methyltransferases). CpG islands, meaning DNA sequences that contain higher number of 5' CG 3' than the rest of the genome, are usually close to genes. This way gene expression is controlled: higher degree of methylation of CpG islands inhibits access of transcription factors (since C5 is exposed at the major groove) and thereby blocks gene expression. Approximately 70% of CpG islands in mammalian genomes are methylated. Deamination of 5-methylcytosine converts it to thymine; so CpG sequences are subject to mutation to TpG. Natural selection and evolution have, however, maintained high density of CpG sequences between promoter and enhancer regions of regulated genes, to control expression. A tumor-suppressor gene product, E-cadherin, and its undercoat proteins, catenins, which connect cadherins to actin filaments, are located at lateral borders, concentrating on adherens junctions of epithelial cells and establishing firm cell-cell adhesion.

Cell-cell adhesion determines the polarity of cells and participates in the maintenance of the cell societies called tissues (8). Cell-cell adhesiveness is generally reduced in human cancers. Reduced intercellular adhesiveness, results in destruction of histological structure, which is the morphological hallmark of malignant tumors. Reduced intercellular adhesiveness is also indispensable for cancer invasion and metastasis. The E-cadherin cell adhesion system in cancer cells is inactivated by various mechanisms that reflect the morphological and biological characteristics of the tumor. Silencing of the E-cadherin gene by DNA hypermethylation around the promoter region occurs frequently, even in precancerous conditions. Transcriptional inactivation of E-cadherin expression was shown to occur frequently in tumor progression. E-cadherin expression in human cancer cells is regulated by CpG methylation around the promoter region.

Enzymes that Catalyze Conformational Changes of DNA and their Physiological Role

As the two DNA strands are complementary to one another, and DNA synthesis may use both strands as a template for replication, resulting double strands contain one parental and one daughter strand (1).

Enzymes called helicases hydrolyze ATP, opening the DNA double helix briefly for the replication fork to proceed. Hydrogen bonds between bases, which keep the two strands that form the DNA double helix together, are briefly broken. As the replication fork proceeds, DNA supercoil has to be locally unwound. To prevent overwinding in the regions which surround the replication fork, enzymes called topoisomerases introduce supercoils. Certain drugs that kill cancer cells are topoisomerase inhibitors (21).

Other enzymes ensure correction of errors in DNA synthesis (21). Errors arise by incorporation of wrong bases in the newly synthesized DNA strands. As synthesis proceeds with a speed of approximately 2000 bases per second, about two wrong bases are incorporated every second. While one daughter strand is synthesized in a continuous mode, the other is synthesized in a discontinuous fashion. Enzymes called ligases connect the fragments of this strand. As both strands of a DNA helix are copied, the replication fork moves along. The ends of chromosomes, however, are GC-rich linear hairpin structures. These are maintained by an enzyme called telomerase, which uses RNA as a template for DNA synthesis. It thereby belongs to the category of reverse transcriptases.

While DNA content is almost identical for most cells of a given organism (1,8), there are several cell types that either lose their nuclear DNA (red blood cells) or undergo recombination (eg. antibody producing immune cells). Recombination involves cutting and rejoining double strands, and results in the exchange of genetic material between two parental strands. Cells of the germline rearrange their DNA during formation of gametes. In the female reproductive tract more intense recombination takes place than in the male reproductive tract. Another phenomenon that involves recombination is the integration of a virus DNA into a host genome, and transposition, the mobilization of autonomous genetic elements.

Structure of DNA within Genes

Most Eukaryotic Genes are Split into Exons and Introns

A basic principle of heredity is the colinearity of a gene with the gene product it codes for, due to the basic chemical structure of DNA that gives the genome a linear conformation (1). The coding region, however, of most genes of higher eukaryotes, such as birds and mammals, is not continuous, but rather split. It is interrupted by noncoding regions, which, during gene expression are transcribed together with coding regions into RNA as a whole, and subsequently separated from noncoding regions in the process of splicing. Lower eukaryotes, such as yeast, have a much higher proportion of continuous genes. In prokaryotes, split genes are extremely rare. The portion of a gene that is produced after

splicing of RNA contains sequences that direct translation of RNA into protein, and sequences that themselves will be translated, and code for parts of a protein. All the DNA regions that code for parts of the final product of splicing are termed exons. Regions of DNA that code for parts of RNA that are discarded after splicing are termed introns.

DNA sequences of introns may be cut and recombined without loss of function of the exons that are located between introns. This fact allows for a potential mechanism of protein evolution via recombination between introns from different genes.

Large sets of different proteins may be encoded by one single gene: either by initiation of transcription by alternative start sites, or by alternative splicing (which involves omission of different exons from the spliced transcript). Another way of product variation is utilization of alternative translation start sites from a given transcript.

DNA Sequences within, Adjacent to, and in the Vicinity of Genes, Influence Gene Expression

As we have already encountered, promoter sequences are immediately precedent of gene coding sequences in the 5' to 3' direction and control the process of gene transcription. Enhancer sequences, which may lie within genes, or at a distance of up to several thousand basepairs away from genes, influence DNA transcription of genes via control of chromatin accessibility.

Alterations in DNA Content May Lead to Cancer

DNA is a relatively stable macromolecule, which stores hereditary information in living cells (1). Even viruses that carry RNA as genetic material, reversely transcribe RNA information into DNA, once they enter the reactive environment of a living cell. Inside a living cell, a great number of reactive molecules are present, and as RNA contains ribose, which has an additional hydroxyl group, it is significantly chemically less stable than DNA (1). DNA, therefore, being relatively stable and serving as an information storage, facilitates normal function and reaction of a living cell to environmental changes. As we mentioned, gene expression, which requires DNA transcription into RNA, is controlled by proteins (1,3). Concentration of certain proteins, therefore, in relation to DNA quantity of a living cell, determines the subsets of genes the cell will express. Changes in ploidy (chromosome number) and exchange of DNA between different chromosomes, as well as changes in the number of copies of a given gene, may alter expression of the gene(s) directly affected, and of many other genes, whose expression is controlled by the directly affected genes (10,22). Directly affected genes that have the power to cause widespread changes in gene expression code for transcription factors, enzymes that activate them, or intracellular and extracellular signal transducers, which activate such enzymes (10,22).

Complex interplay between gene products, under influence of environment, determines cell phenotype (8). Result of cellular phenotypic alterations determines tissue function and thereby affects organism physiology. If growth signals exceed certain temporal and

quantitative thresholds, any normal cell will react to changes by activation of feedback mechanisms that control cell division. Failure of such mechanisms will activate further pathways of growth arrest. Cumulative failure, combined with persistent signals for cell division, can result in cell death. All this signal network is designed to prevent the evolution of cells that have uncontrolled growth. Such cells have the potential to cause cancer, and therefore give disadvantage to the host organism. It is easy to understand that a combination of defects in key control mechanisms will eventually lead to tumor formation. Due to the number of regulatory mechanisms, such a change is difficult to happen without an irreversible change in cellular DNA content (22), which allows propagation of the regulatory defect in daughter cells, and accumulation of regulatory changes over several cell generations. As the immune system surveillance has the potential to remove cancer cells, a signal that increases proliferation increases the potential for cancer to grow, as sheer number may allow some cancer cells to escape the immune system, by increasing their potential to accumulate regulatory changes and spread faster inside the host organism.

Three major factors therefore contribute to cancer: changes in DNA content (21), signals for cell proliferation (23), and suppression of the immune system. DNA damaging agents can cause cancer both by increasing chances of the evolution of malignant cells, and by death of cells of the immune system, because as normal cells, the latter will die when subject to irreversible DNA damage.

In the following paragraphs we discuss in detail the contribution of changes in DNA content to the evolution of cancer cells.

Conditions that Lead to Qualitative and Quantitative Changes in DNA Content

Total DNA content of a cell may be altered either by changes in chromosome number, by recombination between different chromosomes, by recombination between chromosomes and viral DNA (8,10). The DNA content of a cell may also be altered at the level of the chemical structure. The basic chemical structure of DNA can be altered by reactive chemicals and radiation. DNA damaging chemicals include several environmental carcinogens (24) and lead to alterations in DNA sequence, thereby affecting gene regulation or function of the gene product, when alterations take place within the coding sequence.

The term carcinogen is very broad, and includes substances that have a great number of different effects on the basic chemical structure of DNA (21), including the formation of bulky aromatic-type adducts, alkylation (generally small adducts), oxidation, dimerization, and deamination. Chemical carcinogens may also cause epigenetic changes. A major epigenetic change is alteration in DNA methylation status. This type of change can lead to silencing of expression for specific genes. Carcinogenic DNA adducts vary in their mutagenic potential; the binding of benzo[a]pyrene-7,8-diol 9,10-epoxide to the exocyclic (N2) amino group of deoxyguanosine results in formation of an aromatic adduct that resides within the minor groove of the double helix, typical of polycyclic aromatic hydrocarbons. Although this benzo[a]pyrene-7,8-diol 9,10-epoxide adduct may turn out to be the most common form, by far, of DNA damage induced by benzo[a]pyrene in mammalian systems,

other types of DNA damage are possible, including covalent binding of metabolites to deoxyadenosine. Aromatic amine adducts are more complex, not only because they have both acetylated and nonacetylated metabolic intermediates but also because they form covalent bonds at the C8-, N2-, and sometimes O6-positions of deoxyguanosine as well as deoxyadenosine. The major adducts, however, are C8-deoxyguanosine adducts, which reside predominantly in the major groove of the DNA double helix.

Sites of DNA damage caused by chemical carcinogens are acted upon by DNA repair enzymes (1,25-30). Six major mechanisms of DNA repair are known: direct DNA repair, nucleotide excision repair, base excision repair, double-strand break repair, mismatch repair, and postreplication repair (10). These have been characterized in lower organisms such as yeast and bacteria. However, among recent advances is the cloning of more than 70 human genes involved in five of these DNA repair pathways.

DNA Damage Induced By Alkylating Agents and Thiopurines

Alkylation of DNA can occur at many sites (21), either following the metabolic activation of certain N-nitrosamines or directly by the action of the N-alkylureas (N-methyl-N-nitrosourea) or the N-nitrosoguanidines. The protonated alkyl-functional groups that become available to form lesions in DNA generally attack the following nucleophilic centers: adenine (N1, N3, and N7), cytosine (N3), guanine (N2, O6, and N7), and thymine (O2, N3, and O4). Some of these lesions are known to be repaired (O6-methyldeoxyguanosine), while others are not (N7-methyldeoxyguanosine). Furthermore, O6-methyldeoxyguanosine is a promutagenic lesion, whereas N7-methyldeoxyguanosine is not.

Direct DNA repair is carried out by DNA-alkyltransferases. These enzymes catalyze translocation of the alkyl moiety from an alkylated base (e.g., O6-methyldeoxyguanosine) to a cysteine residue at their active site, in the absence of DNA strand scission. Thus, one molecule of the enzyme is capable of repairing one alkyl lesion in DNA. The human O6-methyldeoxyguanosine DNA-methyltransferase has been mapped to chromosome 10q24.33-qter.

Reactive Oxygen Species and 8-Oxoguanine

Oxy-radical damage can result in the modification of DNA to form thymine glycol or 8-hydroxydeoxyguanosine adducts. Three major pathways have been identified. Exposure to organic peroxides (catechol, hydroquinone, and 4-nitroquinoline-N-oxide) leads to this type of oxyradical damage; however, oxyradicals and hydrogen peroxide can be generated in lipid peroxidation and the catalytic cycling of some enzymes. Cells also can be stimulated to produce peroxisomes by treatment with certain drugs and plasticizers.

The DNA base excision repair is a ubiquitous mechanism for removing damage from the genome induced by spontaneous chemical reaction, reactive oxygen species (ROS) and also DNA damage induced by a variety of environmental genotoxicants.

Another potentially mutagenic source of DNA damage is due to the presence of 5-methylcytosine in the genome. 5-methylcytosine transition from the methylated to the unmethylated state is one of the mechanisms that control gene expression, as we elaborated before. Mutagenic is the deamination of methylated cytosine residues in DNA. 5-methylcytosine comprises approximately 3% of deoxynucleotides. In this case, deamination

at a CpG dinucleotide gives rise to a TpG mismatch. Repair of this lesion most often restores the CpG; however, repair may also cause a mutation (TpA). Deamination of cytosine also can generate a C-to-T transition, if uracil glycosylation and G-T mismatch repair are inefficient. Oxyradicals can enhance the rate of deamination, so the activity of inducible nitric oxide synthase and production of high concentrations of nitric oxide could contribute to DNA damage by this mechanism.

Radiation-Induced DNA Damage

Double-strand DNA breaks may occur from exposure to ionizing radiation and oxidation (31-33). Consequences of double-strand DNA breaks are the inhibition of replication past the break site, the inhibition of transcription of the affected gene, and the loss of heterozygosity in case of a gene whose other allele is defective. Double-strand DNA break repair occurs through homologous recombination (34), joining of the free ends is mediated by a DNA-protein kinase in a process that also protects the ends from nucleolytic attack. The free ends of the DNA then undergo ligation by DNA ligase IV. Genes known to code for DNA-repair enzymes that participate in this process include: xrcc4, xrcc5, xrcc6, xrcc7, hrad51b, hrad52, RPA and ATM.

Between adjacent thymine residues on the same DNA strand, ultraviolet radiation may induce a cyclic dimer product.

Ways DNA Damage Causes Cancer

DNA Damage Increases Mutation Rate

Cell cycle control is coupled to mutagenesis: mechanisms that sense damaged DNA induce signals which culminate in cell cycle arrest, until damaged DNA is repaired. Whenever a defect causes cells to bypass this checkpoint, mutations accumulate and regulation of cell growth is lost. A tumor is likely to follow, and when further mechanisms fail, tumor cells may invade tissues and cause cancer. Mechanisms fail because loss of cell cycle regulation (35) and its connection with DNA repair result in higher mutation rates. Accessible regions of chromatin, which determine functional genes of a given cell type, determine also the subsets of carcinogenic mutations that will have decisive impact in the progression of cells toward malignancy.

The critical cell cycle regulatory timepoint of the transition between G1 and S phases is controlled by a complex network of factors, some of which are ubiquitous, others appear to have an essential function in certain subsets of tissues.

The majority of genes mutated in human cancers encode factors that influence the transition of the cell cycle from the G1 phase to the S phase, which is the phase when DNA replication occurs. The precocious G1/S transition may result in an S phase deficient in critical regulatory factors that the cellular apparatus normally uses to ensure fidelity of DNA replication. Mounting problems generated due to lack of critical regulatory factors in such an S phase can escape detection by the cellular surveillance systems, allowing undeterred mitosis (21).

Cancer is basically thought to consist of two essential phenomena: first, tumor initiation, which is the point when a cell will lose control of cell cycle, and tumor promotion, when stimuli which cause cell proliferation will cause a significant increase in the growth of deregulated (initiated) cells. Cancer formation can be therefore viewed as the result from an imbalance between the mechanisms of cell-cycle control and mutation rates within the genes. Tumor initiation results from irreversible genetic damage. For mutations to accumulate, they must arise in cells that proliferate and survive the lifetime of the organism. A chemical carcinogen causes a genetic error by modification of the molecular structure of DNA that can lead to a mutation during DNA synthesis. Most often, this is brought about by formation of an adduct between the chemical carcinogen or one of its functional groups and a nucleotide in DNA. The number of carcinogen DNA adducts correlates with the number of tumors that the carcinogen causes to a susceptible animal. Tumor initiation by carcinogen adducts is due to activation of proto-oncogenes (conversion to oncogenes) or inactivation of tumor suppressor genes.

In a broad sense, genomic instability could be either manifestation of microsatellite instability, which exhibits a mutator phenotype, or manifestation of chromosome instability, which can be seen as chromosomal abnormalities at a metaphase chromosome spread.

Nucleotide excision repair, base excision repair and mismatch repair (36) are three intracellular mechanisms involved in DNA damage repair that may lead to mutations. Nucleotide excision repair, base excision repair and mismatch repair, when they result in mutations, may occasionally silence a tumor suppressor gene. Inactivation of the other allele, even by epigenetic means such as hypermethylation of the gene promoter, may result in a tumor, as increased cellular proliferation has a higher chance to escape cell cycle arrest or coupling to the cell's own apoptotic apparatus.

Nucleotide excision repair of DNA involves five steps: lesion recognition, preincision, incision, gap-filling, and ligation. Nucleotide excision repair is executed by a multiprotein complex consisting of 16 or more different proteins. Large distortions caused by bulky DNA adducts (e.g., BPDE-dG [7r, 8t-dihydroxy-9t, 10t-epoxy-7,8,9,10-tetrahydrobenzo[alpha] pyrene N2-deoxyguanosine] and AAF-dC [2-acetylaminofluorene deoxycytosine]) are recognized by specific proteins that recognize DNA damage (e.g., XPA [xeroderma pigmentosum A]). Removal of the DNA damage is achieved by the action of the endonucleases (e.g., XPF, XPG and FEN [Flap endonuclease-1]). With the intact strand serving as a template, a patch is constructed by 5' to 3' polymerization, and ligation of the free ends then takes place. Transcription-coupled DNA repair is strand specific: the transcribed strand in a gene is preferentially repaired by comparison to the nontranscribed or DNA coding strand. Nucleotide excision repair is a vital mechanism in humans and lack of this function results in the genetic disease xeroderma pigmentosum (XP). Markedly reduced rates of excision repair are found in individuals with xeroderma pigmentosum, and these individuals are at known risk of ultraviolet light-induced skin cancer. Based on the underlying genetic defect, the disease can be divided into the seven complementation groups XPA through XPG. XPF, in association with ERCC1, constitutes a structure-specific endonuclease that makes an incision 5' to the photodamage. XPF-ERCC1 has also been implicated in both removal of interstrand DNA cross-links and homology-mediated recombination. In addition to the above, XPF-ERCC1 has been implicated in a phenomenon

that is unrelated to DNA repair on the one hand, and very specific in respect to cell type on the other: immunoglobulin class switch recombination.

Base excision repair generally removes a segment of DNA containing a small adduct: small adducts (e.g., 3-methyladenine) are generally the target of base excision repair so that there is overlap with direct repair. Removal of the adducted base is brought about by a glycosylase (e.g., hOgg1 [human 8-oxoguanine DNA N-glycosylase], UDG [uracil DNA glycosylase]). Repair of the damaged strand is accomplished by the combined action of an apurinic endonuclease (e.g., HAP1) that degrades a few bases on the damaged strand and a polymerase that synthesizes a patch in the 5' to 3' direction, using the undamaged strand as a template (e.g., polb). The patch is then ligated by one of a number of ligases (DNA ligases: I, II, IIIa, IIIb, IV).

Excision repair processes occasionally result in DNA mismatches. DNA mismatches involve incorporation of unmodified or conventional, but noncomplementary, Watson-Crick bases opposite each other in the DNA helix. Mismatch repair process acts upon transition mispairs (G-T or A-C) more efficiently than on transversion mispairs (G-G, A-A, G-A, C-C, C-T, and T-T). Probable cause is differential recognition of the mispairings by the repair apparatus. Repair efficiency of mispairings also depends on their oligonucleotide environment for the same reason. Mispairings in the G-C-rich regions are therefore repaired more efficiently than those in the A-T-rich regions. The mechanism for the correction of mispairings is similar to that for nucleotide excision repair and resynthesis described earlier, but it generally involves the excision of large pieces of the DNA containing mispairings. Because the mismatch recognition protein is required to bind simultaneously the mismatch and an unmethylated adenine in a G-A-T-C recognition sequence, it removes the whole intervening DNA sequence. The parental template strand is then used by the polymerase to fill the gap. Relatively common mutations in hPMS1, hPMS2, hMLH1, hMSH2, hMSH3 have been shown to predispose to human hereditary nonpoliposis colon cancer and glioma.

DNA mismatch repair (MMR) serves to avoid replication errors and to prevent mutations. The association of defective MMR with familial and sporadic gastrointestinal and endometrial cancer has been acknowledged for some years. It has relatively recently become apparent that MMR defects are common in acute myeloid leukaemia/myelodysplastic syndrome. Acute myeloid leukaemia/myelodysplastic syndrome may follow successful chemotherapy for a primary malignancy. Therapy-related haematological malignancies are often associated with treatment with alkylating agents. Their frequency is increasing and they now account for at least 10% of all AML cases. There is also evidence for an association between MMR deficient AML/MDS and immunosuppressive treatment with thiopurine drugs. MMR interacts with alkylating agent and thiopurine-induced DNA damage. The human DNA mismatch repair (hMMR) system plays an important role in reducing mutation and maintaining genomic stability. The MMR system in human cells is composed of at least six genes (hMSH2, hMLH1, hMSH3, hPMS1, hPMS2 and GTBP/hMSH6). In particular, hMSH2 and hMLH1 are expressed in cells undergoing rapid renewal; their reduced expression has been reported in several tumors.

Specific DNA repair proteins recognize a damaged template when the apparatus of DNA replication encounters it. Postreplication repair will occur. One mechanism has the DNA polymerase stopping at the replication fork when DNA damage is detected on the parental

strand. Another mechanism has the DNA polymerase advancing past the lesion on the template, leaving a gap in the newly synthesized strand. A repair process ascribed to the RecA protein involves filling the gap by recombination of the homologous parent strand with the daughter strand. This process may lead to recombinational events. In a different repair process, when a single nucleotide gap remains, mammalian DNA polymerases insert an adenine residue. As can be expected, this process may result to base mispairing, when the gap involves a different residue than adenine.

Adduct removal or unscheduled DNA synthesis is measured to define the rate of DNA repair. Such measurements have encountered substantial variations among individuals. An approximately five-fold variation in the rates of excision repair has been found in lymphocytes treated with carcinogens in vitro, among the general population. Markedly reduced rates of excision repair are found in individuals with xeroderma pigmentosum, and these individuals are at known risk of ultraviolet light-induced skin cancer. An association also has been found between the reduced capacity of mononuclear leukocytes in vitro to repair aromatic amine adducts in individuals who have first-degree relatives with cancer. Up to 40-fold variations among humans in the activity of O6-alkylguanine-DNA alkyltransferase have been reported as well. DNA repair rates are inhibited by aldehydes, alkylating agents, and some chemotherapeutic drugs. Decreased DNA repair capacity also has been noted in the fibroblasts of patients with lung cancer, compared with those patients with melanoma or noncancer controls. For benzo[a]pyrene-7,8-diol 9,10-epoxideDNA adducts, a unimodal distribution of repair rates is observed in lymphocytes, but interindividual variation has been found to be substantial.

As ultraviolet radiation can initiate carcinogenesis by means of mutation, it can also supply tumor promotion by activating cell division. Phosphatases can get inactivated by UV, allowing for prolonged activation of kinase enzymes such as JNK, which activates some transcription factors which turn on cyclin D gene expression and thereby facilitate G1/S transition.

DNA Damage Kills Immune Cells

Mutagenic agents may also lead to cancer formation via suppression of the immune system, which normally can remove defective cells before the formation of large tumors. Mechanisms which kill cells that accumulate mutations, may cause also widespread death of immune cells, and leave an organism unprotected from cancer. This is evident in nude mice, which are highly susceptible to tumors (21,22).

We can view an example of a successful strategy for confrontation with tumors in the case of skin cancer. Knowledge of both aforementioned pathways of carcinogenesis is applied to inhibit cancer formation.

How is Knowledge of Cancer Development Due to DNA Damage Applied: Two Anticancer Compounds, Imiquimod and Dimericine

The example of skin cancer prevention and treatment illustrates well two major mechanisms of carcinogenesis. The drug imiquimod, originally prescribed for genital warts, is an effective activator of the immune system of the skin. As sun rays damage a variety of cell types on the skin, imiquimod prevents development of malignancy by activating the immune system which kills tumor cells. Another drug, dimericin, contains an enzyme that breaks thymine dimers, the cyclic product of the sun's ultraviolet radiation on DNA. Dimericin prevents mutations, thereby inhibiting one major factor of sun-induced carcinogenesis.

Gene Aberrations that Lead to Cancer: Generation of Oncogenes and Inactivation of Tumor Suppressor Genes

If alterations in DNA cause cancer, then how does it happen? Deregulation of the cell cycle may happen either by activation of oncogenes, which activate cell growth, or by inactivation of tumor suppressor genes, which cause cell cycle arrest or death (10,21,22). Oncogenes are generated by changes in the structure or function of normal cellular genes called protooncogenes.

Normal Function of Protooncogenes

Protooncogenes are genes which under conditions of overexpression or uncontrolled activity lead to cancer. Protooncogenes usually code for proteins which naturally function as stimulus-responsive switches towards cell proliferation.

Conversion into Oncogenes

A protooncogene may be converted to an oncogene by mutations that alter the levels of expression of the gene product, or by mutations which alter the function of the gene product, abrogating certain restrictions on the activity.

A classic case of a known conversion of a gene into an oncogene is the Finkel Biskis Jinkins murine osteosarcoma gene. The corresponding virus carries this oncogene, which has a cellular counterpart termed c-fos. Another case is the avian myeloblastosis virus gene 17, termed v-jun (from junana, japanese word meaning 17). Its cellular counterpart, c-jun, codes for a protein that forms heterodimers with c-fos protein. These two gene products are expressed rapidly in response to a number of stimuli, and c-jun is posttranslationally modified by phosphorylation which activates it. c-jun heterodimers with c-fos, or c-jun homodimers, may bind to promoters of target genes, as constituents of the transcription factor AP-1 (4-7). AP-1 target sequences are present on the promoter DNA of many genes that are expressed in response to inflammation and a variety of other conditions. AP-1 target genes modulate the cell cycle and influence phenomena related to the development of malignancy.

Substrates of Protooncogenes (and Oncogenes)

Protooncogenes generally code for various signal transducers which transmit signals that activate cell division.

Some protooncogenes code for enzymes that in response to a number of stimuli activate cell growth.

Other protooncogenes code for transcription factors which in response to phosphorylation by growth-stimulus responsive enzymes activate expression of genes which are involved in cell cycle progression and immortalization.

Activators of Protooncogenes

Natural activators of protooncogenes include mitogenic stimuli and downstream effectors thereof. Several protooncogenes serve also as signal pathway components for processes not directly related to cell growth, such as inflammatory events (secretion of cytokines, adhesion factors).

Effects of Protooncogenes on Cell Fate

Activated protooncogenes may contribute to inhibition of cell death and activation of cell proliferation. Aberrantly activated protooncogenes, as is the case by tumor promoters, may thereby contribute to cancer without a mutation on the gene itself. Effects of activated protooncogenes include cell cycle progression beyond checkpoints, relief of repair requirement, loss of attachment, immortality, tissue invasion, and competition of cancer growth against detection and destruction by the immune system.

Definition of Tumor Suppressor Genes

As tumor suppressor genes are termed genes whose loss of both copies of a cell is associated with acquisition of the malignant phenotype.

Inactivation of Tumor Suppressors in Cancer

In cancer, tumor suppressor genes are either inactivated, or the signal networks through which they control cell growth are disabled. Familial cancer syndromes are usually marked by a defect in one copy of a tumor suppressor gene. In somatic cells the other copy may be inactivated either by mutagenic (genotoxic) conditions or by methylation. Absence of both copies of a tumor suppressor gene is conducive to irregular growth of the cell.

Normal Function of Tumor Suppressor Genes

Tumor suppressor genes may be activated by deregulated cell cycle progression and cause cell cycle arrest, cell death, or both. Alternatively, tumor suppressor genes may ensure genomic stability.

Tumor suppressor gene products are proteins that may halt cell cycle, when a cell has acquired irreversible changes that lead to malignancy, or may work to prevent such irreversible changes.

Specific Examples

p53, Rb, BRCA1 are tumor suppressor genes (21,37-41). The function of each one is unique, even though they belong to families of genes with partially overlapping functions. A different category of tumor suppressor genes, includes those that code for proteins involved in specific downstream effects of DNA repair, such as XPA. Those genes have not yet been strongly implicated in core functions of intracellular signal pathways. Mutations in DNA repair genes serve mainly to increase the frequency of tumor initiation, while mutations in genes that regulate the cell cycle result in tumor promotion.

p53

p53 is a well known gene, and has several homologues. There may be a certain degree of partial redundancy between p53 and its homologues termed p73 and p63 (42).

p53 is a gene with many functions. It codes for a protein that is able to repress or activate transcription, depending on the circumstances and the genes observed. From all genes that play a role in the regulation of programmed cell death, p53 has been the most intensively studied. Potential p53 effects include activation of cell cycle arrest, senescence, and differentiation. Most research until now has been focused on the role of p53 in apoptosis of senescent or stressed cells.

p53 itself is positively regulated by the gene ARF and negatively regulated by MDM2. Mice without ARF (ARF knockout mice) develop tumors at a very fast pace. MDM2, on the other hand targets p53 for ubiquitination (and subsequent degradation). p53 activates transcription of MDM2. This way, in the absence of an apoptotic stimulus, p53 levels are kept low. Activation of p53 in response to cellular stress such as DNA damage, oncogene activation, telomere erosion and hypoxia is mediated, at least in part, by inhibition of MDM2 and rapid stabilization of the p53 protein.

Although DNA transcription-independent effects of p53 have been described, the best understood function of p53 is that of a transcription factor (38,43) for genes that mediate DNA damage repair, growth arrest and apoptosis (39-41). Mice or embryonic stem cells in which p53 wild-type protein was substituted by a transcriptionally inactive mutant lost cell cycle arrest and apoptotic functions. On the other hand, the ability of p53 to repress transcription might well explain some of its effects, particularly the induction of apoptosis. The mechanisms and requirements for p53-mediated transcriptional repression are under intense study.

Cell cycle arrest by p53 can be explained by the transcriptional activation of the p21WAF1/CIP1 cyclin-dependent kinase inhibitor. Apoptosis, on the other hand, does not appear to involve one single mechanism. Notably, transformed cells have a greater difficulty to recover from cell cycle arrest than primary cells. Some normal cells (e.g., normal gut epithelium and hematopoietic), however, cannot recover from cell cycle arrest and undergo p53-induced death. Selective inhibition of p53 in such tissues might alleviate some side effects of chemotherapy. Conversely, reactivation of p53 may be used as a way to sensitize some resistant cancer cells to radiation or chemotherapy.

18 different sites in human p53 are reported to be posttranslationally modified. Most are modified in response to genotoxic stress. p53 is modified in three different ways: by phosphorylation, by acetylation, and by sumoylation. Key sites appear to be targeted by more than one signaling enzyme, and there is growing evidence that modifications to several sites

are linked in cascades that may provide signal amplification and integration. One example of p53 regulation is provided by the enzyme JNK (2,37,38). JNK, when inactive, binds to p53 between residues 97 and 116 and targets p53 for proteasomal degradation. When JNK is activated by apoptotic stimuli, it phosphorylates p53 at residue threonine 81 and thereby activates it. It is important to note here that JNK and p53 apoptotic pathways do not overlap extensively. Multiple different pathways for p53 activation exist, and conversely, JNK has multiple proapoptotic substrates. Epigenetic studies have shown that mutating or inactivating putative or established 'tumor-suppressor' proteins that play a role in the DNA damage response, including ATM, ATR, DNA-PK, CHK2, breast-cancer-susceptibility gene-1 (BRCA1) or PKR, can prevent or attenuate the activation of p53 by DNA damage. Key perturbations that are thought to lead to p53 activation include the production of oxidant radicals (i.e. oxidative stress), damaged DNA structures or repair intermediates, and the inhibition of enzymes that affect RNA polymerase II-dependent transcription.

p53 is also essential for the senescence response to a variety of signaling factors. Senescence might have evolved to suppress tumor development. p53 may initiate the senescence response partly through the induction of p21.

Activity at of Tumor Suppressor Genes at Cell Cycle Checkpoints

An entirely different tumor suppressor gene from p53 can be illustrated in the case of BRCA1; germline mutations of this gene are known to confer susceptibility to breast and ovarian cancer in high-risk families (21). BRCA1 as a tumor suppressor plays an important role in maintaining genomic stability. BRCA1 has the ability to interact with numerous proteins and to form complexes that are involved in recognizing and subsequently repairing DNA. BRCA1 contains several functional domains that directly or indirectly interact with a variety of proteins via protein-protein interaction; these include tumor suppressors (BRCA2, p53, Rb and ATM), oncogenes (c-Myc, casein kinase II and E2F), DNA damage repair proteins (RAD50 and RAD51), cell cycle regulators (cyclins and cyclin dependent kinases), transcriptional activators and repressors (RNA polymerase II, RHA, histone deacetylase complex and CtIP), DNA damage-sensing complex and mismatch repair proteins (BRCA1-Associated Surveillance Complex; BASC) and signal transducer and activator of transcription (STAT) among others. Throughout the cell cycle BRCA1 protects genomic DNA. Further effects of BRCA1 include control of the fidelity of DNA replication (to prevent mutations that would be passed on to daughter cells) and control of the separation of sister chromatids during mitosis (to prevent unequal distribution of genetic information to daughter cells). Gene knockout and overexpression models indicate that BRCA1 is an integral component of both of these checkpoints. Elimination of BRCA1 from the mouse genome results in early embryo death at day 78. On the other hand, overexpression of BRCA1 causes cell cycle arrest.

Cell cycle arrest caused by overexpression of BRCA1 requires the presence of p21WAF1 or protein Rb, both proteins that are involved in the G1/S checkpoint. In the case of the G1/S checkpoint, BRCA1 is (in contrast to p53) not required to halt the cell cycle in the case of damage. BRCA1 is apparently necessary for proper repair of damage that may occur during or prior to S phase. Human cancers caused by mutation of BRCA1 may be due to accumulation of mutations in somatic cells that result in a cascade of events which culminate

in uncontrolled cell proliferation. Formation of foci containing BRCA1 by inherited mutations, or epigenetic mechanisms (promoter methylation) in sporadic cancers leads to a loss of DNA repair ability, disrupts the potential to form complexes with other proteins that are crucial for DNA repair pathways. Thus, BRCA1 plays a significant role in maintaining genomic stability and serves as a tumor suppressor in breast cancer tumorigenesis.

Almost exclusive for developing embryos, albeit to a much lower level than full length, a truncated form of BRCA1, lacking the exon 11, is expressed in cells. A transgenic mouse that only expressed this truncated form of BRCA1 gave embryo fibroblasts that senesced much faster than wild-type cells and harbored a plethora of genomic abnormalities. Observed chromosomal alterations could be the result of unequal recombination and breakage, two anomalies that predominantly occur during mitosis. Indeed, these fibroblasts treated with DNA damaging agents failed to enact their G2/M checkpoint, and progressed into mitosis as if there was no chromosomal damage. A possible cause for this increase of chromosomal breakage may be the amplification of centrosomes in mitotic cells. Centrosomes are a prime location for BRCA1 during mitosis. Pulling on chromatids in several directions as opposed to just the polar ends of the dividing cell could certainly have an effect on the state of the chromosomes after anaphase. Lack of full length protein at these complexes could account for the amplification. The overall lack of G2/M checkpoint control pointed clearly at the involvement of the exon 11 region of BRCA1 as an essential factor in qualifying cells for division. Overexpression of the C-terminus of BRCA1 in normal breast epithelial cells has also been shown to adversely affect G2/M checkpoint control.

Rb

The tumor suppressor gene Rb (21) is an important component in the G(1)/S transition and its function is abnormal in most human neoplasms. The RB gene family includes three members: Rb/p105, p107 and RB2/p130. The regulation of all three members of the retinoblastoma family of proteins is complex and unique for each single member. Each member of the RB family of growth suppressive nuclear phosphoproteins elicits G1 growth arrest upon ectopic expression in sensitive cell lines, and their phosphorylation status is regulated in a cell cycle-dependent manner.

The retinoblastoma protein (RB) plays a key role in the control of cell proliferation and mediates the terminal differentiation of certain cell types. Increasing evidence suggests that RB functions by contacting and modifying the behaviour of transcription factors. RB can form complexes with E2F and MyoD in vivo, and complexes with a number of other transcription factors have also been demonstrated in vitro. Following DNA damage, the p53-dependent induction of p21CIP1 regulates cyclin E/Cdk2 and cyclin A/Cdk2 complexes both of which phosphorylate pRB, leading to E2F-mediated activation.

Another effect of Rb concerns RNA polymerase III. RNA polymerase (pol) III synthesizes a range of essential products, including tRNA, 5S rRNA and 7SL RNA, which are required for protein synthesis and trafficking. High rates of pol III transcription are necessary for cells to sustain growth. A wide range of transformed and tumour cell types have been shown to express elevated levels of pol III products. Some transforming agents have been shown to stimulate expression of the pol III-specific transcription factors TFIIIB or TFIIIC2. In addition, TFIIIB is bound and activated by several oncogenic proteins, including

c-Myc. Conversely, TFIIIB interacts in healthy cells with the tumour suppressors RB and p53. Indeed, the ability to limit pol III transcription through TFIIIB may contribute to their growth-suppression capacities. The function of p53 and/or RB is compromised in most if not all transformed cells.

Progress in molecular biology leads to a growing picture of the interaction network between different tumor suppressor genes. Currently p53 and Rb may be the best studied. pRB and p53 are involved in the regulation of the G1/S transition and its checkpoints, to a large extent under the control of the E2F transcription factor family. Following DNA damage, the p53-dependent induction of p21CIP1 regulates cyclin E/Cdk2 and cyclin A/Cdk2 complexes both of which phosphorylate pRB, leading to E2F-mediated activation. Similarly, E2F1-dependent induction of p19ARF antagonizes the ability of mdm2 to degrade p53, leading to p53 stabilization and potentially p53-mediated apoptosis or cell cycle arrest. From the existing mouse models discussed above, we also know that proliferation, cell death and differentiation of distinct tissues are also intimately linked through entrance and exit from the cell cycle, and thus through pRB and p53 pathways. Virtually all human tumors deregulate either the pRB or p53 pathway, and often times both pathways simultaneously, which is critical for crippling cellular defence against neoplasia.

Oncogenes, Tumor Suppressor Genes and Aging

Mammalian aging is partly due to a decline in the restorative capacity of tissue stem cells. Stem/progenitor cells ensure tissue and organism homeostasis and might represent a frequent target of transformation. Although these cells are potentially immortal, their life span is restrained by signalling pathways (p19-p53 and p16-Rb) that are activated by DNA damage (telomere dysfunction, environmental stresses) and lead to senescence or apoptosis. Tissue stem cells are rendered malignant by a small number of oncogenic mutations. Overlapping tumor suppressor mechanisms (e.g., p16(INK4a)-Rb, ARF-p53, and the telomere) have evolved to ward against this possibility. Execution of these antitumor checkpoint pathway programs, however, appears to limit the stem cell life span, and may thereby lead to stem cell depletion. Stem cell depletion can result to organism aging.

DNA Manipulation

At least 2 principal ways of DNA manipulation are available (1,8). The one relies on purified enzymes or chemistry and involves assembly or modification of DNA sequences in vitro (in the test tube). The other is expressing DNA-modifying enzymes inside living cells. DNA modifying enzymes, are expressed under the control of tissue-specific or inducible promoters, and can carry out the same functions that they perform in a test tube. Expressing them into a living cell allows study of the effects that modification of the genome inside a living organism has. A specific gene, for example can be knocked out in selected tissues of a transgenic animal.

In vitro synthesis of DNA can be carried out using template-driven DNA polymerase enzymes which make copies of an existing DNA sequence. DNA can be cut using nucleases, which selectively cut either double-stranded or single-stranded nucleic acid. Exonucleases degrade nucleic acids starting from ends, while endonucleases cleave within the strand. A powerful tool for biology emerged with the discovery of sequence-specific endonucleases, which normally are utilized by bacteria to degrade (restrict) foreign DNA. Sequence-specific endonucleases recognize specific DNA target-sites and cut DNA within the site or at a given distance from it. Some restriction endonucleases cut double stranded DNA in an asymmetric fashion, leaving overhanging ends of few bases.

Within a certain range from neutral pH, DNA has a negative charge, therefore cleaved DNA strands are separated by electrophoresis in agarose gels, and visualized by staining with dyes (e.g. ethidium bromide) which intercalate between the stacked base pairs.

End-modifying enzymes exist, that allow adapting ends of synthesized or cleaved DNA fragments to become substrates for further manipulation. Such manipulation may be carried out by ligases, which may join ends of DNA fragments. Overhanging ends of cleaved DNA may also be filled-in using enzymes such as bacterial DNA polymerase I. Such a reaction allows labeling a DNA fragment using incorporation of radioactively labeled nucleotides, usually carrying phosphor isotopes.

Aims of DNA Manipulation

Aims of DNA manipulation include, but are not limited to, study of the properties of gene products (1,8), generation of proteins with altered properties and study of biophysical aspects of macromolecular interactions (DNA-protein, DNA-RNA, etc).

Mutational Dissection Allows for Study of Gene Products

A number of different parts of any given protein can be modified via modification of the gene that codes for it. Through recombinant DNA technology a gene is modified via the change of one or more amino acid encoding triplets. Any part of a gene can be removed or exchanged with parts from another gene. Such work has as result the generation of a correspondingly modified gene product. This facilitates research into the activities of gene products. Proteins that arise from gene manipulation are highly informative and provide great insight into the function of the gene, because recombinant DNA can be introduced into virtually any organism, allowing a dissection of their function. A gene of an organism can also be manipulated or deleted from the host genome. Lack of the gene, results in an organism deprived of a great part of its function, with the exception of cases that products of other genes are activated to replenish partially or completely the functions of the deleted gene. Input from disease models is also used for gene characterization and analysis, and gives first indications of the role of genes. Gene manipulation and further study of introduced mutations in experimental models helps characterize the role of gene products in cell physiology, and in the case of cancer, regulation of cell growth.

mRNA Can be Reverse-Transcribed into Cdna, Giving Information on Expressed Genes

To clone a gene that is expressed in a mammalian cell, we isolate the RNA that is contained in the cell. RNA includes mRNA, which is the intermediate of gene expression. Through precipitation using polyT sequences (which hybridize the polyA tails of mRNA) one can enrich for mRNA. Then, using the enzyme reverse transcriptase (isolated from retroviruses and commercially available), the mRNA is reverse transcribed into DNA, called cDNA (copyDNA), using polyT primers.

Enzymes That are Utilized to Produce and Propagate Recombinant DNA

The discipline of manipulation of genes through planned DNA sequence modifications is called recombinant DNA technology. The basis of recombinant DNA technology relies on the ability to manipulate DNA molecules in the test tube. This ability for planned DNA manipulation involves utilization of purified enzymes whose activities and substrates are characterized and can be controlled. Purified enzymes can therefore be used to make specified changes to the DNA molecules that are being manipulated. The enzymes available to the molecular biologist mostly originate from bacteria which possess certain identified properties, and can be classified into four broad categories:

DNA polymerases, enzymes that synthesize new polynucleotides complementary to an existing DNA or RNA template.

Nucleases, which degrade DNA molecules by breaking the phosphodiester bonds that link one deoxynucleotide to the next.

Ligases, which join DNA molecules by synthesizing phosphodiester bonds between deoxynucleotides at the ends of two different molecules, or at the two ends of a single molecule.

End-modification enzymes, which make changes to the ends of DNA molecules, adding an important dimension to the design of ligation experiments, and providing one means of labelling DNA molecules with radioactive and other markers.

Results of DNA Manipulation

While mostly used to study genes through the analysis of the properties of their products, recombinant DNA technology has other aims as well. It is used to develop novel treatments for incurable diseases and to address problems of crops and livestock: recombinant proteins that arise from expression of genes with modified DNA are used to convey altered properties to genetically modified organisms, with desirable results and sometimes undesirable side effects.

Progress in recombinant DNA technology has allowed the characterization of the human genome and promises to have an important part to contribute to characterization of the far more complex proteome.

Perspectives from DNA Manipulation

Advances in therapeutic strategies aimed to cure currently untreatable syndromes are expected to arise from the application of recombinant DNA technology (44). Chemotherapeutic drugs for cancer treatment have been traditionally originated by the isolation of natural products from different environmental niches, by chemical synthesis or by a combination of both approaches thus generating semisynthetic drugs. Genetic manipulation of antitumor biosynthetic pathways may offer an alternative in the near future. Novel antitumor derivatives have been generated by targeted gene disruption and heterologous expression of one or few genes in another host, and also by combination of genes that code for components of different, but structurally related biosynthetic pathways.

References

[1] Jeremy M. Berg, John L. Tymoczko, Lubert Stryer, Neil D. Clarke. *Biochemistry*. 2002 Michelle Julet, W. H. Freeman and Company, New York.

[2] Vlahopoulos S and V Zoumpourlis. "JNK": a key modulator of intracellular signaling. *Biochemistry* (Mosc) 2004 Aug; 69(8): 844-54.

[3] Vlahopoulos S, Boldogh I, Casola A, Brasier AR. Nuclear factor-kappaB-dependent induction of interleukin-8 gene expression by tumor necrosis factor alpha: evidence for an antioxidant sensitive activating pathway distinct from nuclear translocation. *Blood* 1999; 94:1878-1889.

[4] Domann FE Jr, Levy JP, Finch JS, Bowden GT. Constitutive AP-1 DNA binding and transactivating ability of malignant but not benign mouse epidermal cells. *Mol Carcinogenesis* 1994; 9:61-66.

[5] Huguier S, Baguet J, Perez S, van Dam H, Castellazzi M. Transcription factor ATF2 cooperates with v-Jun to promote growth factor-independent proliferation in vitro and tumor formation in vivo. *Mol Cell Biol* 1998; 18:7020-7029.

[6] Zoumpourlis V, Papassava P, Linardopoulos S, Gillespie D, Balmain A and Pintzas A. High levels of phosphorylated c-jun, Fra-1, Fra-2 and ATF-2 proteins correlate with malignant phenotypes in the multistage mouse skin carcinogenesis model. *Oncogene* 2000; 19:4011-4021.

[7] Papassava P, Gorgoulis VG, Papaevangeliou D, Vlahopoulos S, van Dam H, and Zoumpourlis V. Overexpression of activating transcription factor-2 is required for tumor growth and progression in mouse skin tumors. Cancer Res 2004 Dec 1; 64(23): 8573-84.

[8] Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell JE. Molecular Cell Biology. 4th ed. 2000, Freeman and Company, New York.

[9] Lee TC, Zhang Y, Schwartz RJ. Bifunctional transcriptional properties of YY1 in regulating muscle actin and c-myc gene expression during myogenesis. *Oncogene* 1994; 9:1047-1052.

[10] Tricot G. New insights into role of microenvironment in multiple myeloma. *Lancet* 2000; 355:248-250.

[11] Rahman I. Regulation of nuclear factor-kappa B, activator protein-1, and glutathione levels by tumor necrosis factor-alpha and dexamethasone in alveolar epithelial cells. *Biochem Pharmacol* 2000; 60:1041-1049.

[12] Boulares AH, Zoltoski AJ, Yakovlev A, Xu M, Smulson ME. Roles of DNA fragmentation factor and poly(ADP-ribose) polymerase in an amplification phase of tumor necrosis factor-induced apoptosis. *J Biol Chem* 2001; 276: 38185-38192.

[13] Posadas I, De Rosa S, Terencio MC, Paya M, Alcaraz MJ. Cacospongionolide B suppresses the expression of inflammatory enzymes and tumour necrosis factor-alpha by inhibiting nuclear factor-kappa B activation. *Brit J Pharmacol* 2003; 138:1571-1579.

[14] Carpenter LR, Moy JN, Roebuck KA. Respiratory syncytial virus and TNFalpha induction of chemokine gene expression involves differential activation of Rel A and NF-kappaB1. *BMC Infect Dis* 2002; 2:art. no. 5.

[15] Katsanakis,K.D., Gorgoulis,V., Papavassiliou,A. and Zoumpourlis,V. The progression in the mouse skin carcinogenesis model correlates with ERK1/2 signaling. *Mol Med* 2002; 8:624-637.

[16] Lo YY, Wong JM, and Cruz TF. Reactive oxygen species mediate cytokine activation of c-Jun NH2-terminal kinases. *J Biol Chem* 1996; 271:15703-15707.

[17] Verrecchia F, Wagner EF and Mauviel A: Distinct involvement of the Jun-N-terminal kinase and NF-{kappa}B pathways in the repression of the human COL1A2 gene by TNF-{alpha}. *EMBO Rep* 2002; 3:1069-1074.

[18] Volloch V, Gabai VL, Rits S, Force T and Sherman MY. HSP72 can protect cells from heat-induced apoptosis by accelerating the inactivation of stress kinase JNK. *Cell Stress Chaperones* 2000; 5:139-147.

[19] Chang HY, Nishitoh H, Yang X, Ichijo H and Baltimore D. Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science* 1998; 281:1860-1863.

[20] Di Mari JF, Mifflin RC, Adegboyega PA, Saada JI, Powell DW. IL-1 alpha-induced COX-2 expression in human intestinal myofibroblasts is dependent on a PKC zeta-ROS pathway. *Gastroenterology* 2003; 124:1855-1865.

[21] Bast RC Jr., Kufe DW, Pollock RE, Weichselbaum RR, Holland JF, Frei E, Gansler TS. Cancer Medicine. 5th ed. 2000 Hamilton, BC Decker, Ontario.

[22] Balmain,A. and Pragnell,I.B. Mouse skin carcinomas induced in vivo by chemical carcinogens have a transforming Harvey-ras oncogene. *Nature* 1983; 303:72-74.

[23] Chramostova K, Vondracek J, Sindlerova L, Vojtesek B, Kozubik A, Machala M. Polycyclic aromatic hydrocarbons modulate cell proliferation in rat hepatic epithelial stem-like WB-F344 cells. *Toxicol Appl Pharmacol* 2004; 196:136-148.

[24] Digweed M. Response to environmental carcinogens in DNA-repair-deficient disorders. *Toxicology* 2003; 193:111-24.

[25] Thompson LH, Schild D. The contribution of homologous recombination in preserving genome integrity in mammalian cells. *Biochimie* 1999; 87-105.

[26] Wood RD, Mitchell M, Sgouros J, Lindahl T. Human DNA Repair Genes. *Science* 2001; 291:1284-1289.

[27] Yang J, Xu Z-P, Huang Y, Hamrick HE, Duerksen-Hughes PJ, Yu YN. ATM and ATR: Sensing DNA damage. *World J Gastroenterol* 2004; 10:155-160.

[28] Christmann M, Tomicic MT, Roos WP, Kaina B. Mechanisms of human DNA repair: an update. *Toxicology* 2003; 193:3-34.

[29] Le Page F, Kwoh EE, Avrutskaya A, Gentil A, Leadon SA, Sarasin A, Cooper PK. Transcription-Coupled Repair of 8-oxoGuanine: Requirement for XPG, TFIIH, and CSB and Implications for Cockayne Syndrome. *Cell* 2000; 101:159-171.

[30] Glasunov AV, Glaser VM, Kapultsevich YG. Two pathways of DNA double-strand break repair in G1 cells of Saccharomyces cerevisiae. *Yeast* 1989; 35:131-139.

[31] Olive PL. The Role of DNA Single- and Double-Strand breaks in Cell Killing by Ionizing Radiation. Radiat Res Suppl 1998; 150:S42-51.

[32] Ward JF. DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. *Progress in Nucleic Acid Research* 1988; 35:95-125.

[33] Frankenberg-Schwager M. Induction, repair and biological relevance of radiation-induced DNA lesions in eukaryotic cells. *Radiat Environ Biophys* 1990; 29:273-292.

[34] Brenneman MA, Weiss AE, Nickoloff JA Chen DJ. XRCC3 is Required for efficient repair of chromosome break by homologous recombination. *DNA Repair Mutat Res* 2000; 459:89-97.

[35] Behrend L, Henderson G, Zwacka RM. Reactive oxygen species in oncogenic transformation. *Biochem Soc T* 2003; 31:1441-1444.

[36] Friedberg EC, Walker GC, Siede W. DNA Repair and Mutagenesis. 1995, ASM Press, Washington, D.C.

[37] Cheng WH, Zheng X, Quimby FR, Roneker CA and Lei XG. Low levels of glutathione peroxidase 1 activity in selenium-deficient mouse liver affect c-Jun N-terminal kinase activation and p53 phosphorylation on Ser-15 in pro-oxidant-induced aponecrosis. *Biochem J* 2003; 370:927-934.

[38] Buschmann T, Potapova O, Bar-Shira A, Ivanov VN, Fuchs SY, Henderson S, Fried VA, Minamoto T, Alarcon-Vargas D, Pincus MR, Gaarde WA, Holbrook NJ, Shiloh Y and Ronai Z: Jun NH2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress. *Mol Cell Biol* 2001; 21:2743-54.

[39] Hupp TR, Lane DP, and Ball KL. Strategies for manipulating the p53 pathway in the treatment of human cancer. *Biochem J* 2000; 352:1–17.

[40] Appella E. Modulation of p53 function in cellular regulation. *Eur J Biochem* 2001;268:2763.

[41] Vousden KH. p53: Death Star. *Cell* 2000; 103:691-694.

[42] Nenutil R, Ceskova P, Coates PJ, Nylander K, Vojtesek B. Differential expression of p73alpha in normal ectocervical epithelium, cervical intraepithelial neoplasia, and invasive squamous cell carcinoma. *Int J Gynecol Pathol* 2003; 22:386-392.

[43] Pospisilova S, Brazda V, Kucharikova K, Luciani MG, Hupp TR, Skladal P, Palecek E, Vojtesek B. Activation of the DNA-binding ability of latent p53 protein by protein kinase C is abolished by protein kinase CK2. *Biochem J* 2004; 378:939-947.

[44] Abraham NG. Therapeutic applications of human heme oxygenase gene transfer and gene therapy. *Curr Pharm Des* 2003; 9:2513-2524.