Instituto Juan March
de Estudios e Investigaciones

68 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

DNA Repair and Genome Instability

Organized by

T. Lindahl and C. Pueyo

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INTRODUCTION

T. Lindahl and C. Pueyo

INTRODUCTION

Tomas Lindahl and Carmen Pueyo

A multitude of endogenous and environmental mutagens cause structural damage to cellular DNA, and DNA replication errors also occur as infrequent chance events. Three different DNA excision-repair pathways (base excision-repair, nucleotide excision-repair, and mismatch repair) provide a first line of defense against such alterations, and these pathways have been largely conserved from microorganisms to man. In recent years, cloning of human cDNAs encoding key repair factors, and their functional expression leading to reconstitution of the repair pathways, have greatly improved our understanding of these cellular defense systems.

The association between defective DNA repair and certain forms of human cancer has stimulated research in this area. Defective nucleotide excision-repair in man is detected clinically as xeroderma pigmentosum, a syndrome associated with greatly elevated levels of skin cancer after sun exposure. Ultraviolet light from the sun is, by far, the most important environmental mutagen (except for chain smokers) and the nucleotide excision-repair system evolved early to counteract ultraviolet-induced DNA damage. The recent reconstitution of the complex human pathway with purified proteins has defined the major steps in the process, but less well understood accessory protein factors may be required for optimal repair. Base excision-repair is primarily employed to remove DNA damage caused by endogenous events, and the human pathway(s) have also been reconstituted with purified proteins.

The recently established correlation between defective mismatch repair and a common form of colon cancer in man resulted in broadened interest in this form of DNA repair, and related problems of gene instability. It is an intriguing possibility that an early cellular event during tumorigenesis might be the generation of a mutator phenotype. An emerging important factor here is the epigenetic methylation of DNA. Tumor cells often have complex altered methylation patterns, and the introduction of new technology such as the bisulphite method for sequencing methylated DNA now allows comprehensive studies of this form of DNA perturbation. It is already known that 5-methylcytosine residues in DNA are hot spots of mutation, as strikingly observed in mutated p53 sequences, because the hydrolytic deamination product of 5-methylcytosine is thymine

- the resulting guanine-thymine base-pairs are corrected only with difficulty by a DNA glycosylase present at very low levels, because the mismatch repair system requires a strand discrimination signal only available in newly replicated DNA.

Transgenic mice are becoming increasingly important for studies of genomic instability, and knockout mice defective in one or the other of most well characterized DNA repair functions are now available. Excellent animal models of different complementation groups of xeroderma pigmentosum, Cockayne's syndrome, and trichothiodystrophy have been constructed. Crosses between different strains to produce double knockouts are carried out in several laboratories, and are further elucidating the various steps and controls of repair events. These projects include the large protein kinases which have been shown recently to be involved in control of growth arrest, ATM being most intensely studied. A return to biochemical experimentation is now required to address key questions such as the identification of the relevant substrates for the ATM and DNA-PK_{cs} protein kinases, and the mechanism(s) by which DNA damage causes an increase in cellular levels of p53.

Rejoining of DNA double-strand breaks occurs largely by non-homologous recombination in mammalian cells, and the factors involved are being characterized. It is important to minimize translocation events, but on the other hand broken DNA requires rejoining. The mechanisms that control and fine-tune such processes will be scrutinized over the next years. Such investigations should also help to clarify special problems such as the preservation of telomere integrity, which might require recombinational repair and telomere binding proteins in addition to telomerase. Unique, highly important biological processes such as processing of antibody genes also may depend on special repair and recombination events. In this regard, recent characterization of V(D)J joining has provided detailed understanding of this process, partly thanks to the availability of a cell line that carries out this form of DNA joining. The fascinating events of hypermutation and class switching still await similar detailed explanation, but key facts about the mechanisms involved are gradually being unravelled.

The Juan March workshop on "DNA repair and genome instability" held on June 9-11, 1997, provided a timely and stimulating review of this major field of cancer research.

Session 1

Chairperson: Errol C. Friedberg

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"Pathways of Mutagenesis and Repair in Bacteria, Humans, and Archaea"

We have used mutators to detect new pathways of mutagenesis and repair. Mutators are cells that have a higher mutation rate than the wild type. Such mutators have been extensively studied in bacteria, and this has led to the elucidation of a number of important DNA repair pathways, as well as revealing new pathways of mutagenesis. Repair defects in humans that lead to mutator phenotypes are responsible for a number of cancer susceptibilities. In some cases, these repair systems are the close counterparts of the equivalent bacterial repair system. Therefore, characterizing bacterial mutators and the repair systems that are deficient can aid in discovering the human homolog of these systems. We described the discovery of the GO sysptem in bacteria and cloning the counterparts from the human genome as well as examining repair pathways revealed by sequencing the 2.2 Mb genome of the hyperthermophilic archaeon Pyrobaculum aerophilum.

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Probing the roles of the *umuDC* gene products in SOS mutagenesis. Graham C. Walker, Angelina Guzzo, Sumati Murli, Bradley Smith, and Timothy Opperman. Massachusetts Institute of Technology, Department of Biology, Cambridge, MA 02140

SOS mutagenesis in *Escherichia coli* requires the participation of a specialized system involving the *umuDC* gene products, the RecA protein, and DNA polymerase III (1). Following SOS induction, the UmuD protein is synthesized and subsequently converted to UmuD', the active form in SOS mutagenesis (2). This activation involves interaction of UmuD with activated RecA resulting in a facilitated autodigestion of UmuD that removes the N-terminal 24 amino acids. Both UmuD and UmuD' form homodimers while the the UmuD•UmuD' heterodimer is more stable than either of the homodimers (3). $UmuD_2$ is preferentially degraded by the Lon protease while the UmuD•UmuD' heterodimer is preferentially degraded by the ClpXP protease (4).

In order to probe the various protein-protein interactions that are relevant to the biological functions of the *umuDC* gene products, we constructed a series of monocysteine derivatives of UmuD and UmuD' and then exploited the unique thiol group in each derivative to obtain structural information (5). Following up on our initial study, an additional set of monocysteine UmuD derivatives, with single cysteine substitutions at positions 30 to 42, was constructed. Based on the observation that these purified UmuD protein derivatives formed disulfide bonds quantitatively upon addition of iodine and yet were poorly susceptible to reaction with iodoacetate we suggested that the pairs of residues at positions 37 and 38 are located at the $UmuD_2$ homodimer interface (6). In the crystal structure of the $UmuD'_2$ homodimer reported by Peat *et al.* (7), the region of UmuD' corresponding to positions 30-42 of intact UmuD forms an extended amino terminal tail that is hypothesized to be involved in interdimer interactions, not $UmuD'_2$ homodimer interface interactions. We hypothesized that our $UmuD_2$ crosslinking data might be due to differences in the quaternary structures of the $UmuD_2$ and $UmuD'_2$ homodimers. To test this hypothesis, the corresponding UmuD' monocysteine derivatives were created and their solvent accessibility, and ability to be oxidized into disulfide bonds following exposure to iodine was tested. In contrast to $UmuD_2$, a cysteine located at amino acids 37 or 38 in $UmuD'_2$ was accessible to iodoacetate and did not readily form disulfide bonds after addition of iodine. Conversely, a cysteine at position 57 in $UmuD_2$ did not form disulfide bonds well and was readily reactive to iodoacetate whereas in $UmuD'_2$ the corresponding cysteine formed disulfide bonds well and was readily reactive to iodoacetate. These results strongly suggest that substantial differences exist between the quaternary structures of the $UmuD_2$ and $UmuD'_2$ homodimers.

This conclusion is particularly interesting in light of our discovery of a novel function for intact UmuD and UmuC that is distinct from their role in SOS mutagenesis. We have obtained evidence that these proteins regulate cell growth and division in response to DNA damage. This activity increases DNA damage tolerance in *E. coli* by allowing time for DNA repair to be completed before damaged cells attempt to grow and divide, ensuring that DNA replication and chromosome segregation are completed with high fidelity. Several aspects of the regulation and activity of these proteins are similar to the effector molecules of DNA damage checkpoint control mechanisms in eukaryotic cells.

This novel function of UmuD and UmuC was suggested by our analyses of UmuDC-mediated cold sensitivity. This phenomenon is caused by constitutive expression of the *umuDC* operon from a multicopy plasmid, which results in growth inhibition at 30°C (8). We have demonstrated that UmuDC-mediated cold sensitivity is genetically distinct from SOS mutagenesis (9). In addition, we have found that high levels of intact UmuD and UmuC specifically inhibit cell division by a novel mechanism that is SulA-independent (9), inhibits DNA synthesis at 30°C [(10); Murli *et al.*, manuscript in preparation], and inhibits the ability of the cells to make the transition from stationary phase (quiescence) to rapid growth [Murli *et al.*, manuscript in preparation]. These results suggested that overexpression of UmuD and UmuC results in the exaggeration of a normal activity of these proteins in the regulation of cell division and growth.

We have shown that this novel activity of intact UmuD and UmuC increases DNA damage tolerance. *ΔumuDC* mutations increase sensitivity to UV (11) which has been attributed to the loss of the translesion synthesis activity of UmuD' and UmuC. In a genetic background that abolishes RecA*-mediated cleavage of UmuD (*recA430*), we have shown that intact UmuD and UmuC increase UV resistance. We have also demonstrated that intact UmuD and UmuC are involved in regulating the rate of DNA replication in response to DNA damage. In *E. coli*, exposure to UV results in a rapid inhibition in the rate of DNA synthesis followed by recovery, a process known as induced replisome reactivation (IRR) (12). We have shown that intact UmuD and UmuC regulate the kinetics of recovery in the rate of DNA synthesis after UV. A mutation in *umuC* (*umuC125*) has been isolated that does not cause cold sensitivity for growth, but is proficient in SOS mutagenesis (10). In addition, this mutation causes increased UV sensitivity (10). Interestingly, UmuD in combination with UmuC125 abolishes the inhibition of DNA synthesis after UV, suggesting that increased UV sensitivity produced by the *umuC125* mutation is due to the inability to regulate DNA synthesis after UV.

Another mechanism by which the *umuDC* gene products regulate growth in *E. coli* in response to DNA damage was suggested by the finding that the inhibition of growth at 30°C by high levels of the *umuDC* gene products is highly dependent of the growth phase of the culture. In particular, UmuDC-mediated cold sensitivity is specific for the growth phase transition from lag phase into exponential growth. The *fis* gene product (Fis) is known to promote this growth phase transition after a stationary phase culture experiences a nutrient upshift (13). *Δfis* mutations greatly exacerbate UmuDC-mediated cold sensitivity suggesting that the *umuDC* gene products inhibit growth by counteracting the activity of Fis. Physiologically relevant levels of intact UmuD and UmuC inhibit the transition into exponential growth by UV-irradiated stationary phase and exponentially growing cultures, presumably by counteracting Fis activity. Interestingly, the UV sensitivity of *ΔumuDC* mutants is suppressed by *Δfis* mutations, while *Δfis* mutations are not UV sensitive. This suggests that the UV sensitivity of *ΔumuDC* mutants is due to unregulated Fis activity.

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DNA Methylation, Mutation and Cancer. P.A. Jones, J-M. Zingg, M.L. Gonzalgo, A.S. Yang, J.C. Shen, and C. Schmütte, USC/Norris Comprehensive Cancer Center, 1441 Eastlake Ave., Los Angeles, CA 90033.

DNA methylation is essential for mammalian development yet adds a substantial mutagenic load to organisms which methylate their DNA. The inherent mutagenic activity of 5-methylcytosine is thought to be due to the hydrolytic deamination of 5-methylcytosine in DNA, resulting in the formation of T:G mismatches which are inherently more difficult to recognize and repair than U:G mismatches formed by the deamination of cytosine in DNA. Methylation has probably led to the 80% suppression of the CpG methyl acceptor site in mammals and contributes strongly to the generation of polymorphisms and germ-line mutations. It has also become apparent that 5-methylcytosine participates in the generation of transition mutations that inactivate tumor suppressor genes in various types of human cancer. Analysis of the mutational spectra in tumors shows that the frequency of deamination mutations varies considerably with tumor type and with the causative agent in many types of cancer. For example, approximately 50% percent of all inactivating mutations in the p53 tumor suppressor gene in colon cancer occur at methylation sites whereas only about 15% of the mutations sustained in lung cancer are due to this mechanism. We have investigated alternative methods by which methylation may contribute to the formation of these mutations and have shown that the DNA methyltransferase enzyme itself is capable of directly inducing cytosine deamination and may contribute to this mutagenic pathway in certain circumstances. We have also shown that inhibitors of DNA methylation particularly sinefungin can markedly increase the rate of deamination of cytosine, providing an additional pathway to mutagenesis at methylation sites. We have extended our analysis of the role of methylation inducing mutations in the p53 tumor suppressor gene to the p16 tumor suppressor gene which is frequently inactivated in a wide variety of human cancers. The p16 gene is different than the p53 tumor suppressor gene in that the coding sequences of the gene have the properties of CpG islands and hence are protected from methylation in the germ-line and in somatic tissues. Interestingly, we have found that abnormal methylation events which occur in tumor cells result in the methylation of the coding sequence of the tumor suppressor gene and this may increase the rate of mutagenesis at CpG sites in somatic cells. This is the first example of the abnormal methylation of a CpG island leading to an increased rate of mutagenesis by this endogenous pathway to the formation of transition mutations.

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DNA Repair and Mutagenic Specificity. C. Pueyo. Dpto. de Bioquímica y Biología Molecular. Avda de Medina Azahara s/n. Universidad de Córdoba. Córdoba.

O^6-alkylguanine-DNA alkyltransferases are repair proteins that remove alkyl groups from DNA, mainly from the O^6-position of guanine. During the process, the alkyl group is covalently bound to the protein and so the reaction is stoichiometric and autoinactivating, further repair requiring *de novo* synthesis of more ATase. *E. coli* has two ATases: the products of the *ogt* gene (constitutively expressed) and the *ada* gene (inducible). Ogt is the predominant ATase in wild type, non-adapted bacteria. By monitoring the influence of the inactivation of the *ogt* gene on the lethal and mutagenic effects induced by the bifunctional alkylating drug CCNU (Chloroethyl-Cyclohexyl-NitrosoUrea), we have shown that, of the two bacterial ATases, only the *ogt*-encoded protein has a protective effect. The mechanism of Ogt protection against CCNU mutagenesis and lethality is likely to be the removal of the chloroethyl group from the O^6-position of guanine. By quantifying the induction of mutations and lethal events in *ogt*$^-$ *ada*$^-$ double mutant cells transformed with an exogenous bacterial ATase gene or mammalian cDNA, we have also shown that the recombinant human ATase is close to the bacterial Ogt protein in suppressing the deleterious actions of CCNU. None of the other ATases (rAT, Ada and Tada) were as effective as these two proteins, though they all were expressed at a high level in the corresponding strains. The nucleotide excision repair, which is performed in *E. coli*, by the UvrABC system, shows a synergistic effect with the Ogt ATase in protecting cells against CCNU. The results obtained with CCNU were extensive to the monofunctional alkylating compounds ENU (EthylNitrosoUrea), PNU (PropylNitrosoUrea) and BNU (ButylNitrosoUrea). Near 2,000 independent forward mutations were sequenced for the analysis of the mutational spectra induced by CCNU, and MNU, ENU, PNU and BNU in repair-proficient and repair-deficient genetic backgrounds. DNA repair by Ogt alkyltransferase and (A)BC excinuclease plays an essential role in removing bulky alkyl lesions responsible for G:C→A:T transitions, influencing their ultimate distribution with respect to DNA sequence context and, in some cases, the strandedness of mutagenesis. These preferences were not seen in the case of smaller methyl damages. By means of the traditional shuttle-vector approach and by carrying out parallel studies with mammalian and bacterial cells, we have also shown important similarities between the CCNU-induced mutational spectra recovered in both types of cells. The spectra showed a strong bias towards the untranscribed strand and a clear dependency on dose, which could be attributed to NER and ATase repair, respectively. Interestingly, we have recently showed that bacterial and mammalian ATases greatly sensitise (instead to protect) *E. coli* to both the toxic and mutagenic effects induced by carcinogenic dibromoalkanes. This sensitization has also been observed in ATase-defective human cells transfected with an expression vector containing the human cDNA. The effect, that is not shared by other proteins with redox-active Cys residues, might be related with the postulated glutathione-dependent activation pathway of dibromoalkanes.

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Session 2

Chairperson: Alan Lehmann

Reconstitution of the branched pathway of base excision-repair with human enzymes

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DNA base damage by spontaneous hydrolysis or oxidation contributes to endogenous genomic instability. Altered bases are usually excised by a DNA glycosylase. We (and others) have recently cloned and expressed cDNAs that encode two distinct human DNA glycosylases which remove 8-oxoG and thymine glycol, two major forms of oxidative base damage.

The major form of base excision-repair in mammalian cells involves a DNA glycosylase, AP endonuclease, DNA polymerase b, and the DNA ligase III/XRCC1 heterodimer. A single nucleotide is replaced in this reaction, which has been reconstituted with purified enzymes. An alternative pathway for the latter steps of the reaction, seen in particular after incision by the AP endonuclease at an oxidized or reduced AP site, employs replication factors such as DNase IV(FEN1) and PCNA, and may use DNA polymerase d/e and DNA ligase I instead of Polb and Lig III/XRCC1.

The distinct human DNA ligase IV has a counterpart in S. cerevisiae. This enzyme does not appear to be involved in DNA excision-repair but is required for repair of double-strand breaks by an illegitimate end-joining pathway.

Y. Kubota, R.A. Nash, A. Klungland, P. Schär, D.E. Barnes and T. Lindahl
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R.A. Nash, K.W. Caldecott, D.E. Barnes and T. Lindahl.
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EMBO J. **16**, 3341-3348 (1997)

T. Roldan-Arjona, Y.-F. Wei, K.C. Carter, A. Klungland, C. Anselmino, R.-P. Wang, M. Augustus and T. Lindahl
Molecular cloning and functional expression of a human cDNA encoding the antimutator enzyme 8-hydroxyguanine-DNA glycosylase.
Proc. Natl. Acad. Sci. USA **94**, in press

DNA repair and genome instability: Session 2.

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BIOCHEMISTRY OF G/T MISMATCH REPAIR IN HUMAN CELLS.

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The G/T mispair in mammalian DNA is addressed by two distinct repair pathways. If it arose as a polymerase error, then it will be corrected either to a G/C or to an A/T, the repair event being directed to the newly-synthesized strand. If, on the other hand, the G/T mispair is the result of the hydrolytic deamination of 5-methylcytosine, then it must be corrected back to a G/C and this process is initiated by TDG, a thymine-specific mismatch DNA-glycosylase. What do we know about these two repair pathways that share a common substrate?

The common cancer predisposition syndrome, hereditary non-polyposis colon cancer (HNPCC) has been linked to a deficiency in the postreplicative mismatch correction. Six human genes found to encode mismatch repair proteins have been identified to date, but only four, hMSH2, hMLH1, hPMS1, and hPMS2, have been shown to be mutated in the germlines of HNPCC kindreds and their mutations been shown to co-segregate with the disease. Moreover, the mutations in two of these genes, hMSH2 and hMLH1, segregate with around 70% of HNPCC kindreds, while only three kindreds (around 5%) with mutations in the hPMS1 and hPMS2 loci and none with mutations in the hMSH3 or GTBP loci have been reported so far. The biochemical reasons underlying this phenomenon remain unclear. It is likely that several key components of the mismatch repair process have eluded detection, as these six genes encode homologs of only two of the key bacterial mismatch repair proteins, MutS and MutL. The mechanism of strand discrimination remains obscure, as does the identity of the obligate exonucleases, helicases, DNA polymerase(s), and other ancillary factors. Although the mutations in two more proteins, PCNA and FEN1, have been shown to bring about mutator phenotypes, their link to HNPCC remains to be established. We are studying the biochemistry of the postreplicative mismatch repair process, with the hope of being able to identify at least some of the missing components of this complex pathway.

Many cancers display C to T mutations in CpG dinucleotides in the p53 tumour suppressor gene. These arise presumably by the deamination of 5-methylcytosine. We have shown that G/T mispairs associated with this hydrolytic event are corrected by base-excision repair process, which is initiated by a thymine-specific mismatch DNA-glycosylase, TDG. The *TDG* gene has been mapped to chromosome 12q24, but so far no cancer-associated mutations have been detected in this locus. Are the C to T transitions in p53 really due to the malfunction of TDG or are other factors at play?

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Nucleotide excision repair of DNA by proteins from human cells

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A versatile strategy for repairing damaged DNA, termed nucleotide excision repair (NER), is found throughout the natural world in organisms ranging from mycoplasma to mammals. In humans, NER is a major defence against the carcinogenic effects of ultraviolet light from the sun, and also repairs DNA damage caused by some chemotherapeutic agents such as cisplatin (1). The complete mechanism involves more than 25 polypeptides, and we and our collaborators have been studying the process in cell-free systems. Repair of UV-damaged or cisplatin-damaged DNA has been reconstituted with purified proteins (2). The XPA protein and the single-stranded DNA binding protein RPA interact and bind preferentially to damaged base pairs. An opened DNA structure is then created around the site of damage (3), in an ATP-dependent reaction that involves TFIIH, a dual function repair/transcription factor that contains the XPB and XPD DNA helicases. The single-stranded binding protein XPC and possibly other factors are also in the open complex. Incisions are then introduced on either side of a damaged site, by "structure-specific" endonucleases that cleave near junctions of duplex and single-stranded DNA (4). XPG nuclease cleaves on the 3' side of the open region and then ERCC1-XPF nuclease cleaves on the 5' side, to release an oligonucleotide usually 24-32 residues long (5). A repair patch is formed by a DNA polymerase ε or δ holoenzyme that includes the sliding clamp factors PCNA and RFC, and the gap is sealed by a DNA ligase, resulting in a repair patch of about 30 nucleotides (6). Additional factors participate in NER by modulating the activity of these core components, for example by phosphorylation (7).

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MANY MUTATIONS IN CANCER

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Based on the high frequencies of chromosomal abnormalities and mutations in human cancers, we offered the hypothesis that cancer is manifested by a mutator phenotype (1). This hypothesis is based on the argument that the spontaneous mutation rate in normal cells is insufficient to account for the high frequency of mutations in human cancer cells. We argue that the normal background mutation rate of 1.4×10^{-10} can account for only two or three mutations in each tumor, and not the much larger number that are reported, or the even greater numbers that are likely to be found as methods for detection become more sensitive. The recent demonstrations of microsatellite instability has provided the first strong evidence for a mutator phenotype (2). Relaxed genomic stability could be initiated by mutations in genes involved in DNA replication, DNA repair, or chromosomal segregation. Interference with processes that cause these mutations provides the possibility of preventing the clinical occurrence of cancer by delay.

As an example of genetic instability we will consider Werner Syndrome (WS). It is an uncommon inherited human disease manifested by premature aging (3), genomic instability (4), and a high incidence of unusual cancers (5). The recently cloned WS gene encodes a predicted 1,432 amino acid protein with homology to the RecQ family of DNA helicases. To investigate its catalytic activity, we overexpressed the WS protein as an N-terminal hexahistidine fusion from a recombinant baculovirus construct. We purified the protein from infected insect cells by Ni^{++}-chelation chromatography. The wild type protein displays ATP-dependent, 3'->5' strand displacement activity with DNA substrates and is stimulated by single-strand binding protein. It also exhibits DNA-dependent ATPase activity. Both activities are lacking in a mutant protein (K577M) with a substitution in a highly conserved helicase motif I. The production of this active DNA helicase should facilitate studies on its function in DNA metabolism and the effects of mutants on genetic instability in Werner Syndrome.

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TRACEY BARRET

Structure and mechanism of a G-U mismatch Specific uracil-glycosylase from *E. coli*

The deamination of cytosine and 5-methyl cytosine can result in the generation of guanine-uracil/guanine-thymine (G:U/G:T) mismatched base pairs respectively. Both mismatches are pro-mutagenic leading to adenine-thymine transition mutants if not repaired. In general mismatches in both prokaryotes and eukaryotes are repaired in a nucleotide excision pathway, however, a base excision repair pathway has also been identified in a limited number of organisms. This pathway is initiated by a specific DNA N-glycosylase, however, unlike the well characterised uracil DNA glycosylases (UDG's) will remove uracil (and in the human homologue thymine) from mismatched base-pairs in double stranded DNA only. In addition, this family of DNA glycosylases shows no sequence homology with the UDG's.

The crystal structure of a G:U mismatch double strand specific DNA N-glycosylase (MUG) from E-coli has recently been solved by the multiple isomorphous replacement method to a resolution of 1.8A. The overall topology of the molecule is a Rossman fold, however, there are a number of interesting deviations from this classical architecture. The latest model has an R-factor of 23% (R_{free}=29%) and is undergoing further refinement. A crystal complex has also been obtained with MUG and a DNA fragment consisting of 12 base-pairs. Although this structure is at a fairly preliminary stage, it has so far been possible to identify various groups within the protein that have a key function in DNA recognition, binding and specificity.

Session 3

Chairperson: Jan H. J. Hoeijmakers

THE USE OF MUTANT MICE FOR STUDIES ON DNA REPAIR AND ITS RELATIONSHIP TO CANCER.

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Humans suffering from the hereditary disease xeroderma pigmentosum (XP) are defective in nucleotide excision repair (NER) of DNA. Consistent with the predicted role of this DNA repair mode in mitigating against the mutagenic effects of ultraviolet (UV) radiation, XP individuals are exceptionally prone to skin cancer following exposure to sunlight. It is anticipated that such individuals are also predisposed to cancers in other organs that are typically subject to exposure to environmental chemicals. However, such information is anecdotal since very few XP individuals are available for detailed studies of this type. In order to address this and other aspects of the molecular pathogenesis of cancer in NER-defective mammals we have constructed mouse strains defective in the *XPC* gene, using targeted gene replacement by homologous recombination.

Crosses between XPC heterozygous ($XPC^{+/-}$) animals yielded normal Mendelian segregation of wild-type and mutant *XPC* alleles. Additionally $XPC^{-/-}$ mice develop normally and show no evidence of clinically detectable phenotypes. However, at the cellular level $XPC^{-/-}$ mice are abnormally sensitive to killing by UV radiation at 254 nm and are deficient in NER. Additionally, as has been observed with human *XPC* cells, mouse $XPC^{-/-}$ cells are defective in the excision of 6-4 photoproducts from the non-transcribed strand of the transcriptionally active *p53* gene, but remain proficient in the repair of these lesions from the transcribed strand. $XPC^{-/-}$ mice are highly predisposed to skin cancer induced by UV radiation. Following weekly exposure of mice to UVB radiation of the shaved dorsal skin for 22 weeks, 50% of $XPC^{-/-}$ animals developed skin cancers by ~16 weeks. In contrast, no skin cancers were observed in $XPC^{+/-}$ or $XPC^{+/+}$ animals irradiated for 22 weeks and monitored for a further 7 weeks.

In order to explore possible effects on cancer predisposition of simultaneous genetic defects in NER and in other cellular processes that protect the integrity of the genome in the face of DNA damage, we bred *XPC* mice to mice carrying mutations in the tumor suppressor gene *p53*. $XPC^{-/-}$ $p53^{+/-}$ mice show a greater skin cancer predisposition than that observed in $XPC^{-/-}$ $p53^{+/+}$ animals. We have not detected loss of heterozygosity of the second *p53* allele in skin tumors derived from $XPC^{-/-}$ $p53^{+/-}$ mice by Southern analysis. We are presently sequencing the entire coding region of the *p53* gene in such tumors.

Skin cancer predisposition is further heightened in animals that are homozygous mutant for both genes ($XPC^{-/-}$ $p53^{-/-}$). The latter animals show a 50% cancer incidence by ~9 weeks. Additionally, such animals show a slightly enhanced predisposition to spontaneous testicular teratocarcinomas typically observed in young male $XPC^{+/+}$ $p53^{-/-}$ mice. Of the remaining genotypes, $XPC^{+/+}$ $p53^{+/-}$ animals only begin to show evidence of skin cancer after 22 weeks of radiation exposure. However, double heterozygous animals ($XPC^{+/-}$ $p53^{+/-}$) have an incidence that is intermediate between that of $XPC^{+/+}$ $p53^{+/-}$ and $XPC^{-/-}$ $p53^{+/-}$ animals. This is the first phenotype of which we are aware involving XP heterozygote mammals.

We also have an interest in exploring cancer predisposition in animals carrying genetic defects in more than one DNA repair pathway. To this end we have attempted to generate mutants

defective in the *HAP1* (human AP endonuclease) gene required for base excision repair (BER). Homozygous knock-out mutations are lethal in early embryonic development. However, we obtained unexpected results when we incorporated the *HAP1* heterozygous genotype into various *XPC*, *p53* mutant strains. $XPC^{-/-}$ $HAP1^{+/-}$ animals have a radiation-induced skin cancer incidence curve that is indistinguishable from that observed in $XPC^{-/-}$ $p53^{+/-}$ mice after 15 weeks of irradiation. These curves strictly overlap with those obtained with double heterozygous ($XPC^{-/-}$ $p53^{+/-}$ $HAP1^{+/-}$) mice. $XPC^{+/+}$ $HAP1^{+/-}$ and $XPC^{+/-}$ $HAP1^{+/-}$ mice are indistinguishable from $XPC^{+/+}$ $HAP1^{+/+}$ animals after 15 weeks of irradiation. These observations suggest that inactivation of the *HAP1* and *p53* genes synergize with defective NER by the same mechanism(s). Recent studies by Tom Curran and Carol Prives and their colleagues (*Genes and Devel.* **11:** 558-570, 1997) have provided *in vitro* evidence that HAP1 protein activates p53 protein by a redox-dependent pathway and (to an even greater extent) by a redox-independent pathway.

DNA repair of UV and X-ray induced damage: molecular and clinical aspects.

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To ensure genetic stability all organisms have evolved an intricate network of complementary DNA repair systems, that are capable of removing virtually any type of DNA damage induced by endogenous or exogenous genotoxic agents. Examples of complex multi-step repair pathways are: nucleotide and base excision repair (for removal of a wide variety of lesions), homology dependent and independent recombination repair (for repair of double strand breaks) and post-replication 'repair' (for translesion DNA synthesis). Defects in DNA repair result in hypersensitivity to genotoxins and carcinogenesis. The best studied and most versatile repair process is nucleotide excision repair (NER). The NER pathway eliminates UV- and many chemically-induced DNA lesions. The reaction has been reconstituted *in vitro* with purified components and most genes implicated have been isolated (for recent reviews see Hoeijmakers, *Eur.J.Cancer* 30A, 1912-1924, 1994; Wood, *Ann.Rev.Biochem.* 65, 135-167, 1996).

Inborn defects in NER are associated with three clinically and genetically heterogeneous human syndromes, all characterized by marked sun sensitivity: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). The UV-exposed skin of XP patients shows pigmentation abnormalities and an over 1000x-increased risk of cancer caused by defects in one of at least 7 genes (XPA to XPG). CS displays severe developmental impairment and neurodysfunction due to dysmyelination. Two responsible genes have been identified: CSA and CSB. TTD is characterized by brittle hair and nails due to a shortage of sulphur-rich matrix proteins. The clinical picture also comprises ichthyosis and many symptoms characteristic of CS. Interestingly, the NER-deficient form of TTD is caused by mutations in the XPB, XPD and TTDA genes encoding subunits of TFIIH, a protein complex with a dual function in basal transcription in addition to NER. Remarkably, in contrast to XP, there are no indications for an increased risk of cancer in CS and TTD. Finally, a rare class of patients exists showing a combined XP+CS picture. Such patients carry defects in XPB, XPD or XPG.

The NER reaction entails damage recognition involving XPA and other proteins, dual incision of the injured strand carried out by XPG and the XPF/ERCC1 complex presumably preceded by a local unwinding of the two strands by the TFIIH complex containing the XPB and XPD helicases, removal of the damage-containing oligonucleotide, repair synthesis and ligation. At least two NER subpathways exist: rapid transcription-coupled repair limited to the transcribed strand of active genes and less efficient global genome repair. TTD and most XP complementation groups carry defects that affect both subpathways, CS is specifically deficient in transcription-coupled repair, whereas XP-C is impaired in the global genome repair.

To facilitate purification and functional characterisation of the intricate TFIIH complex and to identify associated factors we have epitope-tagged various subunits. The tags used do not interfere with complex formation and function and permit an efficient two-step, mild purification of TFIIH to homogeneity from cells stably expressing the tagged subunit. The isolated complex consists of 9 subunits, is active in *in vitro* NER and transcription and exhibits all of the established TFIIH enzymatic activities. Presently we are analyzing the effect of mutations in TFIIH on its functions. The specific link of mutations in TFIIH

components with transcription initiation provides a plausible explanation for the non-XP symptoms associated with CS/TTD. Crippling of the TFIIH transcription function may affect the expression of a set of genes causing neuro-developmental CS abnormalities. Mutations that affect the stability of the complex could give rise to the brittle hair and nails of TTD (Hoeijmakers et al., *Curr.Opin.Genet.Developm.* 6, 26-33, 1996). To investigate the complex genotype-phenotype relation associated with TFIIH mutations we have mimicked in the mouse germline a single amino acid substitution in XPD as found in a TTD patient. The TTD mouse reflects to a remarkable degree the phenotype of the human disorder. Importantly, the mice display cancer predisposition suggesting that also patients may have a hitherto unnoticed increased risk of developing cancer.

Similarly, we have generated stable transfectants expressing epitope-tagged, functional CSB and found that this protein resides in a complex with active RNA polymerase II, but not with transcription initiation factors such as TBP, TFIIE, TFIIH and TFIIB. This suggests that CSB has a role in the transcription process and that the non-XP part of the CS features like in the case of TFIIH are due to a transcription-related deficiency. This is in agreement with observations made using a CSB mouse model (van der Horst et al., *Cell*, 89, 425-435, 1997, see also van Gool et al., *EMBO J.*, in press). Also CSB mice exhibit a marked cancer predisposition, whereas a dramatic worsening of the CS features are observed when CSB mice are crossed with XPC mice carrying a defect in the global genome NER subpathway.

One to the mechanisms for the repair of X-ray induced double strand breaks involves homology-dependent recombination repair. To assess the biological impact of this system in mammals we have isolated homologs of several yeast genes involved in this process, including the mouse homolog of RAD54 (mRAD54). mRAD54-/- ES cells exhibit X-ray sensitivity and reduced homologous recombination. mRAD54-/- mice are viable, fertile and fail to display abnormalities in V(D)J recombination and class switching (Essers et al., *Cell*, 89, 195-204, 1997). Further analysis of these and other repair-deficient mouse mutants is in progress.

Molecular analysis of human disorders with defects in DNA repair and transcription

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Xeroderma pigmentosum (XP), Cockayne Syndrome (CS) and Trichothiodystrophy (TTD) are genetic disorders with widely different clinical features, despite the association in all three with defects in nucleotide excision repair. 85% of CS patients are defective in the *CSB* gene (1). We have determined the sites of the mutations in eighteen CS-B patients. The mutations are spread across the *CSB* gene. Many result in drastically truncated products, demonstrating that the gene is not essential for life. Those mutations resulting in amino acid substitutions are confined to the C-terminal two thirds of the protein, raising the possibility that the N-terminal third may not be required for its repair function. The types and sites of the mutations do not correlate with the severity of the disorder, indicating that some other factor contributes to the clinical features.

The XPD protein is a subunit of the TFIIH complex, which has a dual function both in nucleotide excision repair of DNA damage and in basal transcription. Mutations in the *XPD* gene can result in three distinct clinical phenotypes, XP, TTD, and XP with CS. In order to determine if the clinical phenotype can be attributed to the site of the mutation, we have identified the mutations in a large group of TTD and XP-D patients (2-5 and unpublished results). The results are complex because many of the patients are compound heterozygotes. By using fission yeast to investigate the effects of the individual alleles in the haploid state, we have shown that several of the mutations found in patients are lethal. If we eliminate the lethal mutations, the remaining mutagenic pattern is consistent with the site of the mutation determining the phenotype. We have found that 85% of XP-D patients are mutated at the same site in the gene, and that mutations at four other sites account for the mutations in most of the TTD patients. Our results are consistent with the hypothesis that XP results from mutations affecting the repair function of TFIIH without affecting the transcriptional activity, whereas TTD mutations subtly affect the transcriptional activity.

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Negative and Positive Controls of Genetic Diversity

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Evolution is Life's strategy of survival based on the generation of genetic diversity which is there to face uncertain future, and is thus the substrate for selection. Diversity is created by mutations. We have studied the mechanisms and the genetic controls of mutagenesis in bacteria. The results suggest that the mutation rates are subject to control systems both at the level of individual cells and at the level of populations. The former involve a lowering of the efficiency of the constitutive error correction mechanisms and the induction of error-prone systems such as the mutagenic SOS response. The latter involves the variations in the frequency of mutator mutants within the population of cells and/or organisms. Under certain conditions, the contributions of mutators in creating successive rare adaptive mutations can be so important that the mutators can take over the entire population in spite of their massive deleterious genetic effects. These strategies may be important in the understanding of the powerful adaptability of bacteria to changing environments (e.g., to new hosts, or new compartments within the old hosts, and to new antibiotics) accounting thus for the emergence of new pathogens. The same formal analysis may apply to tumour development and account for the accumulation of multiple mutations in the invasive tumours.

We are also exploring the origins of two biological clocks: the universal evolutionary clock accounting for similar time-related germ line mutation frequencies and the somatic clock accounting for the species-specific kinetics of the incidence of disease and death, i.e., life span. The available results in bacterial experimental systems suggest that the accumulation of oxidative damage to guanine (8-oxo-G) in the DNA, RNA, and their respective nucleotide pools, may account for the somatic biological clock.

Experimental results with aging bacterial colonies show that the starving and degenerating cells undergo massive load of errors at the level of DNA (mutations), RNA and protein biosynthesis due to the accumulation of 8-oxo-G mediated mistakes. Such mistakes are largely suppressed by the hydrolysis of 8-oxo-dGTP and 8-oxo-rGTP (nucleotide pool cleaning) catalysed by the MutT protein of *Escherichia coli*. If similar situation occurs in non-dividing somatic cells, such as neurons and heart muscle cells, then the MutT acivity may be among the most imporant factors determining their functional longevity.

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THE FIRST ORF OF THE L1Tc RETROTRANSPOSON OF T. CRUZI CODES FOR A PROTEIN WITH APURINIC-APYRIMIDINIC ACTIVITY.

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The deduced aminoacid sequence of the ORF1 of the L1Tc non site specific non-LTR retronstransposon of *T. cruzi* exhibits a significant homology to the consensus sequence of the class II family of the endonuclease AP proteins. The analysis of the activity of the 40 Kd recombinant protein, named NL1Tc, obtained from the expression of the L1Tc ORF1 in an *E. coli* "in vitro" expression system, revealed that the sequence codes for a protein with apurinic-apyrimidinic (AP) endonuclease activity. The NL1Tc protein was shown to make single-strand breaks at partially depurinated supercoiled plasmids. Data are also presented showing that in vivo expression of the NL1Tc protein conferred viability by complementation to *E. coli* exonuclease III deletion mutants (BW286 strain). We propose that the biological role of the AP endonuclease activity of the NL1Tc protein may be to introduce into the DNA free 3' ends that could be used as primers for the integration, along the *T. cruzi* genome, of the L1Tc element and that the nicking could be a general mechanism for the retrotransposition of non site-specific non-LTR retrotransposons.

Genome Instability and Position Effect of Translocations Involving the Inactive X-chromosome

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Gene inactivation through spreading of X-chromosome inactivation might be a cause of genome instability with important consequences for cell viability and cancer. We have analyzed *de novo* induced translocations between the inactive X-chromosome (Xi) and autosomes to study maintenance and spreading of X-inactivation with respect to the position of the XIST gene (a master candidate for controlling X inactivation[1,2]) and thus to gain some insight into the mechanism of X-inactivation in differentiated somatic cells. Translocations involving the X-chromosome were detected by means of fluorescence *in situ* hybridization (FISH) with X-chromosome specific painting probes. The activation status of the chromosomes involved in the translocation was determined by simultaneous immunocytogenetics with antibodies against either acetylated histone H4 (cytogenetic marker of gene expression[3,4]) or BrdU incorporated at late S-phase (cytogenetic marker of the late-replicating Xi). Xq13 band carrying the XIC and XIST gene was localized by computer-assisted generation of the DAPI banding pattern. Alternatively, the position in *cis* or *trans* of the XIST gene in the reciprocal products of the translocation was determined by simultaneous XIST gene specific FISH and computer enhancing. Our study in differentiated somatic cells provides visual demonstration that (i) the X-inactivation is not spread to the translocated autosomes irrespective of the position the XIST gene, (ii) the XIST gene in *cis* is not required for the maintenance of X-inactivation, (iii) the XIST gene in *cis* is not sufficient to induce X-inactivation, (iv) the maintenance of X-inactivation is an entirely epigenetically controlled process, and (v) X-inactivation is highly stable throughout several cell cycles.

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Session 4

Chairperson: Miroslav Radman

MUTATIONAL ANALYSIS OF THE COMPONENTS OF DNA-PK

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DNA-dependent protein kinase (DNA-PK) is a protein complex comprising a DNA binding component, Ku, and a catalytic component, DNA-PKcs. The Ku protein is a heterodimer composed of a 70 and 80 kDa subunit, but has no obvious domains or motifs. DNA-PKcs is a member of the phosphatidylinositol (PI) 3-kinase superfamily with a PI 3-kinase domain at its carboxy-terminus. Mutants defective in any of these subunits are sensitive to ionising radiation and defective in ability to carry out V(D)J recombination. To help identify functional domains within the components of DNA-PK we have analysed Ku80 and DNA-PKcs defective mutants at the biochemical and molecular level, and have initiated a structure/function analysis of Ku80. Mutations in three Ku80 mutants have been identified. Two are mutations at splice sites resulting in large deletions in the cRNA, and a third mutation results in an amino-acid change in a conserved residue which abolishes the ability of the protein to interact with Ku70. Site-directed mutagenesis within Ku80 has led to the identification of sequences important for the interaction with the Ku70 subunit. Ku80 truncated at the N-terminus by seven amino-acids is able to complement a Ku80 defective mutant but larger deletions fail to interact with Ku70 and therefore lack function. In contrast, deletions in the C-terminus are functional in their ability to complement Ku80 defective mutants. Three mutants defective in DNA-PKcs (the *scid* cell line, irs-20 and V-3 cells) have been characterised at the molecular and biochemical level. Mutations in DNA-PKcs have been identified and the results show that the extreme carboxy-terminus of DNA-PKcs is important for kinase activity but not for the ability of the protein to bind to DNA.

Cell lines from patients with impaired immune responses have been analysed for radiosensitivity and a number of sensitive lines have been characterised. Overlaps between immune deficiency and radiosensitivity will be discussed.

THE DETECTION AND REPAIR OF DNA DOUBLE-STRAND BREAKS.

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DNA-dependent protein kinase (DNA-PK) must bind to DNA double-strand breaks (DSBs) to be active and we and others have shown that it is a multiprotein complex comprising a ~465 kDa catalytic subunit (DNA-PK_{CS}) and a DNA binding component, Ku (e.g. 1,2). Ku is itself comprised of two tightly-associated polypeptides of approximately 70 kDa and 80 kDa (Ku70 and Ku80, respectively). Notably, cells defective in DNA-PK are hypersensitive to ionising radiation due to a DNA DSB repair defect (3,4). Thus, X-ray sensitive hamster *xrs-6* cells are mutated in Ku (e.g. 5,6), and rodent V3 cells and cells of the severe combined immune-deficient (Scid) mouse lack functional DNA-PK_{CS} (7,8). The Scid phenotype arises through an inability to generate mature antibody and T-cell receptor genes by V(D)J recombination. DNA-PK is thus a crucial component of the DNA DSB repair and V(D)J recombination systems. Some of our current work is directed towards learning the precise roles of Ku, DNA-PK_{CS}, and DNA-PK_{CS} relatives in DNA repair and DNA damage recognition in mammalian systems. In this regard, we are defining functional domains of DNA-PK_{CS} and the Ku polypeptides, identifying factors that interact with DNA-PK components, and are detecting physiological substrates for this enzyme.

In addition, we are studying the functions of Ku homologues in yeast. Notably, this work has lead us to demonstrate a crucial role for yeast Ku70 and yeast Ku80 (Yku70p and Yku80p, respectively) in DNA non-homologous end-joining (NHEJ; 9,10). In addition, we and others have discovered that abrogation of yeast Ku function leads to dramatic telomeric shortening, revealing an important role for Ku in telomere homeostasis (10,11). Furthermore, we have recently analysed several other proteins that function in the Ku-dependent NHEJ pathway, which include a yeast homologue of mammalian DNA ligase IV. The results of our studies on these factors will be discussed.

By cloning the DNA-PK_{CS} cDNA (12), we discovered that it falls into the phosphatidylinositol (PI) 3-kinase family of proteins and is most similar to a subgroup of these proteins that function in cell cycle control and DNA repair (13). These include the product of the *ATM* gene, mutations in which lead to the human neurodegenerative and cancer predisposition syndrome ataxia-telangiectasia (A-T). Given its sequence similarity to DNA-PKcs, we speculate that ATM may function in DNA damage detection by mechanisms akin to the detection of DNA DSBs by DNA-PK. With this in mind, we have recently raised antisera against ATM and have used these to characterise its distribution and sub-cellular localisation, and to analyse its expression in A-T patients (14). In addition, we have purified ATM to essential homogeneity and have initiated its biochemical characterisation. The results of these studies will be presented.

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MODULATION OF P53-MEDIATED GROWTH ARREST AND APOPTOSIS

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A variety of cellular responses to DNA damage influence cellular fate, such as whether heritable genetic alterations are passed on to daughter cells and whether the cell survives the damaging insult. Efficiency of repair of the damage and the timing of this repair relative to critical cellular processes, such as DNA replication or chromosome segregation, are two important parameters dictating cellular outcome. P53, the most commonly mutated gene in human cancers characterized to date, is a critical participant in cellular responses to various cytotoxic stresses and induction of p53 can result in either cell cycle arrest or in apoptotic cell death. It is currently thought that many cells mutate p53 during tumor progression so that certain signals which usually result in programmed cell death (apoptosis) will be eliminated and tumor growth will be enhanced. Loss of p53 function also appears to enhance certain types of genetic instability and has been linked to enhanced aneuploidy, gene amplifications and recombination events. Thus, loss of p53 function may be selected for during tumorigenesis to reduce programmed cell death, but p53 mutations may then contribute further to tumor progression by the additional mechanism of enhanced genetic instability. Since loss of p53 function impairs certain apoptotic signalling mechanisms, the selection for mutations in p53 during tumorigenesis can result in some tumors being inherently resistant to chemotherapy- or radiation therapy-induced apoptosis.

DNA damage-induced DNA strand breaks appear to initiate the p53-dependent signal transduction pathway. Recent publications have also suggested that hypoxia and alterations in ribonucleotide pools can induce p53 in the absence of detectable DNA strand breaks. The increases in p53 protein levels after DNA damage appear to result from a combination of increased translation of p53 mRNA and increased half-life of the protein. We have recently identified a specific alteration in the phosphorylation of p53 protein which is introduced by ionizing irradiation and are currently trying to elucidate the functional significance of this phosphorylation change in p53. The gene defective in Ataxia-telangiectasia, ATM, influences the kinetics of p53 induction after ionizing irradiation and studies are underway to characterize the mechanism by which ATM alters p53 induction after irradiation. Once p53 protein levels increase, a number of genes get transactivated, including GADD45, MDM2, $p21^{WAF1/CIP1}$, and cyclin G. Induction of p21 appears to be a critical mediator of the G1 cell cycle arrest after DNA damage.

Since most of our cancer therapeutic agents are DNA damaging agents, they are capable of inducing p53 and intitiating growth arrest or apoptotic cell death. Understanding the cellular determinants of these physiologic end-points could have a significant impact on being able to selectively enhance tumor cell death in patients. We are thus trying to elucidate the molecular determinants of these endpoints. We recently demonstrated that certain cytokines can determine whether a cell undergoes apoptosis or cell cycle arrest after DNA damage and are currently investigating the biochemical/genetic determinants of this decision fork. Both hematopoietic and solid tumors illustrate the anti-apoptotic nature of many growth factors. It appears that many growth factor signals provide survival signals, not only for normal growth in culture, but also following cellular stresses such as DNA damage. Identification of a common survival signal might provide a

reasonable target for further enhancing the selective killing of tumor cells with cytotoxic therapies. It is possible that the main reason we can cure lymphomas and germ cell tumors with current cytotoxic regimens, but are not so successful with carcinomas of the colon, breast, lung, etc., may relate to the ability of the former group of tumor cells to undergo rapid apoptosis following exposure to the cytotoxic drugs we currently use in the clinic. It is conceivable that concurrent blockade of constitutive survival signals in epithelial tumors at the time of cytotoxic exposure will provide a novel way to enhance cure rates.

Another approach to enhancing tumor cell kill with currently available therapies is to modulate other cellular responses to DNA damage, such as DNA repair or DNA lesion processing. The gene which is mutated in the rare, cancer-prone disorder, ataxia-telangiectasia, apppears to be an important determinant of radiosensitivity. Recent studies of the functional domains of the ATM protein have identified two domains within the protein which could serve as potential targets for inhibition to enhance the radiosensitivity of tumor cells. These studies of the functional domains of ATM also demonstrated that the radiosensitivity and chromosomal instability of AT cells is not due to abnormalities in either the G1 or G2 checkpoints, but rather appears to result from difficulties in repair or processing the DNA lesions introduced by ionizing irradiation.

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SPONTANEOUS AND INDUCED RECOMBINATION BETWEEN LONG DNA REPEATS IN *S. cerevisiae* AND ITS CONNECTION WITH TRANSCRIPTION

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DNA repeats are ubiquitous in the genome of eukaryotes and serve as substrates for recombinational DNA repair in mitosis. As a consequence, DNA repeats may be an important source of genomic instability. In this work we analyzed the different recombinational repair mechanisms that can lead to deletions between long direct repeats. We present genetic evidence that homologous recombination between direct repeats leading to deletions can be initiated in the non-homologous DNA region located between the repeats. We also provide genetic and molecular evidence that transcriptional activity is an important source of genomic instability associated to recombination between repeats.

The yeast *HPR1* gene plays an important role in genome stability as indicated by the observation that *hpr1* mutants have high frequencies of DNA repeat recombination and chromosome loss. Based on studies on this gene we suggest a novel and specific mechanism of induction of recombination by trancription. Mutations in the *SRB2* gene encoding a component of the RNA polII mediator completely suppress hyper-recombination in *hpr1Δ*. *HRS1/PGD1*, identified as another full suppressor of the hyper-recombination phenotype of *hpr1* has positive and negative roles in transcriptional regulation. Many of the transcriptional phenotypes associated to *hrs1Δ* have also been described for mutants in *GAL11*, *SIN4* or *RGR1*, which encode proteins of the RNA polII mediator. Reciprocally, *gal11Δ* and *sin4Δ* mutants suppress the hyper-rec phenotype of *hpr1* mutants. These results indicate that hyper-recombination in *hpr1Δ* is linked to transcription.

Finally, we show that hyper-recombination and chromosome instability in *hpr1Δ* cells is caused by blockage of transcriptional elongation. Molecular analysis of different direct-repeat constructs reveals that deletions induced by *hpr1Δ* are specific of repeat constructs in which transcription initiating at an external promoter traverses particular regions of the DNA flanked by the repeats. Transcription becomes *HPR1*-dependent when elongating through such regions. Both, the induction of deletions and the *HPR1*-dependence of transcription, are abolished when a strong terminator is used to prevent transcription to proceed through the DNA region flanked by the repeats. Therefore, our work provides evidence for a new source of genomic instability, associated to transcriptional elongation.

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Session 5

Chairperson: Andrés Aguilera

EARLY AND LATE STEPS IN V(D)J RECOMBINATION. Martin Gellert, Dale A. Ramsden, Tanya T. Paull, Dik C. van Gent, J. Fraser McBlane, and Kevin Hiom. Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, Maryland 20892-0540.

The RAG1 and RAG2 proteins act together to initiate V(D)J recombination by making a DNA double-strand break between each recombination signal sequence (RSS) and the adjacent coding sequence. The break generates a blunt end on the RSS and a hairpin end on the coding DNA. The purified proteins first cooperate to bind the RSS in a "stable cleavage complex" that is highly resistant to competitor DNA. If the pair of RSSs needed for recombination is present, the RAG proteins apparently remain bound to both signal ends and coding ends even after cleavage. The reactions leading to cleavage and hairpin formation are shown to have chemical similarities to the reactions of phage Mu transposase and HIV integrase, as revealed by a stereochemical test and other evidence. Binding and cleavage can be greatly stimulated by the ubiquitous HMG1 or HMG2 proteins, which bind DNA non-specifically and introduce a sharp bend.

Later steps of recombination then share many factors with the normal pathway for repairing double-strand breaks in DNA. Our recent work has focused on setting up complete V(D)J recombination in a cell-free system. Incubation of a DNA substrate with the RAG proteins, followed by a second incubation with a HeLa cell fraction, leads to the formation of both coding joints and signal joints. The continued presence of the RAG proteins after cleavage is absolutely required to make coding joints, but inhibits signal joints. Coding joints often contain self-complementary "P nucleotide" tracts that arise from the asymmetric opening of hairpin ends. There is a strong preference for joining at sites of short DNA homologies; the addition of human DNA ligase I (but not other ligases) leads to a more diverse set of junctions, similar to those found in vivo.

This system may allow the identification of individual factors required for joining.

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Hypermutation of antibody genes

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During the course of immunization to a specific antigen, the affinity of the antibodies produced steadily increases, a process known as affinity maturation (1). Affinity maturation is driven by Darwinian selection of somatic mutants of antibody producing B-cells, depending on antigen for their proliferation and survival, generated by specific hypermutation. Indeed the mutation rate is estimated to be in the order of 10^{-3} per base pair per generation. Hypermutation is short lived and takes place at a very specific stage of B-cell development . The hypermutation target area extends from ~135 bases downstream of the promoter, and gradually decays after ~1 kb (reviewed in 2). The 5'hypermutation boundary is independent of the DNA sequence, but defined by the distance from the initiation of transcription (Rada and Milstein, in preparation).

We have derived transgenic mice containing an artificial kappa chain gene capable of hypermutating at approximately the same rate as the endogenous counterpart (3). Modified transgenes allowed analysis of cis elements required for hypermutation. The transgenes contain a constant segment (CK) of rat origin, so that the derived antibodies can be easily distinguished from the endogenous products. The intrinsic hypermutation process is not random but targets preferential short DNA stretches and changes in such stretches had dramatic effect on mutation rates (4, 5). Specific sequences seem to have been selected during evolutionary time to target for hypermutation hot spots in the antigen binding segments(6).

As with "transcription coupled repair", there seems to be a broad correlation between hypermutation and gene expression. Not only removal of enhancer elements or flanking sequences required for full expression are also required for full hypermutation, but the fraction of cells that best expressed the transgene, were more mutated than the poorly expressed counterpart. Most interesting, mutation in transgenes carrying regulatory element deletions was manifested by an increased proportion of B cells in which the transgene had not been targeted at all rather in the extent of mutation accumulation once

targeted. This leads us to propose a connection between transcription initiation and ***clonal*** recruitement of hypermutation, with hypermutation being more fastidious than transcription in requiring the full complement of regulatory elements (7).

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Chromosomal translocations involved in tumour development; mechanisms, models and genes

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Chromosomal abnormalities, particularly translocations, are consistent features of leukaemic cells and these mutations are thought to be involved in tumour aetiology. The cloning of the breakpoints of many chromosomal translocations has been achieved. This has allowed the identification of novel oncogenes, which encode proteins aberrantly expressed in the tumour cells and leading to the appearance of overt disease. Detailed analysis of the translocation-activated oncogenes has revealed two notable features [1].

[1] Gene activation

Oncogenes activated in lymphoid tumours, by association with immunoglobulin or T cell receptor genes, are usually placed under new transcriptional controls which lead to abnormal patterns of expression (ie. mRNA synthesis). These chromosomal translocations typically do not alter the structure of the gene after the translocation, but rather alter their expression.

In T cell acute leukaemias, translocations often involve breakage within the T cell receptor α or β chain genes on one chromosome, and the activation of a gene encoding a transcription factors on the other. Studies of the function of these proteins indicate that protein-protein interaction is an important feature of their normal and leukaemic functions. As a paradigm, we have studied the *LMO2* gene, which encodes a LIM-only protein, in the T-ALL-associated t(11;14)(p13;q11); some features of the translocation which activates this gene and the role of the *LMO2* gene in leukaemia and haematopoiesis will be discussed [2-4].

[2] Gene fusion

An alternative to gene activation by chromosomal translocation is that the translocation breakpoints occur within a gene on each of the two involved chromosomes leading to a fusion of the two genes, which in turn causes a fusion protein to be made.

To study the influence of protein fusions on tumour formation, experimental models are required which mimic, as nearly as possible, the tumorigenic process after the chromosomal translocation has occurred. As a first step, we have used homologous recombination to introduce a gene fusion into the *MLL* gene in the germ-line of mice [5]. The consequence of the fusion will be described.

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The role of telomeres in the proliferative lifespan of human cells.

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Telomeres are essential DNA-protein structures that protect eukaryotic chromosomes from degradation and fusion and ensure their mitotic stability. In nearly all eukaryotes, telomeres consist of arrays of short G-rich DNA (TTAGGG in vertebrates) complexed with sequence-specific DNA binding proteins. Thus, formation of the complex and hence telomere function have stringent DNA sequence requirements. Telomeric DNA is synthesized de novo by telomerase, a ribonucleoprotein that utilizes a region of its integral RNA molecule as a template for synthesis. This process compensates for the incomplete replication of chromosome termini by DNA polymerases and contributes to the maintenance of telomere length. Human somatic cells have a limited proliferative capacity and are inherently resistant to immortalization. Telomerase is not expressed in most normal cells and, consequently, telomeric DNA is lost and telomeres shorten with each round of DNA replication. Erosion of telomeres has been proposed as the molecular process that monitors and ultimately prevents somatic cell proliferation. Conversely, telomere maintenance may be required for the unlimited proliferation of malignant cells (1).

In previous studies with transformed cells (2) we have shown that transformation does not activate telomerase or arrest the loss of telomeric DNA, and that critically short telomeres correlate with chromosome instability and with proliferative crisis of transformed populations. However, in immortal cells surviving crisis, telomeres become stable and telomerase is expressed, compatible with a requirement for telomere repair by this enzyme for the unlimited proliferation of these cells. We have further documented the presence of short but stable telomeres and telomerase activity in malignant cells in vivo (3, 4).

Mutations of the template domain of the structural RNA of telomerase can reverse the immortal phenotype of unicellular eukaryotes (5). We have used this approach to investigate whether mutant telomerase affects the proliferative potential and viability of immortal human cells. Plasmids encoding mutant or wild type template RNA (hTR) of human telomerase were transfected into fibrosarcoma HT-1080 cells to generate stable transformants. Expression of mutant hTR led to production of mutant telomerase activity and to incorporation of mutant sequences into telomeres. This in turn correlated with impaired cell growth and viability. Our results are compatible with loss of cell survival due to formation of aberrant and non functional DNA/protein telomere complexes.

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DNA replication proteins with functions in DNA repair and DNA recombination

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Before a cell divides into two identical daughter cells the entire genome has to be replicated faithfully. The mechanistic events of this complex macromolecular process are called DNA replication and their details have become more clear in the last years. DNA synthesis at the advancing replication fork requires the coordinated action of different enzymes which appear to be in a high ordered structure to ensure the precise and rapid duplication of more than 10^9 base pairs in mammalian cells. Inaccurate replication would result in mutation while incomplete replication would lead to chromosome breakage upon division.

DNA polymerases (pols) are the main actors for polymerization of deoxyribonucleoside 5'-monophosphates during DNA replication. Five different pols are known and called α, β, γ, δ, and ε and three thereof, namely pol α, δ, and ε are involved in DNA replication.

In preparation for DNA replication the DNA has to become single-stranded to serve as a template for the three replicative pols. It is this form of the DNA that is especially prone to damage of any kind. Nature has provided a set of proteins that support the replicative pols in performing processive, accurate and rapid DNA synthesis and they are called replication accessory proteins. Furthermore these proteins prevent damage to the transient single-stranded DNA. DNA replication accessory proteins provide particular functions that are mandatory for replicative pols. Such functions include the recruitment of particular pols when needed, the facilitation of pol binding to the primer terminus, the increase in pol processivity, the prevention of non-productive binding of the pol to the DNA, the release of the pol after DNA synthesis and the bridging of pol interactions with other replication proteins. Thus it is not surprising that these auxiliary proteins are universally found in nature. Two proteins, proliferating cell nuclear antigen (PCNA) and replication factor C (RF-C) build the so called moving platform. The former has a function as a mobile clamp and the latter is a molecular matchmaker. Many of these proteins, such as pol δ, pol ε, RF-C, PCNA and replication protein A have not only roles in DNA replication *per se*, but in addition also in DNA repair and recombination.

By using a complementation assay for a RF-C dependent pol activity on a singly-primed DNA template, we have isolated from calf thymus a multiprotein complex active in DNA replication. This complex contains pol α/primase, pol δ and RF-C. It is functionally active in replication of primed and unprimed single-stranded DNA templates. RF-C apparently mediates the interaction of pol δ in the complex with PCNA, through an ATP-dependent mechanism. This interaction appears to stabilize the binding of the complex to a template-primer and to coordinate the activity of pol α/primase and pol δ during replication. Our data suggest the existence of an asymmetric pol complex in mammalian cells.

Finally, we have cloned and functionally expressed many replication proteins (e.g. the two pol δ subunits, PCNA, RF-C subunits, replication protein A) so

that enough proteins are available to perform protein-protein and protein-DNA interaction studies as well as site-directed mutagenesis.

Six own recent publications on this topic:

G. Maga and **U. Hübscher**:
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Biol. Chem., in press, 1997

POSTERS

The Mfd protein of *Bacillus subtilis* 168 is involved in both transcription-coupled DNA repair and DNA recombination.

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Inactivation of *Bacillus subtilis mfd* in an otherwise Rec$^+$ strain reduced genetic exchange and DNA repair. When the mutation was transferred into a set of recombination- and repair-deficient strains, the DNA repair and recombination ability of the double or triple mutant strains was drastically reduced. *In vivo* analysis of UV-induced mutations suggests that *Bsu*Mfd is necessary for strand-specific DNA repair, as is the case for the *Escherichia coli* Mfd (*Eco*Mfd) protein. *B. subtilis* Mfd protein shares substantial homology with the *E. coli* Mfd, RecG and UvrB proteins. The purified *Bsu*Mfd protein has a native molecular mass of 140 kDa (expected molecular mass 133 kDa). The *Bsu*Mfd protein showed to be a sequence-independent DNA binding protein with weak ATPase activity. The *Bsu*Mfd protein was able to displace *in vitro B. subtilis* or *E. coli* RNA polymerase stalled at a lesion. The dissociation of the stalled RNA polymerase from the template DNA leads to the release of the truncated RNA from the stalled elongation complex.

Therefore, *Bsu*Mfd protein appears to target the transcribed strand for repair by recognizing a stalled RNA polymerase and dissociating it from the DNA. In addition, the strong recombination deficient phenotype of *mfd*$^-$ *rec*$^-$ strains suggests that *Bsu*Mfd protein is also involved in homologous DNA recombination.

THE EFFECTS OF SIMPLE REPEATING $d(GA{\cdot}TC)_n$ AND $d(CA{\cdot}TG)_n$ DNA SEQUENCES ON RECOMBINATION.

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Simple repeating $d(GA{\cdot}TC)_n$ and $d(CA{\cdot}TG)_n$ DNA sequences are fairly abundant in eukaryotic genomic DNA, being frequently located at "hot-spots" for genetic recombination. In this paper, the effects of these two microsatellite DNA sequences on DNA recombination has been evaluated. SV40 vectors carrying one or two copies of the microsatellite DNA sequence flanking a 2.2 kb insertion of plasmid DNA were constructed. These oversized (7.5 kb in length) SV40 vectors cannot give rise to plaque-forming viruses unless they are reduced to a packageable size by DNA recombination events resulting in the deletion of most of the inserted plasmid DNA. Therefore, determination of the relative number of plaques of lysis obtained from each construct will constitute an estimate of the effect on DNA recombination of the inserted microsatellite DNA sequence. Our results indicate that $d(GA{\cdot}TC)_n$ sequences have a positive effect on recombination. A 10-fold increase on the number of plaques of lysis was observed in the presence of two copies of a $d(GA{\cdot}TC)_{22}$ sequence. On the other hand, a $d(CA{\cdot}TG)_{30}$ sequence showed only a moderate effect (2-3 fold). The genomic organization of the infectious viral particles was determined by restriction endonuclease and sequence analysis. In the case of vectors carrying $d(CA{\cdot}TG)_n$ sequences, infectious viral particles arise principally by homologous recombination events involving the two repeated sequences. On the other hand, in the case of the $d(GA{\cdot}TC)_n$ sequences, a significant proportion of the infectious particles (~50%) arises from non-homologous recombination events involving only one of the two repeated sequences. These results are discussed in terms of the known high degree of structural polymorphism of $d(GA{\cdot}TC)_n$ DNA sequences. Depending on the environmental conditions, this type of repeated DNA sequences can form a variety of non-B DNA conformations which include different types of intramolecular triplexes (either YRY or RRY triplexes) and hairpins (RR hairpins). These altered DNA conformations contain regions of single-stranded DNA which could be responsible for the increased frequency of non-homologous recombination events associated to the presence of $d(GA{\cdot}TC)_n$ sequences.

SOS factors involved in error free versus error prone translesion synthesis at frameshift mutation hot spots.

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Abstract : Mutations are permanent DNA sequence changes that can be induced when replication occurs on a damaged DNA template. In *E. coli.*, the process of translesion synthesis past a lesion that hinders replication requires the induction of SOS-controlled gene products among which the *umuDC* operon. To study translesion synthesis *in vivo*, we have constructed single stranded vectors containing single N-2-acetylaminofluorene (AAF) adducts located within -1 and -2 frameshift mutation hot spots formed by short repetitive sequences. These adducts strongly hinder DNA replication as only 2-5% of the molecules give rise to progeny under non-SOS induced conditions. Induction of the SOS response lead to a ten fold increase in survival. Adducts present within repetitive sequences trigger the formation of misaligned primer/template replication intermediates which, upon elongation, will result in the fixation of frameshift errors (error-prone translesion synthesis). Surprisingly we find that error-free translesion synthesis depends upon functional *umuDC*$^+$ gene products while error-prone translesion synthesis is *umuDC*$^+$ independent but requires another, as yet biochemically uncharacterized, SOS function. These data are discussed in terms of the different steps involved during translesion synthesis through a replication blocking lesion.

Complementation of DNA repair-deficient *Escherichia coli* by apurinic/apyrimidinic endounuclease genes from the trypanosomatidae *Leishmania major* and *Trypanosoma cruzi* .

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Apurinic/apyrimidinic sites in DNA are considered to be highly mutagenic and must be corrected to preserve genetic integrity. cDNA expression libraries form the typanosomatidae *Leishmania major* and *Trypanosoma cruzi* were used to investigate the presence of sequences encoding for enzymes responsible for repair of AP sites in parasitic protozoa. Screening was performed by isolating clones capable of complementing the deficiency of exonuclease III in the *Escherichia coli* mutant BW286 which is also defective in dUTPase activity. This double mutant is nonviable at 42°C due to an accumulation of unrepaired sites following excision of uracil from DNA. We have isolated by this method cDNA clones from both organisms that show pronounced homology, between others, with exonuclease III, a major AP endonuclease involved in base excision. The *Trypanosoma* and *Leishmania* sequences vary in the length of their amino terminus but are highly homologous between each other and present sequences that correspond to probable nuclear transport signals. Expression of the enzymes in AP endonuclease deficient *Escherichia coli* conferred signficant resistance to killing by methyl methanesulfonate and peroxides. Protozoan AP endonuclease may provide a useful tool for studying molecular mechanisms underlying oxidative stress in these pathogenic organisms.

Two regions of allellic imbalance in 1q32 in primary breast cancer coincide with two putative recombination hotspots

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Sixteen microsatellite markers were used to construct a high resolution map of the 1q32 region encompassing the regulator of complement activation (*RCA*) gene cluster. This region had been previously described as a candidate for allelic imbalance (AI). We used 13 of these markers to analyze AI in 21 pairs of primary breast tumours. Our results demonstrate the existence of two critical regions of AI in 1q32. One proximal to *REN* spanning less than 10 cM and another distal to *REN* that covers 5 cM. Interestingly, these two regions of AI coincide with two putative female-specific recombination hotspots detected in the high resolution map of this region.

Recombinational repair in *S. cerevisiae*: Interactions of Rad54 protein with other proteins

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In *S. cerevisiae* repair of radiation induced DNA damage is carried out by several pathways. The *RAD52* group genes (*RAD50-59*, *XRS2*, *MRE11*) are involved in the recombinational repair pathway.

RAD54 belongs by sequence homology to the Swi2/Snf2 protein family of putative DNA dependent or stimulated ATPases. Mutations in the *RAD54* gene lead to extreme sensitivity to ionizing radiation, to a slight UV sensitivity and to various recombination phenotypes. Furthermore, *rad54Δ* cells are unable to undergo mating type switching, a double strand break mediated recombination event.

As recombinational repair is thought to be carried out by a multi-protein complex, we used the two-hybrid system to identify proteins that interact with Rad54p.

An interaction between Rad54 and Rad51 proteins could be observed in the two-hybrid system. To corroborate this, coimmunoprecipitation experiments were performed. The two proteins can be coprecipitated using either an anti-Rad54p Ab or an anti-HA Ab that recognizes the tagged Rad51 protein. Genetic experiments gave further evidence for the biological relevance of this interaction. Cooverexpression of Rad51p and Rad54p in wild-type cells leads to a reduced survival in the presence of MMS (methylmethanesulfonate). Furthermore, overexpression of Rad54p suppresses DNA repair related phenotypes of *rad51Δ* cells in a Rad55p and Rad57p dependent manner.

Two new candidates for the putative repair/recombination complex have been identified performing two-hybrid screens with Rad54.

Deletion mutants of these novel genes, named *D69* and *B81*, exhibit DNA repair related phenotypes like MMS sensitivity. *B81* is also slightly UV sensitive. Interestingly, only the diploid *B81* mutant has a slightly increased sensitivity to γ- irradiation. None of all the other haploid and diploid mutants in these genes shows an enhanced γ-ray sensitivity. Neither *D69* nor *B81* mutants exhibit defects in spontaneous intragenic mitotic recombination. The *B81* gene is essential for meiosis, leading to a strong sporulation defect. Currently, the role of this gene in meiosis is being analyzed by physical, genetic and cytological methods.

Kinetics of excision of α-R-hydroxy-β-ureido-isobutyric acid, a ring fragmentation product of thymidine C5-hydrate, by *E. coli* DNA repair enzymes

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N-(2-deoxy-β-D-erythro-pentofuranosyl)-*N*-3-(2*R*-hydroxyisobutyric acid)urea (α-R-hydroxy-β-ureido-isobutyric acid) is formed in DNA by the action of free radicals or UV light. It is generated by hydrolysis of thymidine C5-hydrate. Although its structure is similar to that of other lesions categorized as non instructive, it is able to form hydrogen bonds in duplex DNA, suggesting potential miscoding properties. This thymine fragmentation residue, when present in DNA, at either 3' or 5' phosphates end inhibits phosphodiesterases activities. We have investigated, using different *Escherichia coli* DNA repair enzymes, the excision of α-R-hydroxy-β-ureido-isobutyric acid residue when present in double stranded oligonucleotide. Excision was measured in double-stranded 30-mer oligonucleotides containing a single lesion by the formation of a 10-mer oligonecleotide product as a function of enzyme concentration, substrate concentration and time. Among the various purified DNA-glycosylases tested, we have shown that α-R-hydroxy-β-ureido-isobutyric acid is substrate for the *E. coli* Fpg protein (Formamidopyrimidine-DNA glycosylase) and Nth protein (endonuclease III). Single and double-stranded oligonucleotides containing this residue were not cleaved by *E. coli* exonuclease III or endonuclease IV.

Duplex oligonucleotide containing single α-R-hydroxy-β-ureido-isobutyric acid residue positioned opposite T, G, and C were also cleaved by Fpg and Nth proteins respectively. Kinetic constants show that the Fpg protein removes the modified thymine from DNA about 100-fold more efficiently than Nth protein does. We have shown that high concentrations of substrate inhibit the Fpg protein activity although Nth protein activity is not inhibited by high concentrations of substrate. The K_m value for the excision of α-R-hydroxy-β-ureido-isobutyric acid residue by Fpg protein was similar to the K_m value for the 8-oxo-Gua, that is considered to be the main physiologycal substrate of the Fpg protein. Comparison of the kinetic constants reveals that K_{cat}/K_m ratio is about 4-fold higher for 8-oxo-Gua than for the ring fragmentation product of thymine. These findings suggest that α-R-hydroxy-β-ureido-isobutyric acid residue in double stranded DNA could be an important substrate for Fpg protein in cells.

INDUCED ECTOPIC RECOMBINATION IN YEAST

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Eukaryotic cells have evolved complex and sophisticated mechanisms to deal with DNA damage. Recombination plays a central role in the repair of double -strand breaks (DSBs) in the DNA. In yeast, recombination between artificial repeats located at different places in the genome (ectopic recombination) occurs at frequencies comparable to those seen for "regular" allelic recombination. Ectopic recombination between repeated sequences leads to the creation of chromosomal aberrations, such as translocations, inversions, deletions, etc. Since a large proportion of the genomes of all eukaryotic cells is composed of repeated sequences, it is not clear what are the mechanisms that prevent genomic instability in normal cells. Cancerous cells, in contrast, show a high level of genomic instability.

A thorough dissection of the mechanism of recombination and of its genetic control has been difficult, due to the low frequency at which recombination takes place. We have constructed yeast strains in which recombination can be induced in a synchronous way in a population of cells. Recombination is initiated by a DSB created by the HO endonuclease, which is controlled by an inducible promoter. We have developed two systems, one involving a naturally occurring repeat (Ty element) and another using an artificial repeat.

With our systems, we have started to analyze the rules by which homology is recognized. What is the minimal length that allows ectopic recombination? What is the role played by homeology in this process? We have also started dissecting the kinetics of the process, and its genetic control. We have found that less than 500 bp of complete homology are efficiently recognized and used in ectopic recombination, but a level of 1% heterology is enough to prevent most of the recombination events and to cause cell death. We are currently analyzing the role played by the mismatch repair system in this homeologous system. So far, deletions of the *PMS1*, *MSH3* or *MSH6* genes did not show any effect. Our results have important implications about the way genomic instability, created by ectopic recombination between repeated sequences, is avoided.

Recombinational repair in the absence of the RecA-like Rad51 protein in the yeast *Saccharomyces cerevisiae* .

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We try to understand recombinational repair (RR) in *Sacharomyces cerevisiae*. The genes of RR were identified by mutations conferring X-ray sensitivity, and subsequently shown to be required in the repair of double-strand breaks (DSBs). *RAD51* is one the DSB-repair genes whose functional role in recombinational repair is most intriguing, since despite the fact that Rad51p is homologous to RecA, *rad51* mutants show near wild-type levels of mitotic recombination, a result that strongly contrasts with the 100-fold reduction in recombination found in *rad52* mutants. It has been suggested that Rad51p is required for recombination of DNA molecules organized in a particular chromatin structure (1), and that *RAD51*-independent events occur by a replicative type of recombination(2).

To determine the role of RAD51 in RR and DSB-repair and the alternative RR mechanisms occurring in the absence of RAD51 we are using different approaches:

1) We have isolated a series of mutations that confer a rec- in a *rad51Δ* background that should allow us to identify genes involved in *RAD51*-independent recombination.

2) We are trying to define the role of chromatin in the requirement of Rad51p, by characterizing *RAD51*-independent recombination in different mutants affected in chromatin.

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MISPAIR EXTENSION FIDELITY OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REVERSE TRANSCRIPTASES WITH AMINO ACID SUBSTITUTIONS AFFECTING Tyr115

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Reverse transcription is error prone and contributes to the high genetic variability of retroviruses. Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is a DNA polymerase which lacks a proofreading function. Site-directed mutagenesis studies on the molecular basis of fidelity of DNA synthesis by the HIV-1 RT have revealed that amino acid substitutions affecting Gly262 and Trp266 in α-helix H render enzymes with a decreased frameshift fidelity. The substitution of Met184 by Val or Ile, Glu89 by Gly or Tyr183 by Phe rendered enzymes with enhanced insertion or mispair extension fidelity. The role of Tyr115 in mispair extension fidelity of DNA-dependent DNA synthesis was analyzed by using a series of 15 mutant enzymes with substitutions involving Tyr115. Six of these mutants (Y115F, Y115W, Y115A, Y115S, Y115D and Y115K) were previously obtained and their characterization revealed the role of Tyr115 in dNTP recognition and misinsertion fidelity of DNA synthesis (Martín-Hernández *et al.* 1996. *EMBO J.* **15**, 4434-4442). Nine additional mutants (Y115V, Y115I, Y115L, Y115M, Y115N, Y115H, Y115C, Y115G and Y115P) have now been obtained and characterized. Their kinetic parameters for elongation using homopolymeric RNA-DNA and heteropolymeric DNA-DNA complexes showed major effects of the amino acid substitutions on the K_m value for dNTP. Enzymes with large hydrophobic residues at position 115 displayed lower K_m values than enzymes with small and charged amino acids at this position. The influence of all these amino acid replacements was analyzed in mispair extension fidelity assays using three different mismatches (A:C, A:G and A:A) at the 3'-terminal position of the primer DNA. For the A:C mispair, a 2.6-33.4-fold increase in mispair extension efficiency (f_{ext}) was observed as compared with the wild-type enzyme, using a DNA duplex formed by a 38mer template mimicking an HIV-1 *gag* sequence and a complementary 16mer primer. Unexpectedly, all the mutants tested as well as the wild-type RT were very efficient in extending the A:G and A:A transversion mispairs ($f_{ext} \approx 0.1$-0.2). This effect was not observed with the wild-type HIV-1 RT and a different template-primer complex formed by M13 ssDNA and a 20mer primer ($f_{ext} = 2.9 \times 10^{-5}$ for the A:G mispair). However, in the case of the A:C mispair, the extension efficiencies of the wild-type RT and several Tyr115 mutants were similar with both template-primers. In all cases enzymes with smaller residues at position 115 (e.g., Y115G) were more efficient in extending the A:C mismatch. The results support a role of Tyr115 in accommodating the complementary nucleotide into the nascent DNA while polymerization takes place.

CHARACTERIZATION OF AN AFRICAN SWINE FEVER VIRUS 20 kDa-DNA POLYMERASE INVOLVED IN DNA REPAIR

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African Swine Fever Virus (ASFV) O174L gene encodes a 20 kDa protein homologous to the members of the family X of DNA polymerases. We have cloned, expressed and purified ASFV polX, demonstrating, by different criteria, that this protein is a DNA polymerase repairing enzyme. This is the smallest DNA polymerase described to date, with a molecular weight half of that of the related DNA polymerase β (40 kDa), also involved in DNA repair.

We cloned the ORF O174L into the bacterial expression vector pRSET-A to place an hexahistidine tag at the N-terminus of the protein which allows the partial purification of the overexpressed ASFV polX by affinity chromatography through Ni-NTA agarose beads. An *in situ* polymerase gel analysis detected a DNA polymerase activity coincident with the 20 kDa overexpressed new band in *E.coli* extracts. The intrinsic ASFV polX activity corresponded to a monomer, when assayed in solution after sedimentation through a glycerol gradient. Polymerization by ASFV polX is highly distributive and depends on a template and a 3´-OH primer terminus. As expected, the polymerase activity required either Mg^{2+} or Mn^{2+} as metal activators, being insensitive to aphidicolin, but very sensitive to ddNTPs. In fact, the insertion efficiency of ddNTPs is virtually the same compared to that of dNTPs. The enzyme also incorporates NTPs but with a reduced efficiency.

We have also analyzed some of the properties that could contribute to the fidelity of DNA synthesis. The enzyme lacks a proofreading 3´-5´ exonuclease activity, but it is not able to extend a mismatched primer terminus. Moreover, and unlike DNA polymerase β, ASFV polX discriminates very precisely the incoming base.

To test the capacity of ASFV polX to act in DNA repair, we performed a series of experiments on different gapped substrates. The protein fills 6 base gaps distributively until the length has been reduced to 3 nucleotides. This short gap is then filled by a processive mechanism, that becomes more efficient with the presence of a PO_4 group on the 5´-side of the gap. Finally, we have demonstrated that a single nucleotide gap can be efficiently filled by ASFV polX and completely repaired by addition of T4 DNA ligase.

Expression of the human HAP1 and cellular sensitivity to DNA damaging agents

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One of the most common lesions generated in DNA is the apurinic/apyrimidinic (AP) site which results from the hydrolysis of the N-glycosyl bond linking the base to the deoxyribose moiety. Spontaneous hydrolysis occurs to the extent that up to 10^4 purines/cell/day may be lost in humans. This already high spontaneous rate can be increased still further by the action of DNA damaging drugs or radiation. Since these lesions are both cytotoxic and mutagenic, cells of all organisms express dedicated repair enzymes, termed AP endonucleases, to remove AP sites for the maintenance of genome integrity and cells viability. Most AP endonucleases are very versatile enzymes, capable of performing numerous additional repair roles. The major AP endonuclease in human cells (HAP1 or AP protein) also exhibits RNAase H activity and an ability to control the redox state of certain proto-oncogene products such as the transcription factor c-Jun. The DNA repair and redox activities of HAP1 are distinct both structurally and functionally.

We set out to identify whether the HAP1 protein is involved in cellular protection against several stresses. To achieve this, the HAP1 cDNA was cloned downstream of the SV40 promoter in the mammalian cell expression vector pSV2neo, and transfected into the Chinese hamster (CHO) cells. The transfected cells expressed an AP endonuclease activity 6-fold over the constitutive level. To test whether overexpression of the HAP1 protein influenced sensitivity to DNA damaging agents, clonogenic survival was measured after exposure of the cells to DNA damaging drugs, including alkylating and oxidative agents. The resistance of control and transfected CHO cells to the lethal effects of various compounds (MMS, Aziridine, H_2O_2, Bleomicine) has been evaluated. Our data show that HAP1 protein increases the lethal effect of MMC. However, the sensitivity towards MMC was not changed in cells expressing the E. coli FPG protein, that also possesses an AP-nicking activity. To exert its cytotoxic activity, MMC requires reductive activation, becoming an alkylating species that forms monofunctional and bifunctional adducts with DNA.

Therefore, our results suggest that the increased cytotoxicity of MMC in cells overexpressing the HAP1 protein may be due to the reductive activation of MMC by the redox domain of this protein and not by its AP endonuclease repair activity, and experiments are in progress to test this hypothesis.

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ENZYMATIC REPAIR OF 8-OXOGUANINE IN EUKARYOTES

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A damaged form of guanine, 7,8-dihydro-8-oxoguanine (8-OxoG), is a major lesion in DNA exposed to ionizing radiation and oxidative stress. *Escherichia coli* possesses two DNA glycosylase activities that prevent mutagenesis by 8-OxoG: the Fpg protein which excises 8-OxoG in DNA, and the MutY protein which excises adenine residues incorporated opposite 8-OxoG. Inactivation of both the *fpg* and *mut*Y genes results in a strong GC-->TA mutator phenotype.

The *OGG1* gene of the yeast *Saccharomyces cerevisiae* was cloned and sequenced. The *OGG1* gene codes for a protein of 376 amino acids with a molecular mass of 43-kDa and mapped to chromosome XIII. Comparison of the amino acid sequence of the Ogg1 protein and of bacterial Fpg proteins does no reveal significant homology. The Ogg1 protein possesses both a DNA glycosylase activity that excises 8-OxoG and another activity that nicks DNA at abasic site. Studies of the catalytic mechanism demonstrate that the Ogg1 protein is a DNA glycosylase / AP lyase which proceeds via the formation of an transcient covalent imino enzyme-DNA intermediate. The *OGG1* gene has been disrupted yielding the *ogg1::TRP1* mutant. The *ogg1* mutant of *S.cerevisiae* is a mutator that specifically accumulates GC-->TA transversion events. Although the sequences are not clearly related, these results suggest that the yeast *OGG1* gene is a functional homologue of the bacterial *fpg* gene.

Using the *OGG1* sequence we have retrieved several human cDNA clones displaying close to 40% identity with the deduced yeast protein sequence. Northern blot analysis showed that the corresponding gene is expressed in all the human tissues analyzed. The cloned coding sequence was then expressed in *Escherichia coli* carrying a disrupted version of the *fpg* gene. Extracts from induced cultures displayed a DNA glycosylase/AP lyase activity on duplex DNA carrying an 8-OxoG/C base pair. When the human gene was expressed in the hypermutator *Escherichia coli fpg mutY* it reduced the spontaneous mutation frequency to the levels of the single *mutY* mutant. Moreover, when the coding sequence was expressed in a yeast strain mutant in *OGG1* , it was able to complement the spontaneous mutator phenotype. These results make of this novel gene (*hOGG1)* a strong candidate for the human homologue of the yeast *OGG1*.

We conclude that Base Excision Repair (BER) of 8-OxoG residues contributes to the maintenance of genetic stability in prokaryotic and eukaryotic cells.

A stuttering mode of synthesis by HIV-1 reverse transcriptase generates sequence expansion.

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Point mutations and frameshifts are the most frequent errors performed by DNA polymerases during DNA replication. However more complex aberrations, like extensive sequence insertions or deletions, can also take place. Amplification of di or tri-nucleotides is associated with a number of human heritable disorders and cancers and, generally, with genetic instability in eukaryotes. It is probably due to an aberration in the copying process of polymerases.

We have shown that HIV-1 reverse transcriptase (HIV-1 RT), an atypic DNA polymerase which copies both a RNA and a DNA template to produce a double-stranded DNA molecule competent for viral integration, is able to perform a reiterative DNA synthesis. On specific DNA and RNA template motifs, 5-6 nucleotides long, mismatch formation commits HIV-1 RT to a new mode of synthesis which generates repetitive products. On RNA templates these products can be longer than 150 nucleotides. This mechanism, which involves extensive primer misalignment, is strikingly similar to that postulated for telomerases.

The easy expansion of di or tri-nucleotides in several regions of the genome could be also generated by DNA polymerases undergoing a different mode of synthesis during the elongation process. By analogy with HIV-1 RT, where the repetitive synthesis is activated by mismatch formation, we suggest that replication handicaps induce eukaryotic DNA polymerases to undergo such alternative modes of synthesis.

Gerhard Ries

Homologous recombination in plants: Isolation of *Arabidopsis thaliana* mutants with increased recombination frequencies

Homologous recombination in plants is a powerful process by which genetic information can be rearranged and new gene combinations can be established. Whereas the process of recombination itself has been analyzed in some detail, little is known about how the frequency of recombination events is regulated and how it is affected by environmental stimuli. To address these questions, we propose a genetic screen to identify mutations that result in increased frequencies of homologous recombination in *Arabidopsis thaliana*. A transgenic *Arabidopsis* line will be constructed that carries two independent chromosomal recombination substrates consisting of different disrupted reporter genes. Upon recombination events between homologous DNA sequences, the functional reporter genes will be restored. After chemical seed mutagenesis, a large population of M2 plants homo- and heterozygous for the induced mutations will be generated and screened for recombination events. Plants displaying elevated recombination frequencies at both markers simultaneously are candidates for general "recombination-up" mutants. This screen should identify genes and activities involved in several steps of recombination regulation. Genetic, molecular and biochemical analysis of these mutations will enhance our understanding of how plants can sense environmental stimuli and translate this into a genetic response, i. e. increased recombination frequencies. The results should be of interest for basic research as well as for plant genetic engineering.

EUKARYOTIC DNA GLYCOSYLASES THAT REPAIR OXIDATIVE DNA DAMAGE

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In *Escherichia coli* oxidized bases are mainly removed by two DNA glycosylases. Formamidopyrimidine DNA glycosylase (Fpg) repairs damaged purine residues such as 8-oxoG and formamidopyrimidines (fapy), and endonuclease III (Nth-Eco) removes damaged pyrimidine such as urea, thymine glycol and 5-hydroxyuracil. A functional homologue of Fpg has been recently described in *S. cerevisiae*. This protein (Ogg1) does not have sequence similarity to Fpg and removes 8-oxoG more efficiently than fapy residues. Searching in an human EST database we have identified an ORF encoding a protein with a high sequence homology to Ogg1. The human Ogg1 homologue (hOGG1) is a 47.2 kDa protein of 424 amino acids and possesses an enzymatic activity that cleaves a 49-mer oligonucleotide containing a single 8-oxoG opposite a cytosine (8-oxoG/C). However, no cleavage is observed when an adenine is placed opposite 8-oxoG (8-oxoG/A). Unlike *E. coli* Fpg, hOGG1 protein does not show any detectable activity on fapy residues and is not activated by 100 mM KCl. Expression of the hOGG1 cDNA in a *E. coli (fpg, mutY)* double mutant results in a decrease of the spontaneous mutation frequency.

Additionallly, we have cloned *S. pombe* and human genes encoding sequence homologues of Nth-Eco, and the corresponding proteins have been purified to apparent homogeneity and characterized. The substrate specificity of these enzymes for modified bases in oxidatively damaged DNA has been investigated by using the gas chromatography/isotope-dilution mass spectrometry (GC/IDMS) technique. DNA substrates prepared by γ-irradiation or H_2O_2/Fe-EDTA treatment were incubated with Nth proteins and the reaction products analyzed by GC/IDMS. The eukaryotic enzymes excise a number of pyrimidine-derived lesions. Excision of lesions was measured as a function of time, enzyme or substrate concentration, and temperature to determine kinetic constants.

A ROLE OF JNK IN CISPLATIN INDUCED APOPTOSIS

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The effectiveness of cancer chemotherapy is restricted by the failure of some tumours to respond and by the appearance of resistant cell populations in patients after initial response. The elucidation of the molecular mechanisms by which anti-neoplasic drugs induce programmed cell death is essential to improve cancer therapy. Platinum compounds are anticancer drugs widely used for the treatment of various cancers. The *cis* isomer of diaminedichloroplatinum (cisplatin) forms DNA adducts and kills the cells triggering apoptosis. On the other hand the *trans* isomer (transplatin) although being able to damage DNA.has not cytotoxic effect.

In our laboratory we have approached the study of some of the responses induced by treatment of *cis* and *trans*platin in order to establish a correlation among these responses and apoptosis. Using as a model system the mouse keratinocyte cell line Pam 212 we have found that treatment of these cells with these drugs, (cis and transplatin) induces the activation of the Stress-Activated Protein Kinase (SAPsK or c-Jun Kinase ,JNK), but not the extracellular signal regulated kinases ERKs.While JNK activation by transplatin is rapid and transient, cisplatin produces a delayed an persistent activation of the kinase.The activation of JNK by these drugs takes place both in cytosol and in nucleus, being higher in the nuclear compartment. Additionally, we have established a correlation between activation of JNK and cell death. We have been able to modulate the relative toxicity of the drugs simply by molulating the profile of the JNK activation. Taken together these results suggest that activation of JNK is a mediator of the toxicity of these drugs.

The Human hepatitis B virus X protein, HBx, binds the basal transcriptional factor TFIIH and stimulates the DNA helicase activity of TFIIH: Implications for a role of HBx in cellular nucleotide excision repair pathway

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The infection by the human hepatitis B virus (HBV) leads to chronic hepatitis and an association with hepatocellular carcinoma has been documented. HBV encodes a regulatory protein termed HBx, which has been largely assayed as a transcriptional transactivator. The investigations on the mechanism of its action has led to the identification of several cellular targets with whom HBx directly interacts. We have recently identified basal transcriptional factor TFIIH as one of the cellular target of HBx. HBx selectively binds to ERCC2 and ERCC3 subunits of TFIIH. Furthermore, the binding leads to significant stimulation of DNA helicase activity. The HBx binding was also demonstrated for yeast homologous of ERCC2 and ERCC3, the RAD3 and SSL2 subunits. In defining the functional relevance of these infractions, we show that yeast cells expressing HBx display an increased hypersensitivity to UV. These observations lend support to a model in which HBx may influence nucleotide excision repair pathway via its interactions with basal factor TFIIH in infected hepatocytes and contribute to events leading establishment of hepatocellular carcinoma.

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Chromosomal Distribution of Genes Subject to Transcription Coupled DNA repair*

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It is well known that nucleotide excision repair (NER) is preferentially directed to actively transcribed genes and their transcribed strands through transcription coupled mechanisms[1]. Human chromosomes are highly heterogeneous with respect to gene density and transcriptional activity. Thus, chromosomal bands harbouring actively transcribed genes would be expected to be preferentially repaired. Xeroderma pigmentosum group C (XPC) cells are known to be partially deficient in NER since they can only repair the transcribed strands of active genes[2]. UV-induced repair patches in confluent XPC were labelled with BrdU. After EcoRI digestion and CsCl gradient, extracted DNA fragments containing BrdU-repair patches were isolated with antibodies against BrdU and an immunomagnetic system. The extracted repaired fragments were random primed with biotin and used as a probe to perform fluorescence in situ hybridization (FISH) to metaphases from normal cells. This approach allowed us to visualize the chromosomal sites subject to transcription coupled repair. Because of the genetic defect in XPC, these sites correspond to transcription points. The chromosomal distribution of FISH signals were compared to that found with the unrepaired fractions containing non-repaired bulk DNA and the non-transcribed strands of active genes. To further control the distribution of repair sites in human chromosomes we have also generated similar probes with wild type cells and xeroderma pigmentosum group A (XPA) cells, which are known to be completely deficient in NER. Preliminary observations indicate that: (i) there are clusters of DNA repair in some but not all telomeric regions, (ii) the R bands (light G bands) are preferentially repaired and (iii) heterochromatic bands such as pericentromeric region of some chromosomes and the long arm of the Y chromosome exhibit a deficiency in DNA repair.

[1]FHanawalt, P.C. (1994) Science 266, 1957-1958. [2]Venema et al., 1991 Mol. Cell. Biol. 11: 4128-4134.

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Characterization of DinR, the Bacillus subtilis SOS Repressor

KEVIN W. WINTERLING, ARTHUR S. LEVINE, RONALD E. YASBIN, AND ROGER WOODGATE

In *Bacillus subtilis*, exposure to DNA damage and the development of natural competence leads to the induction of the SOS regulon. It has been hypothesized that the DinR protein is the cellular repressor of the *B. subtilis* SOS system due to its homology to the *Escherichia coli* LexA transcriptional repressor. Indeed, comparison of DinR and its homologs from gram-negative and positive-bacteria revealed conserved structural motifs within the carboxyl terminal domain that are believed to be important for autocatalysis of the protein. In contrast, regions within the DNA binding domain were only conserved within gram-negative or positive genera, which possibly explains the differences in the sequence specificity between gram-negative and gram-positive SOS boxes.

The hypothesis that DinR is the repressor of the SOS regulon in *B. subtilis* has been tested through the over-expression, purification, and characterization of the DinR protein. Like *E. coli* LexA, *B. subtilis* DinR undergoes an autocatalytic reaction at alkaline pH at a siscile Ala^{91}-Gly^{92} bond. The cleavage reaction can also be mediated *in vitro* under more physiological conditions by the *E. coli* RecA protein. By using electrophoretic mobility shift assays, we demonstrated that DinR interacts with the previously characterized SOS-box of the *B. subtilis recA* gene, but not with sequences containing single base-pair mutations within the SOS-box. Together, these observations strongly suggest that DinR is the repressor of the SOS regulon in *B. subtilis*.

List of Invited Speakers

Workshop on

DNA REPAIR AND GENOME INSTABILITY

List of Invited Speakers

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