Instituto Juan March
de Estudios e Investigaciones

100 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

The Interface Between Transcription and DNA Repair, Recombination and Chromatin Remodelling

Organized by

A. Aguilera and J. H. J. Hoeijmakers

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Introduction

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The interface between transcription and DNA repair, recombination and chromatin remodelling

Transcription of DNA is a central process in the cell and obviously essential for all living systems. It is a key regulatory mechanism in development and differentiation and the most important focal point of signal transduction and other regulatory networks controlling the response to exogenous and endogenous signals. The transcription process allows fine-tuning of gene expression and is the crucial stage where the "on/off" decision is made. Many disease states, including cancer, involve dysregulation of gene expression.

Due to its fundamental importance, elucidation of the molecular mechanism of the process of transcription and its regulation is one of the most intensively studied fields of research in today's biology. As a result of the massive, world-wide investment of research activities impressive progress has been made concerning the insight into the basal mechanism of transcription initiation and the interplay between the basal transcription machinery and the regulatory circuit. The basic transcription reaction has been dissected and reconstituted in vitro from purified components. Different factors with multiple specialized functions accomplish the complex series of events from transcription initiation to elongation and termination. Numerous interactions with regulatory proteins have been identified.

Recently, converging developments have triggered the notion that transcription has multiple links with other cellular processes that involve DNA homeostasis, notably DNA damage and repair, recombination and chromatin dynamics. Several examples illustrate the molecular intricacies and biological impact of these interphases.

1. An important connection between transcription and DNA repair was revealed by the discovery of a distinct DNA repair pathway focussing on removal of DNA lesions that obstruct ongoing transcription. This transcription-coupled repair process is intimately connected with the nucleotide excision repair system, that eliminates a wide range of lesions from the genome, including damage induced by ultraviolet light and by numerous chemical agents.

2. Another example of a connection between transcription and DNA repair is the dual functional factor TFIIH, that is involved in transcription initiation as well as in nucleotide

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excision repair. Importantly, mutations in this multi-subunit factor can give rise to three distinct conditions all associated with photosensitivity: the skin cancer-prone syndrome xeroderma pigmentosum with or without the hallmarks of Cockayne syndrome and the severe neuro-developmental disorder thrichothiodystrophy characterized by brittle hair and nails. The latter two diseases reveal a novel concept in human genetics: the existence of inborn defects in basal transcription.

3. Another intriguing link between transcription and DNA homeostasis is the observation of increased mutation rates associated with transcription. This phenomenon has a good example in the poorly understood mechanism of hypermutation in active immunoglobulin genes, that enhances antibody diversity. Interestingly, an increase of mutation rates has also been observed associated with transcriptional activation of genes in yeast.

4. Recombination rates are increased by transcriptional activation, in prokaryotes and eukaryotes from yeast to mammals. Intriguingly, several reports suggest that transcriptional elongation defects can also be linked to an increase in recombination.

5. Finally, both DNA repair and recombination have been shown to be modulated by different states of chromatin structure whereas transcription is also intimately linked with chromatin fluidity.

The above examples likely represent the tip of an iceberg. Probably, numerous ramifications of transcription, repair, recombination and chromatin conformation with each other as well as with other cellular events have yet to be discovered. This workshop has provided a forum where recent discoveries have been reported and discussed in an effort to integrate the knowledge of the individual areas with each other and to improve our understanding of the molecular basis and biological impact of these fascinating connections.

A. Aguilera

J.H.J. Hoeijmakers

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Session 1: Transcription Machinery

Chair: Richard A. Young

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Dissecting Transcriptional Circuitry and Mechanisms in Yeast

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Genetic and biochemical studies have identified much of the transcriptional machinery of eukaryotic cells and provided mechanistic insights into the functions of various components at specific promoters. However, knowledge of the contributions of regulators and the transcriptional machinery to the complete transcriptional circuitry is lacking. Genome-wide expression analysis is being used to obtain clues to the roles played by transcriptional regulators, components of the transcription initiation apparatus, histones and chromatin modifying enzymes in gene regulation. Our data describes how expression of the genome depends on over 50 components critical to transcriptional regulation in yeast cells. This information is providing the foundation for understanding the molecular mechanisms involved in genome-wide expression, and new insights into transcriptional regulation will be described.

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Wyrick, J.J., Holstege, F.C.P., Jennings, E.G., Causton, C., Shore, D., Grunstein, M., Lander, E.S., and Young, R.A. Chromosomal landscape of nucleosome-dependent gene expression and silencing in yeast. Nature, in press (1999).

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TFIIH in transcription and repair : J-M. Egly Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, 1 rue Laurent Fries, BP 163, 67404 ILLKIRCH Cédex, C.U. de Strasbourg, France.

Our objective is to understand the relationship between transcription and DNA repair by analysis of the dual function of the transcription factor TFIIH in both mechanisms knowing that some mutations in either XPB or XPD helicases of TFIIH, give rise to three, rare, autosomal recessive disorders previously known as DNA repair disorders xeroderma pigmentosum (XP), Cockayne Syndrome (CS) and trichothiodys-trophy (TTD).

Once initiation was occurred after the formation and assembly of the general transcription factors which position the RNA polymerase II (RNA pol II) on the promoter, RNA pol II will traverse the gene, with the help of some additional factors the role of which being to prevent or overcome pausing sites, resulting from either the DNA sequence itself, the nucleosomes or DNA damage. Indeed, DNA damage induced by carcinogens, antitumor drugs as well as oxidative reactions may structurally modify DNA and in consequence stop the transcription until DNA is repaired.

TFIIH is involved in transcription, DNA repair and likely in transcription coupled repaired as suggested by the observation of the clinical features of the XP, CS and TTD patients as well as from biochemical studies.

Having designed a baculovirus expression system which allows the expression of either wild type or « mutated » recombinant TFIIH and an immuno-purification protocol to obtain TFIIH from derived patients cell lines, we are now in position to understand the function and the role of most of the TFIIH subunits not only in transcription but also in NER.

In transcription :

- XPB helicase is essential for promoter opening and the first phosphodiester bond formation.
- XPD helicase, although not absolutely required in the promoter opening, plays a role in promoter escape and elongation.
- p44 nucleates the formation of the « core TFIIH » and thus regulates XPD helicase activity.
- cdk7 stimulates transcription of certain promoters.
- MAT1 enhances the phosphorylation process but allows CAK to be anchored not only within the CAK but also in the transcription complex.

In DNA repair :

- TIFF is involved in both global genome repair as well as transcription coupled repair.
- XPB and XPD mutations can completely prevent opening and dual incision of damaged strand.
- CAK does not seem to be absolutely required for the NER reaction.

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Recent papers dealing with TFIIH function

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The elongator complex: coupling chromatin modification to transcriptional elongation

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The elongator complex is the major component of the RNA polymerase II (RNAPII) holoenzyme responsible for transcriptional elongation in yeast (Otero et al., 1999). Elp3, the 60 kilodalton subunit of elongator/RNAPII holoenzyme, is a highly conserved histone acetyltransferase (HAT) capable of acetylating core histones in vitro (Wittschieben et al., 1999). Here we report that mutations which impair the in vitro HAT activity of Elp3 also confer typical elp phenotypes in vivo. Mutation of histone H3 and/or H4 N-terminal tails in elp3□ mutants confers sickness or lethality. Like Elp3 in elongator, SAGA-associated Gcn5 protein is a HAT that functions in RNA polymerase II (RNAPII) transcription. gcn5□ elp3□ double mutants display a number of severe phenotypes. These phenotypes are also observed for cells maintaining the structural integrity of elongator and SAGA but lacking the complexes' enzymatic activity due to single point mutations in the respective HAT catalytic domains. Our data demonstrate functional redundancy between elongator and SAGA HAT activities, revealing a much more general requirement for histone acetylation during transcriptional activation than indicated by studies of Gcn5 alone.

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Mechanisms of Transcriptional Pausing and its Suppression by Elongation Factors.

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RNA chain elongation and consequently gene expression by human RNAPII are regulated by elongation factors like TFIIF, Elongin, and ELL that suppress transcriptional pausing and increase the efficiency of pre-mRNA synthesis. Pausing occurs in at least two steps: isomerization into a paused conformation and slow escape from the pause site. Elongation factors could act at either step or both. To elucidate the mechanism of pausing and how elongation factors affect it, we have developed a rapid quench-flow kinetic assay of pausing at the HIV-1 +62 pause site that allows millisecond time resolution and involves only three nucleotide addition steps: C61 to human RNAPII transcription complexes halted at HIV-1 A60, U62 to arrive at the pause site, and G63 to escape the pause site. Because addition of C61 and U62 occur rapidly and because [GTP] can be varied without affecting these steps, we can measure the rates of paused complex formation and escape as a function of [GTP] or time of elongation factor addition. Our results establish that (*i*) formation of HIV-1 paused complexes is governed by the relative stabilities of RNA:DNA hybrids in the paused and elongation-competent complexes; (*ii*) NH4+ ion (which mimics elongation factors) and probably TFIIF and Elongin stimulate pause escape without affecting paused complex formation; and (*iii*) pausing requires steps in addition to rapid equilibration between backtracked and active transcription complexes. Possible structural models of pausing based on the recently determined atomic resolution structure of RNAP and the results of our kinetic analyses will be presented.

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Session 2: Transcription and Chromatin Structure

Chair: Alan P. Wolffe

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HIERARCHIES OF FUNCTION FOR CHROMATIN REMODELING MACHINES AND HISTONE MODIFICATION.

Alan P.Wolffe, Esteban Ballastar, Trevor N.Collingwood, Dmitry Guschin , Axel Imhof, Peter L.Jones , Qiao Li, Fyodor Urnov, Gert J. Veenstra and Paul A Wade.

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We have examined the links between chromatin remodeling and histone acetylation and deacetylation connected with gene activation and repression.

We find that DNA methylation has a causal role in regulating transcription within chromatin by the targeting of chromatin remodeling and histone deacetylation.We have characterised two complexes that link these processes. The Mi-2 complex contains the nucleosomal ATPase Mi-2, together with Mta 1-like and MBD3 proteins which selectively recognise methylated DNA, the histone binding protein RbAp48 and histone deacetylase itself (1,2). The Mi-2 complex is targeted by several gene specific repressors indicative of DNA methylation having a role in promoter specific transcriptional control. A second complex contains the DNA methylation specifc transcriptional repressor MeCP2 together with SIN3 and components of the replication machinery (3).

These results firmly connect DNA methylation to gene silencing through the targeted modification of chromatin structure and histone deacetylation (4). DNA methylation has significant roles in both the etiology of human disease and attempts at therapeutic intervention (5).

The similarities between the two complexes : chromatin remodeling engines, the RbAp48 -histone deacetylase and methylCpG binding proteins also indicates that disruption of canonical nucleosomes will be important for transcriptional silencing. We have suggested that such disruption or partial assembly such as occurs at the relication fork is important for the RbAp48 - histone deacetylase complex to operate effectively (6).

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We have made use of nuclear receptors to examine the connections between chromatin remodeling and histone acetylation. The thyroid hormone receptor targets both gene activation and repression. The chromatin remodeling machines involved in both of these events are being defined. We find that chromatin remodeling as defined by changes in topology and in nucleosome organisation is an essential prerequisite for transcriptional activation by p300 and PCAF (7).

Our results for both gene repression and activation demonstrate that chromatin remodeling is an integral component of the pathways of corepressor and coactivator function through deacetylase and acetyltransferase dependent pathways.

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2) Wade et al (1999) Nature genetics 23: 62-66 (Mi-2 and methylation)
3) Jones et al (1998) Nature genetics 19: 187-191
(DNA methylation and deacetylase)
4) Bird & Wolffe (1999) Cell (November) in press
5) Wolffe & Matzke (1999) Science (November) in press
6) Vermaak et al (1999) Mol Cell Biol 19: 5487-5860
(RbAp48 - histone deacetylase- histone H4 connection)
7) Li et al (1999) EMBO J (November) in press
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HISTONE ACETYLTRANSFERASE COMPLEXES FROM YEAST.

S. John[1], A. Eberharter[1], A. Hassan[1], K. Neely[1], M. Vignali[1], D. Sterner[4], A. Wallberg[2], R. Utley[1], K. Ikeda[1,3], P. Grant[1], M. Pray-Grant[1], D. Steger[1], S. Berger[4], A. Wright[2], and J. Workman[1].

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3: Department of Biology, Jichi Medical School, Japan. 4: The Wistar Institute, Philadelphia, PA.

There are several multiprotein histone acetyltransferase complexes in yeast. The 1.8 MDa SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex and 0.8 MDa Ada complex contain the Gcn5, Ada2 and Ada3, proteins which were originally identified in screens for genes-products acting as transcription adaptors. Gcn5 is the primary if not only subunit possessing catalytic histone acetyltransferase (HAT) activity within SAGA and Ada. The SAGA complex contains additional groups of gene products linked to transcriptional regulation. This includes the TBP group of Spt proteins (Spt3, Spt7, Spt8 and Spt20) which have been genetically linked to TBP (TATA-binding protein) function. The Ada1 protein, which is phenotypcially related to the Spt proteins is also found in the SAGA complex. The SAGA complex contains a subset of TAF_{II} (TBP-associated factors) proteins previously thought to function only in TFIID. These include TAF_{II} -90, -68/62, -60, -25, -20/17. The Ada/GCN5, Spt and TAF_{II} subunits all play important roles in the in vitro activities of the SAGA complex including nucleosome acetylation, transcription activation, and interactions with transcription activators and TBP. The largest subunit of the SAGA complex is the 3,744 amino acid protein Tra1. Tra1 is the yeast homologue of the human TRRAP protein which has been found to function as an essential cofactor for the c-Myc and E2F oncoproteins. Tra1 is related to the ATM/PI-3 kinase family of proteins and is a potential target for interactions with transcription activators.

the SAGA complex and the NuA4 complex (nucleosome acetyltransferase of H4) activate transcription from nucleosome arrays via direct interactions with transcription activation domains. Acidic activation domains of transcription factors can target the SAGA and NuA4 complexes to promoters to bring about acetylation of nearby nucleosomes. By contrast, the ADA and NuA3 (nucleosome acetyltransferase of H3) HAT complexes are able to stimulate transcription from chromatin templates but are apparently not directly targeted via acidic activation domains.

The ADA complex is related to SAGA in that it contains Ada2, Ada3 and Gcn5 but lacks the other subunits found in the SAGA complex (e.g. Spt and TAF proteins). The ADA complex instead contains unique subunits indicating that it is a distinct HAT complex with distinct functions. One of these novel subunits, Ahc1 (ADA histone acetyltransferase component 1) is required for the integrity of the ADA complex, but when disrupted does not give rise to the ADA phenotypes which are observed when SAGA components are disrupted. The NuA3 complex is a GCN5-independent histone H3 acetyltransferase complex that instead contains SAS3 as the catalytic subunit. NuA3 also contains yTAF30. The NuA3 complex interacts with Spt16/Cdc68 via the SAS3 subunit. Spt16/Cdc68 is a component of the abundant yeast CP complex that has been suggested to play a role in both transcription and DNA replication.

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REGULATION OF CHROMATIN REMODELING. Zhaohui Shao, Gavin Schnitzler, Said Sif, Michael Phelan, Florian Raible, Ramin Mollaaghababa, Chao-Ting Wu, Welcome Bender and Robert E. Kingston

Several eukaryotic protein complexes, including the SWI/SNF complexes of yeast and humans, use the energy of ATP hydrolysis to remodel chromatin structure. These alterations in nucleosome structure have been shown to increase the efficiency of numerous different steps required for activation of transcription. Genetic studies suggest that the SWI/SNF family is involved not only in activation of gene expression, but also in maintaining the active state of genes during cell division. We are studying both the way that SWI/SNF activities are regulated and the way that the energy of ATP hydrolysis is used to promote changes in chromatin structure.

The polycomb-group (Pc-G) genes are required to maintain repression of homeotic genes during development. Mutations in these genes are suppressed by mutations in genes of the SWI/SNF family. These genetic studies suggested that complexes encoded by Pc-G genes might directly affect the activity of SWI/SNF family complexes.

An epitope-tag strategy was used to purify a complex from Drosophila that contained the products of the Pc-G genes *polycomb, posterior sex combs, polyhomeotic* and *sex combs on midleg*. The complex, termed PRC1 (Polycomb Repressive Complex 1), also contained several other proteins and ran at greater than 2 MD on sizing columns. Pre-incubation of PRC1 with nucleosomal arrays blocked the ability of these arrays to be remodeled by SWI/SNF. When SWI/SNF and PRC1 were added to arrays at the same time, there was no repression of remodeling. Thus, PRC1 can block SWI/SNF function and SWI/SNF can block PRC1 function in this purified system. PRC1 was active on nucleosomal arrays that had trypsinized histone tails, which differentiates this complex mechanistically from repressive complexes that function via histone de-acetylation or via direct contact with the N termini.

To study the mechanisms that allow SWI/SNF to remodel chromatin structure, we have studied the effects of SWI/SNF on structure of mononucleosomes. When mononucleosomes are used as substrate, SWI/SNF will create, in an ATP-dependent manner, a nucleosomal structure that has an altered DNA path and that contains the DNA and protein components of two nucleosomes. This remodeled species is visualized as two closely packed nuclesomes by microscopy. Surprisingly, SWI/SNF not only catalyzes the formation of this complex, but also catalyzes the formation of a standard mononucleosome when the remodeled species is used as the starting material. Thus SWI/SNF appears to use the energy of ATP hydrolysis to promote a rapid equilibration between a standard and remodeled nucleosomal conformation. In addition, as shown by Lorch and

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Kornberg for the related yeast RSC complex, human SWI/SNF is able to transfer histones from one piece of DNA to another, implying that this complex can shift nucleosome position.

We interpret these studies to suggest that the primary purpose of the SWI/SNF family of complexes is to increase the rate of equilibration between different nucleosome positions and conformations. We propose that these activities are necessary to increase the rate of chromatin rearrangement during various regulatory processes so that these processes can occur in a biologically relevant time frame. Inhibiting these activities (e.g., by Pc-G function) might block nuclear processes by imposing a kinetic barrier to processes that require chromatin remodeling.

Relevant publications from our laboratory:

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Phelan, M.L., Sif, S., Narlikar, G.J. and R.E. Kingston (1999) Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits. Molecular Cell 3: 247-253.

Shao, Z., Raible, F., Mollaaghababa, R., Guyon, J.R., Wu, C-t., Bender, W. and R.E. Kingston (1999). Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell 98:37-46.

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Roles of Cell Division and Gene Transcription in Methylation of CpG Islands

By Peter A. Jones, Christina M. Bender, Carvell T. Nguyen, and Keith D. Robertson

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De novo methylation of CpG islands located in gene promoters is a well established mechanism for transcriptional repression. On the other hand, methylation of CpG islands in the transcribed regions of genes does not block transcription elongation in mammalian cells. We used a quantitative assay for CpG methylation to measure the kinetics of remethylation of the p16 gene promoter and second-exon CpG islands in bladder cancer cells after transient treatment with 5-Aza-CdR to determine the relationship between CpG island methylation and gene transcription. Both CpG islands became remethylated in a process associated with time but not with the rate of cell division and exon 2 became remethylated more quickly than the promoter. The kinetics of remethylation of several other CpG islands located downstream of transcription initiation sites were also examined and remethylation occurred most rapidly in genes which were transcribed prior to drug treatment. The data suggest that de novo methylation is not restricted to the S phase of the cell cycle and show that transcription elongation through CpG islands does not inhibit their remethylation. We also examined the expression of the putative de novo DNA methyltransferases DNMT 3a and DNMT 3b in the cell cycle and compared this with the levels of the more abundant putative maintenance methyltransferase DNMT 1. These experiments showed that both DNMT 1 and DNMT 3b were down regulated in the G1 phase of the cell cycle, whereas DNMT 3a was less subject to down regulation in G1.

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Interactions of Yeast and Mammalian SMC Proteins with DNA and Meiotic Chromatin

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SMC (Structural Maintenance of Chromosomes) proteins interact with DNA in chromosome condensation and gene dosage compensation (SMC2 & 4), and sister chromatid cohesion and DNA recombination (SMC1 & 3, RAD18). We present data on the interaction of individual SMC proteins and functional SMC domains with DNA and meiotic chromatin. The C-terminal and coiled-coil domains of *S. cerevisiae* and bovine SMC1, SMC2 and SMC3 proteins bind DNA in an ATP-independent manner with three levels of specificity: (1) a >100-fold preference for double-stranded versus single-stranded DNA; (2) a high affinity for DNA fragments able to form secondary structures and for synthetic cruciform DNA molecules; and (3) a strong preference for AT-rich DNA fragments of particular types. Contrasting other cruciform DNA-binding proteins like HMG1, the SMC C-terminal and coiled-coil domains do not bend DNA, but rather prevent bending in ring-closure assays. The bovine recombination protein complex RC-1 and its isolated bSMC1/3 heterodimer subunits reanneal complementary DNA strands, and are active in a 3-strand transfer assay, which entails annealing of a single-strand DNA to a plasmid DNA substrate, creating recombinogenic DNA structures. Immunofluorescence on rat spermatocyte nuclei spreads localized SMC1 and 3 proteins in a beaded structure along prophase I chromosomes, where the proteins appear to associate with the axial cores of synaptonemal complexes (SCs). Immunoblotting revealed SMC1 and 3 in rat spermatocytes and enriched in SC preparations. The synaptonemal complex-specific proteins SCP2 and SCP3 co-immunoprecipitate and co-immunopurify with the SMC1/3 heterodimer indicating novel protein interactions and complexes in meiotic cells.

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Cancer, aging and the condition of our genes

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All forms of life have developed sophisticated mechanisms to protect their genes against the noxious effects of ubiquitous DNA-damaging agents, which threaten life, cause inborn defects and induce cancer. An important component of the DNA care-taking apparatus is an intricate network of DNA repair systems that constantly surveys the genome for lesions. One of the most versatile repair pathways is nucleotide excision repair (NER). Inborn defects in NER are associated with three distinct syndromes characterized by marked sun (UV) sensitivity and signs of premature aging. Xeroderma pigmentosum (XP) patients show cutaneous abnormalities, including a >1000x-increased risk of cancer and frequently accelerated neurodegeneration caused by defects in one of 7 genes (*XPA* to *XPG*). Cockayne syndrome (CS) displays severe neurological dysfunction and early developmental arrest and death due to defects in *CSA* or *CSB*, which are specifically involved in cleaning active genes from DNA lesions. Trichothiodystrophy (TTD) is characterized by brittle hair and nails and ichthyosis in addition to CS symptoms. Remarkably, mutations in *XPB* and *XPD* can give rise to XP, XP combined with CS or TTD. These genes were found to encode subunits of the dual functional repair-transcription factor TFIIH and are thus implicated in NER as well as basal transcription. To investigate the striking clinical heterogeneity associated with defects in these genes we have mimicked in the mouse germ line a single amino acid substitution in *XPD* as found in a TTD patient. TTD mice reflect to a remarkable degree the phenotype of the human disorder, including brittle hair and various signs of premature aging: reduced life span (average age ~7 months), early developmental arrest, neurological symptoms, osteoporosis, kyphosis, early depigmentation of hair, starvation etc. Also the NER defect causes cancer predisposition not noted in the human patients. The aging features appear to become dramatically enhanced when the partial NER defect of TTD mice is made complete by crossing with XPA mice that are entirely deficient NER but develop normally. We propose that DNA damage compromises the transcriptional capacity of the cell in aging TTD mice. This suggests that the symptoms of premature aging are due to accumulation of endogenously generated DNA lesions interfering with transcription. These findings link aging with the condition of our genes and have important consequences for the molecular basis of aging.

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Session 3: Transcription and DNA Repair (I)

Chair: Philip C. Hanawalt

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Role of transcription-coupled DNA repair in genomic stability and human genetic disease

Philip C. Hanawalt

Excision repair is an important mechanism by which a wide variety of DNA alterations can be recognized and repaired, to maintain genomic stability and cellular viability. There are two branches of excision repair, global genomic repair (GGR) that deals with lesions throughout the genome and transcription-coupled repair (TCR) that specifically targets lesions in the transcribed strand of expressed genes (1). In mammalian cells only those genes transcribed by RNA polymerase II are subject to TCR and the mechanism is thought to involve the initial arrest of the translocating polymerase at the lesion site. The complex of the polymerase, RNA product, and DNA template is very stable but the polymerase may either reverse translocate or be removed from the DNA to facilitate access of repair enzymes to the lesion (2). Current models for TCR and implicated genes will be reviewed. The efficiency of TCR does not vary over the cell cycle for a gene that is continually expressed (3). Efficient GGR of UV induced cyclobutane pyrimidine dimers (CPDs) has been shown to require activation of the p53 tumor suppressor, although the efficient repair of the more structure-distorting 6-4 photoproduct is much less dependent upon p53 induction (4). There is a remarkable parallelism between the regulation of GGR by the inducible SOS response in bacteria and the p53 dependent pathway in human cells (5). Recent studies have shown that, like p53-defective cells, xeroderma pigmentosum group E cells are deficient in GGR of CPDs, but proficient in TCR. The expression of the p48 xeroderma pigmentosum gene is dependent upon p53 and appears to be the link between p53 and GGR (6). The implications of p53-dependent GGR and constitutive TCR will be discussed in terms of their implications for cell survival, mutagenesis, and oncogenic transformation. These findings may be relevant to the use of rodent models in carcinogenicity testing since the rodent cells are deficient in the inducible GGR of CPDs and perhaps some other types of DNA lesions.

These results, in concert with recent evidence that transcription arrest is an important signal for stabilizing p53 and initiating apoptosis, provide a plausable model to explain the low incidence of sunlight-induced skin cancer in Cockayne's syndrome patients (defective in TCR) compared to the remarkable cancer prone phenotype in xeroderma pigmentosum patients (defective in GGR). The characteristic deficiencies in neurological development and growth in Cockayne's syndrome may be the consequence of inappropriate apoptosis initiated by endogenous oxidative damage in expressed genes (1).

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DNA lesions, transcription blockage and transcription-coupled repair: focus on mechanisms and biological consequences

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DNA damage induced by a broad range of agents threatens cellular integrity largely by affecting vital processes such as replication and transcription. Several findings have indicated an intimate link between DNA damage, repair and transcription (1,2). Upon infliction of DNA damage, transcription is initially inhibited. The restoration of inhibited transcription is thought to be dependent on removal of transcription blocking DNA lesions in expressed genes by a specialized repair pathway termed transcription coupled repair (TCR) (1). DNA lesions in nontranscribed sequences are repaired by a general repair pathway called global genome repair (GGR). Xeroderma pigmentosum (XP) and Cockayne syndrome (CS) are human genetic disorders associated with defects in nucleotide excision repair (NER) either affecting GGR, TCR or both. We have assessed the roles of TCR and GGR in repair of structural different lesions, transcription recovery, mutagenesis and (acute/long term) biological effects in mammalian cells and transgenic mice with defined deficiencies in the NER sub-pathways employing different genotoxic agents

TCR and acute (and long term) effects of DNA damage: Human and mouse *XPC* fibroblasts are defective in GGR of UV photolesions, but exhibit a normal level of TCR. The key role of TCR in alleviating the adverse effects of DNA damage is illustrated by a 10-fold higher resistance of *XPC* mice to acute toxic effects (erythema, killing) of UVB irradiation and DMBA treatment when compared to *XPA* (defective in GGR and TCR) or *CSB* (defective in TCR) mice (3). At least in case of UV B exposure, the differential sensitivity of the various genotypes correlates with the apoptotic response: TCR efficiently counteracts UVB induced apoptosis in the epidermis. In spite of the resistance to acute UVB effects, *XPC* are as prone as *XPA* mice to skin cancer induction by UVB irradiation whereas skin cancer develops more slowly in *CSB* mice. This indicates that expression of GGR (and to lesser extent TCR) is critical for counteracting UVB induced skin cancer in mice.

TCR and mutagenesis: The protection of TCR against skin carcinogenesis in rodents most likely relates to its antimutagenic action. UV induced mutation frequencies in the HPRT gene in hamster cells defective in TCR, are clearly enhanced. The spectrum of mutations is different from repair proficient cells and resembles that of cells lacking both TCR and GGR. Most of the base substitutions in TCR defective cells originate from DNA photolesions in the transcribed strand suggesting that defective TCR of photolesions causes the enhanced UV-induced mutagenesis in these cells (4). A similar bias in strand distribution is seen in P53 mutations in UVB induced skin tumors in mice (5).

TCR and transcription response: Restoration of inhibited transcription is thought to be mediated by transcription coupled repair (TCR) of transcription blocking DNA lesions in expressed genes. However, this model has been challenged by experiments with Cockayne syndrome cells exposed to NA-AAF, in which these cells failed to recover transcription in spite of an efficient repair of NA-AAF induced lesions in transcriptionally active genes (6). Therefore, we have proposed that failure to recover RNA synthesis accounts for the increased sensitivity of CS cells to DNA damaging agents and that transcription inhibition might be partly due to a trans mechanism. To resolve the molecular mechanisms underlying the inhibition and recovery of transcription upon infliction of UV-photolesions, we assay nuclear extracts from human cells exposed to UV for their transcription activity using an in vitro transcription system with undamaged DNA as substrate. We observe an inhibition of transcription activity in nuclear extracts prepared from UV-exposed normal human

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fibroblasts and fibroblasts directly after exposure. The transcription activity is restored in normal extracts but not in Cockayne syndrome extracts prepared 6 hours after UV-exposure; this is imilarly to the situation in intact cells. These findings provide evidence that the initial inhibition of transcription by UV might occur by trans mechanisms rather than by inhibition of elongation of transcription. Western analysis of the nuclear extracts reveals that the inhibition of the transcriptional activity correlates with the loss of the hypo-phosphorylated RNA polymerase II (pol IIa) in the extracts and an increase in the level of the hyper-phosphorylated RNA polymerase II (polIIo). Following post UV-incubation, RNA polIIa reappears in extracts from normal cells but not in those from CS cells, suggesting that the defect in CS cells to recover RNA synthesis may be due to an inability of these cells to recycle or resynthesize RNA polIIa. In addition, components of basal transcription factor TFIIH e.g., XPB and XPD are known to be associated with a CS phenotype and it is conceivable that modifications of TFIIH may play a role in transcription restoration after DNA damage. One possibility is that upon DNA damage induction, transcription factor TFIIH becomes involved in repair and that CS gene products are somehow involved in the conversion of TFIIH back to transcription initiation. In this view CS proteins act as repair-transcription uncoupling factors. We have investigated the nuclear distribution of TFIIH by employment of specific antibodies and immunofluorescence microscopy. The results of this approach clearly suggest profound changes in the nuclear organization of TFIIH upon exposure to UV.

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The interface between DNA repair and transcription: *in vivo* molecular dynamics and mechanisms.

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Numerous environmental and endogenous agents damage DNA and thus interfere with its primary functions: transcription and replication. In addition, genotoxins induce mutations leading to inborn defects and cancer. One of the mechanisms counteracting the deleterious effects of DNA injury is nucleotide excision repair (NER) which eliminates UV-induced lesions as well as bulky chemical adducts and intra strand crosslinks in a 'cut and paste'-type of reaction. The biological impact of this system is unveiled by several human NER syndromes characterized by cutaneous symptoms (including photosensitivity and skin cancer susceptibility), as well as accelerated neurodegeneration. Two NER subpathways exist: global genome repair covering the entire genome, and transcription-coupled NER for fast repair of transcription-blocking lesions to allow rapid resumption of transcription. Gene cloning and establishment of an *in vitro* assay have culminated in a detailed model for the core of the NER reaction. However little is known about the mode of action, dynamics and organization of NER *in vivo* as well as its cross talk with transcription, chromatin remodeling and cell cycle control. To understand the functioning of this system in living cells we have tagged NER products with green fluorescent protein. The tag did not interfere with NER function. Confocal analysis and photobleaching revealed that all tagged proteins are mobile and have different diffusion rates and nuclear distribution. This argues against the existence of a preassembled NER 'holocomplex'. After induction of DNA damage a fraction of NER proteins becomes transiently immobile in a dose- and time-dependent fashion, corresponding with one repair event. These findings favour a model involving consecutive assembly of NER factors on the site of a lesion. NER proteins involved in NER and basal transcription exhibited dramatic changes in intra nuclear distribution and mobility in response to DNA damage. These results provide insight into the nuclear dynamics of inducible processes in living cells.

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Interactions between chromatin structure, transcription, nucleotide excision repair and photoreactivation.

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DNA is continuously damaged by intra- and extracellular DNA damaging agents. Unless repaired, DNA-lesions affect gene expression and may lead to mutations, cell death and cancer. In recent years, it has become increasingly evident that damage formation and repair are intimately coupled to structural and dynamic properties of chromatin. We use yeast *S. cerevisiae*, which contains genes with well characterised chromatin structures and transcriptional properties, to investigate the formation of DNA-lesions by ultraviolet light (cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs)), and their repair by DNA- photolyase (photoreactivation, PR) and nucleotide excision repair (NER). The following results will be discussed:

(i) TATA binding protein promotes the selective formation of UV-induced 6-4 PPs and modulates DNA repair in the TATA boxes of the *SNR6* - and *GAL10* - genes, transcribed by RNA-polymerase III and RNA-polymerase II, respectively (8).

(ii) Folding of DNA in nucleosomes affects DNA structure and the formation of CPDs (2).

(iii) Nucleosome structure and positioning modulate CPD repair by photolyase and NER (3-6).

(iv) PR and NER serve complementary roles in repair of active genes transcribed by RNA-polymerase II. DNA-photolyase is inhibited by RNA-polymerases stalled at CPDs and preferentially repairs the non transcribed strand, while NER preferentially repairs the transcribed strand (transcription coupled repair) (4, 5). No TCR, but a mild inhibition of NER and PR, is observed in the transcribed strand of genes transcribed by RNA-polymerase III (7).

(iv) PR is the predominant pathway to repair CPDs in open promoters and origins of replication. The role of NER is to remove 6-4PPs and CPDs that are not accessible to photolyase (5, 8).

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Chromatin assembly and DNA repair

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Sensing DNA damage is crucial for the maintenance of genomic integrity and cell cycle progression. The participation of chromatin in these events is becoming of increasing interest. We identified in various systems a connexion between chromatin assembly and NER. We have now extended this analysis to a wider range of repair pathways. At the molecular level, we found that the human Chromatin Assembly Factor-1 (CAF-1) could participate in these processes. Interestingly, this factor has been involved in linking chromatin assembly to various aspects of DNA metabolism, such as DNA replication and heterochromatin formation. We will present our recent work on the topic and discuss the possible importance of factors connecting various aspects of DNA metabolism.

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Session 4: Transcription and DNA Repair (II)

Chair: Tomas Lindahl

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Cross-talk between the Nucleotide Excision-Repair and Base Excision-Repair Pathways

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The two main pathways for excision of damaged residues from DNA in mammalian cells, BER and NER, in general appear to be completely distinct from each other, recognizing different DNA substrates and employing separate and non-overlapping protein factors. However, several instances of interaction or sharing of activities between BER and NER have come to light recently.

A minor alternative "long-patch" pathway of BER has been analyzed and reconstituted with purified proteins : It employs factors such as PCNA, FEN1, Pol δ/ε, and DNA ligase I also used in the latter stages of NER as well as in lagging-strand DNA replication (1,2).

Studies in other laboratories have indicated that the NER protein XPG stimulates cellular repair of oxidized pyrimidine residues such as thymine glycol by BER, and that loss of this XPG function may account for the severe form of Cockayne syndrome seen in XPG deletion mutants. We have shown in biochemical experiments that purified XPG protein acts as a DNA loading factor for the human thymine glycol-DNA glycosylase, hNth1. Thus, in addition to its essential function in NER, XPG has a second different role as an activity promoting BER of oxidative damage (3). Different regions of XPG are required for its functions in NER *vs.* BER.

DNA damage inflicted by reactive oxygen species is generally removed by BER, but our recent studies with oligonucleotides containing the 8-5' cyclopurines cyclodeoxyadenosine or cyclodeoxyguanosine in both the R and S stereoisomeric forms show that these γ-ray induced lesions are refractory to BER and are only detectably excised by NER (4).

Knockout mice defective in the 8-oxoguanine-DNA glycosylase, OGG1, have been constructed. These mice accumulate 8-oxoG in their genomes but show weak backup repair activity, which is not due to another DNA glycosylase but appears to be caused by transcription-coupled NER of 8-oxoG residues (5).

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The implications of these interactions and shared substrate specificities between BER and NER will be discussed.

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

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Analysis of UV-induced mutations at adjacent pyrimidine showed that mutations in *Bacillus subtilis* wild-type cell arose predominantly from the nontranscribed strand, but arose largely from the transcribed strand in the *mfd* strain. The purified *B. subtilis* Mfd protein has a native molecular mass of 140 kDa (expected molecular mass 133 kDa). The Mfd protein, which is a monomer in solution, showed to be a sequence-independent DNA binding protein with weak ATPase activity, but lacks DNA helicase activity. The Mfd protein was able to displace *in vitro B. subtilis* or *E. coli* major vegetative RNA polymerase stalled at a lesion. Like *E. coli* Mfd [Selby & Sancar (1993) Science 260, 53-58; (1995) J. Biol. Chem. 270, 4882-4889)], *B. subtilis* Mfd protein appears to target the transcribed strand for repair by recognizing a stalled RNA polymerase and dissociating it from the DNA.

Inactivation of *B. 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.

A *B. subtilis* mutant in *mfd* partially relieves carbon catabolite repression (CRR) of the *hut* [Zalieckas *et al.* (1998) Mol. Microbiol. 27, 1031-1038)]. It was proposed that the co-operative binding of the CRR repressor at its target site works as a transcriptional roadblock stalling RNAP which is then displaced by Mfd. A RNAP encountering a pause signal at a hairpin stabilising region could form a DNA:RNA hybrid pause (transcriptional pausing) to allow a Mfd-mediated rearrangement of the transcriptional complex.

This work was partially supported by DGICYT (PB96-0817) to J.C.A. We are very grateful to M. Salas for providing us with *B. subtilis* RNA polymerase, to J. E. Hearst and to A. Sancar for the gift of a psoralen-modified oligonucleotide.

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HYPERMUTATION OF ANTIBODY GENES.

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The somatic integration of V- D- and J- gene fragments introduces the diversity of the primary (naive) repertoire of antibodies which is restricted to the contribution of the germ line genes and to variants at the boundaries where segments are joined. Antigen stimulation induces a selected set of these naive B-cells to multiply and differentiate and as a consequence two independent genetic events take place. One is hypermutation and the other is class switch. Hypermutation is calculated to occur at an average maximum rate which approximates 10^{-3}/base pair/generation. Over 95% of the mutation events are point mutations.

It has long been suspected that hypermutation and gene expression were somehow connected. The first indication came from the observation that the 5'-hypermutation boundary was at or near the promoter. However, it is likely that hypermutation does not start very near the promoter.but about 185 bases from the initiation of transcription (Rada *et al.*, 1997). However, this does not exclude a close connection between hypermutation and transcription. Indeed there are other arguments that points towards such an association.

The efficiency of hypermutation is seriously impaired by deletion of the 3' and intron enhancer elements and in particular, by removal of the MAR segment located in the J-C intron (Betz *et al.*, 1994; Goyenechea *et al.*, 1997). The VJ segment when replaced by other sequences (including bacterial gpt, globin (Yelamos *et al.*, 1995) or the C region gene (Peters& Storb, 1996) is capable of hypermutation if it is expressed in the context of the normal promoter-enhancers. The promoter can be substituted by other promoters e.g. globin (Betz *et al.*, 1994) or even a pol I dependent promoter (Fukita *et al.*, 1998), with relatively low effect on hypermutation.

Possibly the most direct connection between transcription and hypermutation, comes from comparisons between levels of expression and hypermutation. Thus the high expressors of a transgene are the most mutated even though the endogenous gene hypermutation is similar in both subpopulations (Goyenechea *et al.*, 1997). Furthermore mRNA levels expressed in endogenous (or genetically altered) heavy chain genes driven by different promoters correlated with the levels of hypermutation (Fukita *et al.*, 1998).

The possibility of polarity in the hypermutation mechanism has been analysed at the level of the pattern of amino acid substitutions. The largest body of sequence analysed suggests that while both strands are target for hypermutation, the possibility of a certain preference for one of the strands is not excluded (Milstein *et al.*, 1998).

Overall the results suggest that while hypermutation requires the transcription unit, transcription by itself is insufficient. While other factor(s) seem to be required to prime hypermutation (Hypermutation Priming Factor), recruitment of the factor(s) is likely to have a considerable degree of clonal stability (Goyenechea *et al.*, 1997; Neuberger *et al.*, 1998). However this

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may not be the end of the story. Since the boundary is almost 200 bases downstream of the promoter (Rada *et al.*, 1997), something else may be required (e.g. additional factors in the elongation complex).

There are other pieces of evidence that point towards further complications. The hypermutation pattern of KO Msh2$^{-/-}$ discloses a greately increased proportion of mutations affecting G and C than the wild type counterpart. The same is true for constitutively hypermutating cell lines. We have interpreted these results as the consequence of two separate hypermutation stages taking place in normal animals. One, closely connected to transcription, targets the G and C hot spots characterized by the motif GAGCT (and close homologues) which incidentally is also the heavy chain class switch motif (Dunnick *et al.*, 1980; Ehrenstein& Neuberger, 1999). The second stage targets the AT hot spots, possibly with a marginal preference for the polar mutation of A. This second stage would be defective in Msh2$^{-/-}$ mice and in constitutively hypermutating cell lines (Rada *et al.*, 1998).

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THE DETECTION, SIGNALLING AND REPAIR OF DNA DOUBLE-STRAND BREAKS. **Stephen P. Jackson. Wellcome/CRC Institute, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QR, UK.**

A major focus of my laboratory is to understand the molecular basis by which eukaryotic cells detect DNA damage, mediate its repair and signal its presence to the transcription, cell cycle and apoptotic machineries. Of particular interest to us are DNA double-strand breaks (DSBs), which are generated upon exposure to ionising radiation or radiomimetic chemicals, and which also occur as intermediates in certain programmed genomic rearrangements. Previous work has established that the fundamental mechanisms of DNA DSB repair and DNA damage signalling are highly conserved throughout eukaryotic evolution (1). In my presentation, I will discuss two specific examples of our recent work.

First, I will address the *Saccharomyces cerevisiae SIR2*, *SIR3* and *SIR4* gene-products, which are involved in transcriptional silencing at telomeres and the silent mating loci - probably through facilitating the formation of repressive heterochromatin - and which are also required for the non-homologous end-joining pathway of DNA DSB repair to occur accurately and efficiently. I will show that DNA damage, and DNA DSBs in particular, can trigger the reversible loss of telomeric silencing and the intra-nuclear relocation of Sir3p (2). Furthermore, I will show that this response depends on key DNA damage-signalling components. This work suggests a model in which signalling of DNA damage releases Sir3p from telomeres and permits its subsequent association with other nuclear subdomains, possibly in order to regulate transcription, participate in DNA repair and/or enhance genomic stability by other mechanisms.

Second, I will discuss our recent work on the forkhead-associated (FHA) domain, which is present on a variety of proteins involved in nuclear signal transduction pathways, including the key *S. cerevisiae* DNA damage checkpoint-signalling protein Rad53p. Specifically, I will show how our analyses have revealed the FHA domain to be a phospho-dependent protein-protein interaction motif (3). The implications of this mode of interaction for the mechanisms of DNA damage signalling and of other intracellular signal transduction pathways will be discussed.

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Molecular mechanisms of replication restart: processing of replication forks by enzymes of recombination.

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Lesions in DNA frequently lead to stalled replication forks, preventing fork progression and, ultimately, the transmission of genetic material. Stalled forks collapse to generate DNA ends which must be repaired by recombination with the sister chromatid in order for replication to proceed. Plausible enzymatic mechanisms by which such DNA ends are repaired to allow fork reassembly have been proposed based on the known *in vitro* properties of recombination enzymes in *Escherichia coli*. However, illegitimate recombination events may have potentially lethal consequences by promoting chromosome instability. Such problems could be avoided if damaged forks are rescued without the generation of DNA ends.

Recent work has demonstrated that arrested replication forks can form Holliday junctions in *E. coli*, and that these may be processed by recombination enzymes to allow replication restart without chromosome breakage. We have found that replication fork structures can be directly targeted by recombination enzymes *in vitro*. Specifically, RecG forms Holliday junctions from replication forks which can then be acted upon by RuvAB and RuvC. The action of the replisome assembly protein PriA at fork structures also indicates that RecG and PriA both target such DNA structures but with different consequences. Thus recombination and replication enzymes interact to allow replication to proceed in an environment of continual fork damage. Evidence from eukaryotic systems suggests that such mechanisms are conserved throughout all organisms.

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Session 5: Transcription and Recombination

Chair: James E. Haber

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DOUBLE-STRAND BREAK REPAIR IN HUMAN CELLS.

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Double-strand breaks in DNA (DSBs) are induced by the harmful effects of ionizing radiation. These chromosomal breaks can be lethal to the cell unless repaired efficiently, and inefficient or inappropriate repair can lead to mutation, gene translocation and cancer. In lower eukaryotes, such as yeast, DSBs are primarily repaired by Rad52-dependent homologous recombination. In contrast, vertebrates repair most DSBs by Ku-dependent non-homologous end-joining (NHEJ). Using cell-free extracts prepared from human lymphoblastoid cell lines, an *in vitro* system for DNA end-joining has been developed (1). Intermolecular ligation is accurate, dependent upon DNA ligase IV/Xrcc4, and requires Ku70, Ku86 and DNA-PK$_{cs}$, the three subunits of the DNA-activated protein kinase DNA-PK.

Mammalian cells can also repair DSB's by homologous recombination (either by Rad52-dependent single-strand annealing, or by Rad51/Rad52-dependent strand invasion mechanisms). Human Rad51 protein is a structural and functional homolog of the *E. coli* RecA protein and promotes homologous pairing and strand transfer reactions in vitro. Until recently, however, little was known about Rad52. Biochemical studies now show that Rad52 forms ring structures on DNA and can stimulate Rad51-mediated pairing reactions (2, 3). Rad52 also promotes specific DNA-DNA interactions in the absence of hRad51, consistent with a direct role in single-strand annealing (SSA) pathways of recombination.

Using electron microscopy, we found that human Rad52 protein, like Ku, binds directly to DSBs, protects them from exonuclease attack and facilitates end-to-end interactions (4). These observations lead us to propose a model for DSBR in which either Ku or Rad52 binds the DSB. Ku directs DSBs into the NHEJ repair pathway, whereas Rad52 initiates repair by homologous recombination. Ku and Rad52, therefore, direct entry into alternative pathways for the repair of DNA breaks.

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Effect of Chromosome and Chromatin Structure on Recombination in Saccharomyces.

James E. Haber

The accessibility of DNA sequences for recombination is apparently governed by the secondary structure and the chromatin structure of DNA sequences as well as by their localization within the nucleus. We have been investigating a number of aspects of they way chromosome and chromatin structure governs the outcome of repair of a double-strand break (DSB) induced by HO endonuclease.

1. Donor preference during *MAT* switching. After HO cleaves *MAT*, it can be repaired by recombination with one of two normally unexpressed donor sequences, *HML* and *HMR*, that have a heterochromatic structure. Although HO cannot cleave equivalent sites in the donors, the cut ends of MAT DNA can – with the help of recombination proteins – invade this "closed" environment to initiate recombination.

The preferential use of *HML* by *MAT***a** cells and the choice of *HMR* by *MAT*α cells is a fascinating example of control of a very large chromosome region that affects recombination, but not transcription (Wu et al., 1998). The use of *HML* in *MAT***a** depends on the activity of a cis-acting Recombination Enhancer, a sequence we have now whittled down to less than 200 bp and which resides at about kb30 on the left arm of chromosome III. RE controls the ability of a donor placed at several sites along the entire left arm of this chromosome to participate both in HO-stimulated recombination and in recombination between *leu2* heteroalleles. We think of this element as yeast's locus control region, with analogies to the globin locus and immunoglobin loci in humans. RE is "on" in *MAT***a** cells and turned off in MATα cells, with a dramatic change in chromatin structure in the 2.5 kb surrounding RE (but not along the rest of the chromosome arm). Recent studies of the control of RE and the way it may work at such long distances will be presented.

2. Accessibility of donor sequences for recombination. Efficiency of recombination is also governed by chromatin structure. For example, if *HMR* is replaced by similar sequences lacking the cis-acting silencer elements, a *MAT***a** cell uses *HMR* as often as *HML*, providing evidence that the openness of chromatin structure can overcome the normal donor competition between two silent donors. A similar reversal happens with *HML* in *MAT*α cells.

When *MAT* and *HMR* are placed on a plasmid, recombination requires Rad52p, Rad51p and several other important recombination proteins; but when the closed donor sequence is replaced by an "open" donor (with a mutation that prevents HO cutting) recombination can occur (although inefficiently) without Rad51p, Rad54p and Rad57p, but absolutely requires Rad52p.

The idea that there is Rad51-independent recombination was surprising. We also showed that *RAD51*-independent recombination occurs between homologous chromosomes, but with an important difference. Here, in a Rad^+ cell, recombination occurs almost always by gene conversion; but without Rad51p (or Rad54p or Rad55p and Rad57p), recombination occurs by break-induced replication (BIR) (Malkova et al., 1996). We have more recently investigated the way strand invasion can occur in this chromosomal system. A surprising connection between replication and recombination has emerged.

3. DNA structure itself influences the efficiency of recombination. We have recently shown that DNA repair synthesis is substantially less efficient (processive) than normal replication (Pâques et al., 1998). This is evident from the fact that the efficiency of gap repair induced by a DSB falls significantly as the size of the gap increases. Moreover, the efficiency of repair is influenced by the sequences the DNA repair polymerases must traverse. For example, efficiency of copying 300 bp of CAG repeats is only 60% of that for copying CAA repeats or random

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sequences. CAG repeats form hairpin structures and we believe that the repair polymerases are less able to copy these sequences. We also find a dramatic increase in CAG rearrangements in recombination compared to replication. This effect is suppressed by overexpression of Mre11p, which is believed to have a role in cutting or unwinding hairpin structures.

The gap-repair system also will permit us to examine the effect of other DNA structures (silenced regions, centromeres, etc.) on recombination, as well as the effects of transcription. Such studies are underway.

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Transcription elongation-associated recombination.

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DNA recombination may be directly affected by other aspects of DNA metabolism. A relationship particularly intriguing is that observed between recombination and transcription. Induction of recombination by transcription has been documented in prokaryotes and eukaryotes (1,2,3). It is likely that changes in local supercoiling or in chromatin structure associated with transcription may facilitate the initiation of a recombination event, although there may be other explanations. Our aim is to define and to understand the molecular nature of the effects that transcription, and hence chromatin structure, may have on mitotic recombination in yeast.

We have provided genetic and molecular evidence that recombination between direct repeats can be induced by transcriptional elongation impairment. Null mutations of the *HPR1* and *THO2* genes lead to an strong increase in the frequency of deletions occurring by recombination between direct repeats (>2000-fold above wild-type levels) that correlates with an impairment of transcription elongation observed in such mutants (4,5,6). The higher the impairment of transcription caused by *hpr1Δ* and *tho2Δ*, the higher the frequency of deletions. The hyper-deletion phenotype is transcription-dependent and is not observed in DNA repeats that are not transcribed. We have now evidence that reciprocal recombination is also stimulated by these mutations. The *tho2Δ* mutation increases recombination between inverted repeats above 20-fold the wild-type levels. This result suggests that transcriptional elongation impairment may induce all types of recombination.

A new search of mutants with a transcription-dependent hyper-recombination phenotype has led us to identify a new yeast gene, *THO3*, whose null mutation confers identical phenotypes as *hpr1Δ* and *tho2Δ*. We will present data on the genetic and molecular analysis of *tho3Δ* mutants indicating that they are affected in transcription elongation. These results suggest that Hpr1p, Tho2p and Tho3p functionally interact in the same molecular process, which has to be related with transcription elongation. Whether or not the effect of these three proteins on transcription elongation is direct or indirect, they may not be related with other known proteins of transcription elongation, such as Spt4p, Spt6p, Spt16p, Elc1p, TFIIS, since mutations in their structural genes do not lead to the same phenotypes of genetic instability. Therefore, our genetic approach is defining a new class of eukaryotic genes that connect transcription with genetic instability.

To explore whether chromatin structure might have an effect on mitotic recombination that could explain the observed effects of

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transcription, we have analyzed recombination of different mutants related to chromatin structure and transcription. Mutations in *SPT2*, *SPT4* and *SPT6* increases recombination up to 15-fold above the wild-type levels. Interestingly, induction of recombination by *spt6-140* is only observed under transcription conditions. When the DNA region involved in the recombination event is not transcribed, *spt6-140* has no significant effect on recombination. We propose that chromatin structure is a barrier for recombination. The *spt6-140* alters chromatin structure facilitating the initiation of the recombination process. This effect is enhanced by transcription, which opens chromatin structure and, thus, facilitates recombination. Given the recent involvement of *SPT4*, *SPT5* and *SPT6* in transcription elongation (7), it is possible that hyper-recombination in *spt* and *hpr1*, *tho2* and *tho3* mutants share similar mechanisms.

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Mechanistic Intermediates in Immunoglobulin Class Switch Recombination.

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Class switch recombination is the process by which the immunoglobulin heavy chain isotype changes from Igm to Igg, Iga or Ige, thereby permitting the production of IgG, IgA, or IgE, instead of IgM. This genome rearrangement is specific to B-cells of vertebrates, and the manner in which the process is initiated has no relationship to V(D)J recombination. In fact, there is no other prokaryotic or eukaryotic DNA recombination process like class switch recombination. Among its distinctive features are that it can occur anywhere within 2 to 10 kb class switch regions. These regions consist of sequences that C-rich on the template strand and repetitive, with a repeat length of 25 to 80 bp. Despite the fact that switch regions are positioned within introns, all seven of the switch regions have accompanying upstream promoters that have no obvious purpose, since the G-rich transcripts have been shown by others not to generate a protein. The function of the G-rich transcript has remained unclear.

In vitro transcription of any of the switch regions (on supercoiled plasmids) using phage polymerases results in the stable association of the RNA with the DNA to generate an RNA:DNA hybrid. This RNA:DNA hybrid is resistant to E. coli RNase A, high temperature, high salt, and phenol extraction. However, the RNA:DNA hybrid is extremely sensitive to E. coli RNase H. These studies raised the possibility that such a structure might form in vivo.

Recently, we have been able to demonstrate that an RNA remains stably associated with the class switch DNA sequences in the nucleus of murine B cells. The RNA:DNA hybrid could be demonstrated by Northern blot or by RT-PCR. The RNA:DNA hybrid is only formed in B cells stimulated with cytokines that activate the specific promoters upstream of the switch regions. The RNA:DNA hybrid is not detected in the same cells prior to stimulation. The RNA in the hybrid is the one that arises from the intron promoter upstream of each switch region. The RNA is of variable length, consistent with the repetitive nature of the switch regions and with structural data indicating RNase A sensitive interruptions within the several kilobase RNA:DNA hybrid zones.

In order to determine if this structural feature is essential for class switch DNA recombination, we generated a recombinant mouse that expresses the E. coli RNase H gene in all tissues. The mice are grossly normal, have normal numbers of B and T cells, normal lymphoid tissue architecture histologically, and normal blood levels of IgM, IgG, IgA, and IgE. B cells from the mice fail to form the RNA:DNA hybrid at nearly the levels of wild type cells. Direct assessment of the B cells for class switch recombination by using PCR to detect switch recombination demonstrates that it is significantly reduced.

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We further examined the RNase H mice for ability to generate switched isotypes in antigen-specific and in hapten-specific secondary immune response time courses. The RNase H mice produced normal levels of IgM in the primary exposure to antigen. Strikingly, in the secondary immune response, the IgG levels were consistently reduced.

We conclude that the RNA:DNA hybrid suggested by our previous in vitro studies actually forms in the mammalian nucleus and serves as an essential target for the recombination process. We believe that this target is recognized by a nuclease that cleaves both DNA strands during the recombination process.

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Regulation of Intermolecular V(D)J Recombination

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V(D)J recombination, normally an intramolecular process, assembles immunoglobulin and T cell receptor genes from V, D, and J coding segments. Oncogenic chromosome translocations can result from aberrant rearrangements, such as occur in intermolecular V(D)J recombination. How this is normally prevented remains unclear; DNA cleavage, joining, or both could be impaired when the recombination signal sequences (RSS) are located in *trans*, on separate DNA molecules. Several mechanisms guard against inappropriate rearrangements. That efficient cleavage requires synapsis of a 12/23 RSS pair provides one important level of control. A second regulatory mechanism, not directly related to the V(D)J recombination machinery, controls the accessibility of antigen receptor loci to the recombination machinery, which is mediated by chromatin structure.

We have recently found that both *trans* cleavage and joining of signal ends occur efficiently *in vivo*. Unexpectedly, *trans* joining of coding ends is severely impaired (100- to 1000-fold), indicating that protection against intermolecular V(D)J recombination is established at the joining step (1). Perhaps the defect in joining coding ends produced by *trans* cleavage described here serves as a last line of defense–a checkpoint–that functions to remove cells that have initiated potentially dangerous interchromosomal V(D)J recombination events. In this case, the persistence of unrepaired coding ends, detected as free ends by the p53-dependent DNA damage sensing machinery, may signal apoptosis of cells bearing potential oncogenic DNA rearrangements.

The mechanisms responsible for blocking intermolecular coding joint formation are currently under investigation. Interestingly, these events may be related to processes that regulate recombination in *cis*. This possibility was suggested by recent analysis of TCRβ enhancer-deficient mice, which revealed that cleavage and signal joint formation are relatively unaffected, but coding joint formation is severely inhibited (2). Enhancers may shorten the "functional" distance between RSS, perhaps by forming locally constrained chromatin domains. Enhancers and chromatin factors could also play a more direct role in aiding formation of coding joints. This hypothesis is supported by the observation that the yeast Sir proteins, which are important in telomeric silencing, also play critical roles in DNA end joining. Another possibility is that factors recruited by enhancers could stabilize the coding ends in the post-cleavage complex, facilitating coding joint formation. Thus, our ongoing investigations into the mechanisms responsible for blocking coding joint formation in *trans* may yield important insights into the processes by which enhancers and chromatin factors regulate V(D)J recombination.

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Transcription coupled somatic hypermutation studied on an inducible *GFP* transgene.

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Recent observations suggest a critical role of transcription for the somatic hypermutation mechanism at the immunoglobulin (Ig) loci. During somatic hypermutation, a million fold increased mutation rate occures in a 1-2 kb region encompassing the rearranged variable region exon of the Ig genes. The transcription initiation site determines the 5'boundary of the hypermutated region and the transcription level seems to affect the mutant frequency. In order to elucidate the influence of transcription on the hypermutation mechanism, we developed a novel mutation detection assay A premature stop codon has been introduced into a green fluorescent protein (GFP)-reporter gene. GFP becomes expressed in stable transfectant clones only upon stop codon reversion. Revertant cells can be easily and reliable detected by FACS analysis. In addition, the expression level of the GFP can be directly measured for individual revertant cells. Thus, a putative connection between reversion rate and expression level can be studied. We report here that the *GFP* -reporter gene mutates in the hypermutation active cell line 18-81 at a rate of about 10-4 point mutation / bp / generation. The mutation rate is directly linked to the transcription level as studied in a tet operon inducible GFP construct. Increasing the transcription level causes an increased point mutation rate at the stop codon.

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METHYL-CPG CONTAINING PROTEINS STABLY ASSOCIATE WITH NUCLEOSOMAL DNA

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Recently, it has been described a new family of proteins containing the methyl-CpG binding domain (MBD), which was initially identified for the first time in MeCP2. These proteins show specificity for methylated DNA. In particular, *Xenopus* MBD3 has been demonstrated to be a component of the Mi-2 complex, the most abundant form of deacetylase in amphibian eggs and cultured mammalian cells. We report here the interaction between MBD3 and other MBD-containing proteins to nucleosomal DNA. We find that MBD3 forms discrete complexes with nucleosomal DNA associating with methyl-CpGs exposed in the major groove via the MBD. These observations provide a molecular mechanism by which MBD3 can gain access to chromatin in order to target the Mi-2 complex which further modifies chromatin structure.

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Transcriptional elongation and genome stability in *hpr1* and *tho2* mutants of *S. cerevisiae*.

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We have previously shown that impairment of RNA pol II-dependent transcriptional elongation is a source of genetic instability. The *hpr1* and *tho2* mutations confer a strong increase in plasmid instability and recombination between DNA repeats and affect transcription elongation. The inability of the mutants to elongate transcription is linked to both the plasmid instability and the hyper-rec phenotypes (1,2).

Elongation impairment and instability are more frequent in certain sequences, being extreme in the commonly used *lacZ* reporter-gene. In order to understand the roles of Hpr1p and Tho2p in transcription elongation and genome instability we have performed an *in vivo* analysis of this sequence dependence. We have isolated short fragments of *lacZ* that are able to impair transcription elongation in the mutants and we have defined the gene context where this impairment is maximal.

We have also analyzed the effect of mutations in *PPR2/DST1*, encoding TFIIS, in *SPT4*, *SPT6* or *SPT16*, involved in chromatin structure and transcription elongation, and other genes putatively involved in RNA PolII elongation. Our results reveal that *HPR1* and *THO2* belongs to a novel class of genes affecting transcription elongation different to those encoding known elongation factors.

To determine a possible physical interaction between Hpr1p and Tho2p, two-hybrid analysis has been performed. The conclusions of this assay open the possibility for the existence of other elements to explain the epistatic relationships between the *hpr1* and *tho2*.

Up to now, the *hpr1* and *tho2* mutations have been associated with high increases of recombination between direct repeats leading to deletions and with increases in plasmid and chromosome loss. To define the whole range of phenotypes related to genome instability caused by these mutations we have determined their effect on a complete set of recombination systems. Our results extend the types of mitotic genome accidents that can be stimulated by *hpr1* and *tho2* mutations.

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The E1A Associated p400 Complex Consists Of Two Different Proteins: TRRAP and p400, a Novel Swi/SNF2 Related Protein

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The adenovirus E1A polypeptides are multifunctional proteins that induce a number of celllular effects ranging from transcription activation and repression to immortalization, blockade of differentiation, stimulation of cellular DNA synthesis, and, together with actvated ras or E1B, transformation of rodent fibroblasts. Among their nuclear interaction partners are the pocket proteins and the p300/CBP transcriptional coactivators. Previous reports described an additional E1A-associated protein of ~400 kD, which unlike p300/CBP cannot bind the E1A mutant Δ26-35. We have purified, microsequenced and cloned two 400kD proteins which coprecipitate with E1A and migrate in gels as a closely spaced doublet. The lower band was identified by microsequencing as a putative homolog of a yeast PI-3-like kinase and is identical to a recently published, c-myc associated protein, TRRAP (McMahon et al, 1998), TRRAP seems to be essential for c-myc+ras and E1A +ras transformation of primary rodent cells and was, in addition, identified as a component of the pCAF histone actelyase complex (Vassilev et al., 1999). Microsequencing of the p400 kD top band revealed a polypeptide with striking homology to the conserved domain of the Swi2/SNF2 family of DNA-dependent ATPases, which are involved in chromatin remodelling processes. P400 differs from other Swi2/SNF2 species in that its putative helicase domain is separated into two segments by a 500 aa intervening sequence.
TRRAP and p400 form stable physical complexes in vivo in the absence of E1A. p400 - like TRRAP - associates with c-myc and other transcription factors.
We will report current progress in characterizing the enzymatic properties of p400 and its role in transcription andE1A/c-myc mediated transformation .

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UvrAB helicase suppresses illegitimate recombination independently of nucleotide excision repair

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Illegitimate recombination is one of major causes of genetic instability in prokaryote as well as in eukaryote. This recombination usually occurs at a low frequency, but it is greatly enhanced by UV irradiation or other environmental stresses. DNA damages induced by these environmental stresses are thought to induce DNA double strand breaks, leading to illegitimate recombination. Here we show that illegitimate recombination is enhanced by mutations of nucleotide excision repair (NER) genes, *uvrA* or *uvrB*, but not by that of *uvrC* or *uvrD*. Unexpectedly, the recombination was enhanced by the *uvrA uvrB* double mutation even without UV-irradiation, but that *uvrB uvrC* double mutation have not shown this effect, suggesting that illegitimate recombination is suppressed by UvrA and UvrB, independently from nucleotide excision repair. Moreover, illegitimate recombination was synergistically enhanced by the *recQ uvrA* double mutation. These results strongly suggest that UvrAB helicase functions as a suppressor of illegitimate recombination independently of NER, and their helicase activity plays a crucial role in the suppression of recombination, possibly via the same pathway in which RecQ helicase functions synergistically.

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Role of Chromatin Remodelling in Silencing and Genomic Stability in *Ascobolus*

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In fungi, plants and animals, methylation of cytosine residues by methyltransferase is a vitally important DNA modification mechanism that controls gene expression. This process is essential for mammalian development, as determined in the mouse by mutations of methyltransferase and of MeCP2 (a protein that binds methylated CpGs), both resulting in embryonic lethality (6,10). *In vitro* reconstitution studies in mammalian systems (4,8) have led to an attractive hypothesis for the molecular mechanism by which DNA methylation stably represses transcription. The series of events leading to transcriptional inactivation are as follows: MeCP2 binds methylated DNA, and its repressor domain recruits a transcriptional co-repressor complex, consisting of mSin3, histone deacetylases, and possibly other chromatin remodelling proteins (3,5,7). Thus, histone deacetylation and methylation appear to be coordinated processes in mammalian cells.

My objective is to isolate genes involved in chromatin remodelling and assay their effects on methylation-induced silencing and genomic stability *in vivo*. I will use the well-characterized model system in the filamentous fungus *Ascobolus immersus*, called methylation-induced premeiotically or MIP. This epigenetic process causes duplicated sequences to become transcriptionally inactivated by extensive methylation of cytosine residues during sexual reproduction (not only CpGs as in vertebrates), and also inhibits homologous recombination of the duplicated regions (2,9). As in mammalian systems, methylation by MIP causes a change in chromatin, coextensive with the region that is methylated (1). However, it is not known whether the same repressional complex associated with CpG methylation in mammals is also involved in MIP. Therefore, the approach I will take is first, to determine by chromatin immunoprecipitation analysis (CHIP), whether methylated sequences by MIP are associated with deacetylation, and whether the same unmethylated sequences are associated with the acetylated or active fraction. It is known in Ascobolus, that trichostatin A, a well-known inhibitor of histone deacetylase, is able to cause global hyperacetylation as determined by Western blot analysis of nuclear extracts enriched for histones. I will use antibodies to specific lysine residues on histone H4 (the targets of acetylation and deacetylation), to determine the pattern as well as the proportion of acetylation of the met2 and hph reporter sequences in the methylated and unmethylated states. This CHIP analysis is currently in progress, and I intend to present the results at this meeting. If I can demonstrate that methylated sequences are associated with deacetylation, then I will identify genes in *Ascobolus*, encoding proteins for chromatin remodelling in response to CpG methylation, namely MeCP2, and the co-repressor protein, mSin3A, and histone deacetylases, as performed in mammals. Once the genes are isolated, they will be tested for their ability to be methylated by MIP. Mutant strains, carrying the inactivated gene of interest, will be assayed for changes in chromatin structure upon methylation of a reporter sequence, for the extent of methylation and transcript levels and for general genomic stability by testing homologous recombination and cross-over frequencies at the spore color locus, *b2*, a meiotic recombinational hotspot in *Ascobolus*.

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Conditional knock out of the transcription factor Sp1

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Sp1 (specific protein 1) was the first eukaryotic transcription factor to be cloned. Sp1 is the founding member of a growing family of transcription factors that contain a DNA binding domain consisting of three Cys_2-His_2 zinc fingers.
Despite being ubiquitously expressed, Sp1 expression levels vary between different cell types and different developmental stages. Elevated Sp1 levels seem to be associated with the differentiation process, but virtually nothing is known about the roles of Sp1 in vivo.
Being a transcription factor, Sp1 contains domains that are essential for transcriptional activation and for the interaction with other transcription factors, like e.g. the TATA-box protein accessory factor $TAF_{II}110$. Binding sites for the Sp1 protein were found in a large number of ubiquitously as well as tissue-specifically expressed genes. Point mutations in Sp1 binding sites have been implicated in familial hypercholesterolaemia and osteoporosis.
Apart from directly influencing the transcriptional regulation of genes, Sp1 seems to play an important role in other processes as well.
Sp1 binding sites seem to be crucial for the maintenance of a methylation-free status of CpG islands, as was shown directly for the CpG island of the APRT gene. The maintenance of appropriate methylation patterns is absolutely essential for normal development.
Sp1 might be involved in organising chromatin and establishing transcriptional competence, since the function of the 5' hypersensitive site 3 (HS3) of the Locus Control Region (LCR) in the human β-globin locus is absolutely dependent on the presence of Sp1 binding sites. This 5' HS3 is the only LCR element that is able to ensure copy-number-dependent and position-independent transcription of the β-globin locus in a transgenic mouse assay.
Sp1 might also be involved in the regulation of the cell cycle. A direct interaction of Sp1 with the cell-cycle regulated transcription factor E2F was shown in vitro.

To study the role of Sp1 in vivo our laboratory inactivated the gene by targeted deletion of the zinc fingers of Sp1. Homozygous Sp1 knock-out mice die around day 10 of gestation. They are severely retarded in development and morphologically abnormal. Despite an extensive analysis of putative target genes, the only candidate in the early embryo identified so far is the MeCP2 gene. The resemblance of the phenotype of the Sp1 knock-out and the MeCP2 knock-out support this finding. It was suggested that both of these genes are required for the maintenance of differentiated cells but dispensable in cell growth and early development.
Because the early death of the homozygous knock-out embryos prevents the investigation of the role of Sp1 in later developmental stages, we generated conditionally targeted Sp1 mice by making use of the Cre-loxP recombinase system.
The use of this system should enable us to knock-out Sp1 in a tissue-specific as well as time-regulated manner. This can be achieved by using mice transgenic for the Cre-recombinase, which is expressed under the control of a tissue-specific promoter. The timing of the activity of Cre will be controlled through a steroid-hormone dependent version of the recombinase. Injection of the exogenous hormone will result in the inactivation of the Sp1 gene product in those tissues expressing the Cre recombinase.
In the induced knock-out, effects of the loss of Sp1 will be monitored by histological examination and expression analysis of putative target genes.

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DNA damage recognition in eukaryotic nucleotide excision repair

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The nucleotide excision repair (NER) machinery embodies an efficient DNA repair mode capable of removing a wide range of DNA lesions in virtually all organisms. In eukaryotes DNA is nucleosomal and some regions can be in a highly condensed state. Nevertheless, DNA damage is efficiently removed throughout the genome.
Recognition of lesions in DNA proceeds via a poorly understood mechanism. In Yeast several proteins or protein complexes have now been identified which may play a role in the damage recognition step of NER. Originally the Rad14 protein was identified as the recognition factor in NER. However recently the Rad7/Rad16 complex has shown to have affinity for damaged DNA as well. Interestingly the function of this complex can be substituted for by the transcription machinery. Damages in transcribed sequences can be recognized by the RNA polymerase which blocks at the site of the lesion and targets repair factors. The fact that the Rad7/Rad16 complex is dispensible when lesions are recognized otherwise suggests it plays a role in recognition itself.
In search for more candidate proteins involved in damage recognition we recently found the Rad4Rad23 complex to bind preferentially to damaged DNA. Our previous genetic work showed that Rad4, like Rad7Rad16, is not essential for repair of all lesions *in vivo*. Additionally, XPC, the human homologue of Rad4 also has been shown to complex to DNA at the site of damage. These observations suggest a role for Rad4/Rad23 in damage recognition.
By mobility shift analysis we have analyzed the binding characteristics of the Rad4Rad23 complex to DNA and found preferential binding of this protein complex to structurally distinct lesions. Using immobilized DNA substrates we were able to bind Rad4Rad23 complexes damage dependently. Elution of bound complexes and subsequent western analysis of the eluates revealed the presence of both Rad4 and Rad23 in the DNA bound complexes.
Studies on human NER showed that Replication protein A, a protein involved in many DNA metabolic processes, was shown to have affinity for damaged DNA, which places also RPA on the list of candidate damage recognition proteins. We have purified yeast Rpa and found it to bind preferentially to damaged DNA like its human homologue.
Several members of the yeast NER machinery have now been implicated in the recognition of the damaged site. The possible involvement of multiple proteins suggests a complex mechanism of damage recognition in eukaryotes. This may be partly due to the chromatin state of eukaryotic DNA which may influence recognition of damage. Possibly the proteins involved bind cooperativily to damaged DNA. Currently we are analyzing their combined action at the site of the DNA damage.

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Genetic analysis of the effects of transcription and chromatin structure on *RAD51*-dependent and -independent mitotic recombination

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In this work we explore the possibility that changes in chromatin structure, whether or not associated with transcription, affect recombination. We have analyzed recombination of different mutants related to chromatin structure and transcription. Mutations in *SPT2*, *SPT4* and *SPT6* increase the frequency of crossovers (CO) /gene conversions (GC) and deletions up to 15-fold above wild-type levels.The genetic and molecular analysis of recombination in *spt6-140* mutants reveals that a significant proportion of the *spt6*-induced recombination events are long-conversion tract events, suggesting an enhanced capacity of the recombination machinery for heteroduplex extension. In the inverted repeat systems that we used, recombination is reduced by 4-10-fold in the *rad51*, *rad54*, *rad55* and *rad57* mutants, leading to a pattern of GC significantly different from wild type. *spt6-140* suppresses this rec- phenotype and restores the wild-type pattern of gene conversion and reciprocal exchange.

Finally, our analysis reveals that induction of recombination by *spt6-140* in a Rad51+ genetic background is only observed under transcription conditions. When the DNA region involved in the recombination event is not transcribed, *spt6-140* has no significant effect on recombination and there is a stronger dependency on *RAD51*.

We propose that chromatin structure is a barrier for recombination. The *spt6-140* alters chromatin structure facilitating initiation and heteroduplex extension. This effect is enhanced by transcription, that opens chromatin structure and, thus, facilitates recombination. Such recombination events are long conversion events associated with crossing over, and do not require the *RAD51* gene product.

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Discordance between transcription-coupled repair and the recovery of messenger RNA synthesis. Bruce C. McKay, Feng Chen and Mats Ljungman, Division of Cancer Biology, Department of Radiation Oncology, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI 48109-0936.

Ultraviolet (UV) light induces helix distorting DNA lesions which are repaired by the nucleotide excision repair (NER) pathway. These DNA adducts are thought to block elongation by RNA polymerase II. A specialization of NER, termed transcription-coupled repair (TCR), has evolved to deal with the UV lesions within the template strand of RNA polymerase II-transcribed genes but not genes transcribed by RNA polymerases I or III. To gain insight into the relationship between TCR and the recovery of mRNA synthesis, we have assessed the recovery of transcription exclusively from RNA polymerase II-transcribed genes. Using this assay system, we have made several important observations. Firstly, mRNA synthesis recovers more efficiently following exposure to 10 J/m^2 than 5 J/m^2 in normal fibroblasts suggesting that some aspect of the recovery of mRNA synthesis is UV-inducible. Secondly, the complete recovery of transcription appears to precede the complete removal of UV-induced dimers from the transcription strand of active genes in human fibroblasts and Chinese hamster ovary cells. This observation suggests that translesion RNA synthesis occurs *in vivo*. Thirdly, the recovery of mRNA synthesis in TCR-proficient Li-Fraumeni syndrome fibroblasts and other cells lacking normal p53 tumor suppressor function is less efficient than predicted by their DNA repair phenotype. Together, these results suggest that the capacity of cells to recover mRNA synthesis is not solely determined by the repair of transcription blocking UV lesions and further suggests that not all UV lesions are permanent blocks to RNA polymerase II progression. Furthermore, p53 or a p53 regulated protein appears to participate in some aspect of the recovery of transcription.

To gain further insight into the role of p53 in the recovery of mRNA synthesis, we have used two different conditional expression systems. Using stable cell lines expressing a temperature sensitive allele of p53, we have found that expression of functional p53 prior to UV-irradiation stimulates the recovery of mRNA synthesis whereas p53 expressed exclusively during the post-UV incubation period is insufficient to enhance the recovery of transcription. We suggest that p53 regulates the steady state level of one or more proteins important for the recovery of mRNA synthesis and further suggest that this function of p53 is independent of its role in nucleotide excision repair. Current efforts are directed at the identification of proteins regulated by p53 which contribute to this recovery process.

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Hal8p/Crz1p/Tcn1p, A TRANSCRIPTIONAL ACTIVATOR OF *ENA1* GENE

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Four putative yeast transcription factors (Hal6-Hal9p) have been identified which upon overexpression in multicopy plasmids increase sodium and lithium tolerance in *Saccharomyces cerevisiae*. Hal6p and Hal7p are bZIP proteins, whereas Hal8p and Hal9p are zinc finger proteins. Deletions of the four genes reveal that only the two zinc finger proteins are relevant to halotolerance, since *hal8* and *hal9* mutants are sensitive to high salt and show decreased expression of the Na^+/Li^+ extruding ATPase encoded by *ENA1* gene (Mendizabal et al. 1998).

The extremely low *ENA1* expression in a *hal8* mutant suggests that Hal8p is an *ENA1* transcriptional activator. *HAL8* gene disruption mimics calcineurin inhibition effect on *ENA1* expression. Therefore, Hal8p should be considered a target of the calcium-activated calcineurin signal transducing pathway.

A GST-Hal8p fusion protein binds specifically to sequences within *ENA1* promoter which confer calcium-regulated expression to a *CYC1*-lacZ reporter gene.

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A CAF-1 / PCNA mediated chromatin assembly pathway triggered by sensing DNA damage

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Abstract

Sensing DNA damage and signalling an appropriate cellular response is crucial for the maintenance of genomic integrity and cell cycle progression. We demonstrate that DNA damage in the form of single-strand breaks and gaps triggers the propagation of nucleosomal arrays. This nucleosome assembly pathway is stimulated by the histone chaperone chromatin assembly factor-1 (CAF-1). The largest CAF-1 subunit (p150) interacts directly with the outer "front side" of proliferating cell nuclear antigen (PCNA), a repair factor which colocalises with CAF-1 in chromatin after DNA damage *in vivo*. The recruitment of both of these factors is dependent on the number of lesions and requires ATP. We propose that connecting chromatin assembly through PCNA to a variety of DNA damage processing events can be important for DNA damage signalling.

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CREB/ATF transcription factor-mediated chromatin remodeling at a meiotic recombination hot spot *ade6M26* in fission yeast

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The single base substitution *ade6M26* creates a meiosis-specific recombination hot spot in *Schizosaccharomyces pombe*.--The *ade6M26* mutation generates a heptanucleotide sequence, in which any of the base substitution abolishes the hot spot activity, at the 5' region of the *ade6* open reading frame. A hetero-dimeric protein Mts1-Mts2 binds specifically to the heptamer sequences. Mts1-Mts2 proteins are turned out to be identical to CREB/ATF transcription factors (Gad7/Atf1 and Pcr1, respectively) which are involved in stress response and induction of meiosis in *pombe*. We discovered a chromatin remodeling around the M26 mutation during meiosis. This chromatin remodeling is highly dependent upon the integrity of the heptamer sequence and both subunits of Mts1-Mts2 heterodimer. This chromatin remodeling requires several genes and factors responsible for meiosis induction, such as *mei2*, *mei3*, *cgs1*, *wis1*, and heterozygousity at mating-type locus. The results suggest links among recombination activation, transcription activation, chromatin remodeling, and signal transduction pathways for meiosis induction.

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An HMG-I(Y)-like protein that regulates carotenoid synthesis and fruiting body development in the bacterium *Myxococcus xanthus*

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The high-mobility-group (HMG) proteins constitute a family of ubiquitous and abundant non-histone nuclear proteins in eukaryotes. They function as architectural elements that modify the structure of DNA and chromatin, thereby modulating various DNA-dependent activities (1). The HMG-I(Y) subfamily of proteins are characterized by the presence of multiple copies of the positively charged AT hook motif: a motif consisting of the conserved core Arg-Gly-Arg-Pro sequence embedded in a somewhat less conserved cluster of basic and proline residues which bind to the minor groove of AT-rich DNA (1). This laboratory identified, cloned and sequenced the gene *carD* from the bacterium *Myxococcus xanthus*, whose product participates in light- and starvation-induced responses manifested, respectively, in carotenoid synthesis and fruiting body development in the bacterium (2, 3). The amino acid sequence predicted for the product of gene *carD* revealed the presence of four repeats of the mammalian HMG-I(Y) motif at the C-terminal end (3). This, to our knowledge, is the first report of such a protein in prokaryotes. As in the eukaryotic proteins, there is a very acidic region and this lies immediately N-terminal to the HMG-I(Y) region in protein CarD. The acidic region is preceded by a putative leucine-zipper motif, which in turn follows the N-terminal region of the protein (3). Thus this prokaryotic protein has several attributes of eukaryotic transcription factors. We have cloned and overexpressed in *E. coli* the *M. xanthus* gene *carD* using the pET system, and purified the protein using procedures similar to those employed for eukaryotic HMG-I(Y) proteins (4). The results of our ongoing characterization of the protein will be presented.

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STRUCTURAL AND FUNCTIONAL HETEROGENEITY OF Rap1p COMPLEXES WITH TELOMERIC AND UASrpg-LIKE DNA SEQUENCES

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Rap1p binds to a variety of related DNA sequences. We studied complexes of Rap1p and of its DNA binding domain with two of these sequences, the UASrpg sequence (5'-A C A C C C A T A C A T T T-3', RPG) and the S. cerevisiae telomeric consensus sequence (5'-A C A C C C A C A C A C C C-3´, TEL). When cloned in front of a minimal CYC1 promoter, the two sequences differed in their transcriptional potential. Whereas RPG and TEL binding sites activated transcription with approximately the same strength, adjacent RPG sequences showed higher synergistic activity and orientation-dependence than TEL sequences. We also found different sequence requirements for Rap1p binding in vitro to both sequences, since a single base-pair that severely reduced binding of Rap1p to the RPG sequences, had very little effect on the TEL sequence. Both KMnO4 hypersensitivity assay and the hydroxyl radical foot printing analysis showed Rap1p binding domain distorted differently DNA molecules encompassing both sequences. We propose that Rap1p is able to build structural and functionally different complexes, depending on the type of DNA sequence the complex is build on.

The genetic analysis revealed a second aspect of the functional heterogeneity of the different Rap1p binding sites. Two types of mutations affected our constructs: histone depletion resulted on a overactivation of both constructs, with much greater effects in constructs bearing a single RPG and TEL sequeces, gal11- mutations recduced transcription similarly in all cases. Our conclusion is that Rap1p activates transcription by a dual mechanism of action: by overcoming histone repression and by direct or indirect interaction with components of the RNA polymerase holoenzyme. Our results suggest the relative significance of these two ways may change from one promoter to another depending on the base sequence of the Rap1p binding site. The relevance of this functional and structural heterogeneity for the multiple functions Rap1p binding sites appear to have in vivo will be discussed.

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An ISWI complex with similarities to Drosophila CHRAC, isolated from HeLa nuclear extract.

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We have purified an ISWI (hSNF2L) complex from HeLa nuclear extract with similarities in subunit composition and activity, compared to the previously identified Drosophila Chromatin Accessibility Complex (CHRAC) (Varga-Weisz et al., 1997). The hSNF2L complex with a molecular weight around 700 kD was biochemically purified following immunoreactivity to hSNF2L. The complex contains topoisomerase II alpha (topo II alpha), a minor amount of topo II beta, the histone-fold protein p17 and several other putative subunits. ISWI, topo II and p17 were also found in Drosophila CHRAC. The interactions between hSNF2L, topo II alpha and p17 were confirmed with immunoprecipitations using antibodies against hSNF2L and p17. Interestingly, our putative human CHRAC complex contains a 185 kD subunit: a protein of similar size is also part of the Drosophila complex. Transfected GFP-tagged p17 binds transfected HA-tagged p15, the human homologue of another histone-fold protein found in Drosophila CHRAC. Tagged p17 and p15 also bind hSNF2L, suggesting that p17 and p15 form a heterodimer within CHRAC (On the histone-fold proteins in CHRAC, we are in collaboration with D. F. V. Corona and P. B. Becker at EMBL Heidelberg, who supplied us with the protein sequences of Drosophila CHRAC p15 and p17.). The human CHRAC complex shows chromatin- and DNA-dependent ATPase activity, probably due to hSNF2L and topo II, respectively. Similar to Drosophila CHRAC, the human complex stimulates the formation of properly folded and regularly spaced nucleosomes. It also increases the accessibility of nucleosomal DNA to Micrococcus nuclease digest.

Varga-Weisz et al. (1997) Nature **388**, 598-602.

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Premature transcription termination of an Ig transgene dissociates hypermutation and transcription.

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The role of RNA transcription in the process of hypermutation of Ig genes was assessed by introduction of a transcription termination signal in an Ig light chain transgene acting as mutation substrate (SPA-C2). Transgenic lines harboring SPA-C2 substrates only, control substrates, or both integrated in tandem were analyzed. Three out of four single SPA-C2 transgenic lines show background mutation level, but display minimal transcription elongation and, surprisingly, initiation of transcription was also reduced. The fourth line shows normal mutation rate while transcription elongation is reduced ten-fold. In two double transgenic lines, a 50 to 100-fold reduction in transcription elongation is observed for the SPA-C2 transgenes while their mutation level is only slightly reduced compared to the adjacent control transgene copies, and transcription initiation is moderately affected. These results thus clearly dissociate transcription elongation and mutation.

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Title: Higher order chromatin structures are detected in DNA double-strand breaks *in vivo*.

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When mammalian cell cultures or mice are exposed to ionizing radiation in survivable or lethal amounts, histone H2AX immediately becomes extensively phosphorylated on serine 139. Collectively referred to as gamma, these phosphorylated H2AX forms appear *in vivo* after several procedures that introduce double-strand breaks into DNA. Gamma components appear to be the only major novel components detected by mass or $^{32}PO_4$ incorporation on AUT-AUC or SDS-AUC gels after exposure of cells to ionizing radiation. The appearance of gamma-H2AX is rapid; half-maximal amounts are reached by one minute after exposure of cell cultures to ionizing radiation. Stoichiometric calculations show that H2AX molecules on about 0.03% of the chromatin are phosphorylated per DNA double-strand break, a value corresponding to about 2 X 10^6 bp DNA per double-stranded break. Thus, large amounts of chromatin and thousands of H2AX molecules are involved with each DNA double-stranded break An antibody raised to gamma-H2AX detects specifically this phosphorylation and its homologues in Drosophilla, Saccharomyces and other species. In mammalian cells, the antibody detects a number of structures consistent with the number of predicted DNA double-stranded breaks after ionizing radiation, and may be capable of detecting a single DNA double-stranded break within one minute of formation. Furthermore, radiation of mitotic cells revealed that the domains of chromatin that contain gamma-H2AX, closely resemble and may correspond to the megabase long metaphase chromosome bands.

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Characterization of Brg1 and hBrm based human SWI/SNF complexes

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Regulation of transcription in the context of chromatin involves many cellular activities that either promote or hinder the process by which DNA is rendered accessible. The recent discovery and characterization of chromatin remodeling complexes, and histone acetylase and deacetylase complexes suggest that these activities are involved in the regulation of cell growth and differentiation primarily by modifying chromatin structure. Genetic and biochemical studies have shown that complexes, which contain SWI2/SNF2 related proteins, can remodel chromatin in an ATP-dependent manner. SWI2/SNF2, which contains the ATPase domain, is a central component of the SWI/SNF complex and is highly conserved among eukaryotes. Human cells contain at least two homologues of yeast SWI2/SNF2, designated Brg1 and hBrm, which are related but not identical. Both Brg1 and hBrm have been shown to be involved in transcriptional activation and repression, and appear to be regulated differently by phosphorylation during mitosis. There is little information about the role that each one of these proteins and the complexes they associate with play in the regulation of chromatin structure.

To address the mechanisms by which the Brg1 and hBrm-based human SWI/SNF complexes regulate chromatin structure both in vivo and in vitro, I have used a helper-free retrovirus system to establish cell lines that express FLAG-tagged copies of either wild type Brg1 or hBrm. Biochemical characterization of these cell lines reveals that Brg1 and hBrm are found in complex with at least eight subunits: p250, hSWI3(p170), hSWI3(p155), hSwp73 (p60), p50, RbAp48, hArp7(p47), Ini1 (p45). Based on the predicted molecular weight of the Brg1 and hBrm complexes (~1 MDa), and their apparent molecular weight by gel filtration (~2 MDa), we wanted to determine whether Brg1 and hBrm can be found in the same complex. Westren blot analysis of fractions immunopurified from the tagged lines that express either Brg1 or hBrm showed that these two ATPases do not interact. Using these cell lines, I have developed a two step purification scheme to easily purify highly active Brg1 and hBrm-based hSWI/SNF complexes to homogeneity. Both complexes can alter the topology of plasmid DNA that has been assembled into chromatin using either Xenopus egg extracts or Drosophila embryo extracts, although, hBrm complexes appear to be slightly less efficient in their ability to remodel arrays of nucleosomes. When Brg1 and hBrm complexes were tested for their ability to remodel mononucleosomes, the hBrm complex was inactive despite the fact that it was able to hydrolyze ATP. These studies suggest that there are functional differences between these two chromatin remodeling complexes, and raise the possibility that these differences might be reflective of the role that each one of these ATPases paly in vivo in order to regulate cell growth.

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Induction and persistence of chromosome aberrations involving breakpoints in euchromatic (17cen-p53) and heterochromatic (1q12) subchromosomal regions and chromosomes with heterogeneous transcriptional activity

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Two heterogeneous subchromosomal regions were investigated for in vivo and in vitro radiation-induced chromosome aberrations: (i) chromosome 1 heterochromatin, band 1q12, by tandem tabelling FISH with adjacent probes and (ii) 17cen-p53 euchromatic region by double colour FISH with region specific probes. In addition, overall chromosome breakage was monitored by multicolour painting and by the micronucleus (MN) assay complemented with pancetromeric FISH. The in vivo study was performed on buccal cells from thyroid cancer and hyperthyroidism patients sampled before and after radiaoctive iodine therapeutic exposure. No increase in neither centromere negative micronuclei nor 1q12 fragility was observed after radioactive iodine treatment. However, a highly significant increase in 17cen-p53 fragility leading to p53 monoallelic deletions and 17p gains, as well as chromosome translocations involving chromosomes 1, 4 and, above all 10, were detected in the same samples. The in vitro study was perfomed in an irradiated TK6 lymphoblatoid cell line, a highly proliferative cell system resembling the continuously dividing buccal cells. Cytogenetic damage was measured 1, 3, 7, 14, 28, 42 and 56 days after irradiation by micronuclei with pancentromeric FISH, tandem labelling 1cen-1q12 FISH and multicolour painting with libraries specific for chromosomes with heterogeneous length and gene density and transcriptional activity (chromosomes number 1, 4, 18 and 19). A high increase of chromosome breakage was detected in all cases with similar frequences of induction. However both MN and translocations involving the heterochromatic band 1q12 were highly unstable and desappeared 1 week after induction. On the contray, 30% of translocations involving overall chromosomes 1, 4, 18 and 19 were highly stable, irrespective of the gene density or length of the chromosome involved. From our results we conclude that (i) euchromatin is as radiosensitive as heterochromatin, (ii) MN and 1q12 translocations are highly unstable upon cell divisions, probably leading to an undetectable increase of MN and 1q12 breakage in the continuously proliferating buccal cells of thyroid disease patients, and (iii) radiactive iodine induced breakage in 17cen-p53 region leading to an increase of the 17p gains and losses which were apparently stable upon cell divisions and hence detectable in buccal cells from thyroid disease exposed patients. In addition, the induction of chromosome aberrations is proportional to the length of the chromosome involved, gene density makes no measurable contribution to the induction of aberrations, and neither length nor gene density influence the persistence of chromosome aberrations.

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Cells lacking the recombination-repair gene XRCC2 show an increased level of chromosome mis-segregation

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We have previously shown that the human gene XRCC2 complements the hypersensitivity of the V79 hamster mutant cell line *irs1* to mitomycin C (MMC) and X-rays.
This gene shows homology to members of the recA/rad51 homologous recombination-repair gene family. It is probable therefore that XRCC2 plays a role in homologous recombination repair in humans. Studies of recombination genes in other organisms have also suggested a role in maintaining chromosome stability through the correct segregation of chromosomes during mitosis. To assess the role of XRCC2 in chromosome segregation, we have used a cytogenetic assay with fluorescently labelled hamster chromosome specific repeat sequences. The level of mis-segregation was found to be significantly elevated in *irs1* compared to V79, together with an increase in chromosome exchange frequency. Several *irs1* sub-lines transfected with the XRCC2 gene were analysed and they all showed a restored resistance to MMC and X-rays, together with a level of mis-segregation and chromosome exchange frequency equal to that found in the parental cell line.
Losses or gains in whole chromosomes are found in nearly all major human tumour types, and may be a vital step in the path to cancer by dramatically changing gene dosage.

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New insights about the role of the *Drosophila's* protein GAGA in transcription

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GAGA protein was described fifteen years ago as protein able to bind to gaga regions locate in several promoters of different genes(1,2). This activity was described as basic for the correct expression of those genes.

GAGA seems to contain three main domains: a N-terminal domain called POZ, which has been described as a homo/heteromerization (3) domain; a central DNA binding-domain (only contains a unique Zn-finger), and a C-terminal Glutamin-rich domain (Q).

The first role atributed to GAGA was of activation, but several years after it was proposed a role of Antirepressor rather than real Activator (4). The antirepression was an interesting model for GAGA, but it was not able to explain all the aspects of GAGA behaviour.

One explanation could be the existence of activities which are able to bind to the same sequences as GAGA. In fact, endogenous GAGA is a good candidate since the levels of GAGA are high during all the stages of the fly.Moreover, It has been described recently a new protein called Pisqueak which recognizes the same gaga sequences as GAGA(Psq) and it presents a repressor activity (5).

We have studied the role of GAGA in transcription *in vitro*, using an heterologous system like HeLa nuclear extract instead of Drosophila's nuclear extracts in order to avoid the problem of the endogenous GAGA . We found that GAGA presents an activator activity independent of Antirepression . Furthermore, the responsible for this activity is the C-terminal Glutamin-rich domain (Q), and the POZ domain seems to modulate this activity. In both cases Q is able to show a high activity in GAL4 context, and studies of progressive deletions of the Q domain suggest a modular and redundant structure for Q.

Although GAGA failed to activate in vitro in a Drosophila nuclear extract SNF-like extract, GAL4Q was able to mantain this activation. Our studies have included also *in vivo* transient cotransfection assays in *Drosophila's* SL2 cells, confirming the activation activity of GAL4Q, although it was sensibly lower than GAL4VP16.

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A physical complex of the Fanconi anemia proteins FANCG/XRCC9 and FANCA

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Abstract.

Fanconi anemia (FA) is an autosomal recessive chromosomal instability syndrome characterized by progressive bone marrow failure, hyperpigmentation of the skin, developmental abnormalities and an increased cancer risk, particularly acute myeloid leukemia and squamous cell carcinoma. Cells derived from FA patients have an increased level of spontaneous chromosomal damage and a specific hypersensitivity to cross-linking agents, such as mitomycin C (MMC) and diepoxybutane. FA is genetically heterogeneous, with at least eight complementation groups (A-H). Each of these groups probably represents a distinct disease gene. The FA genes *FANCA, FANCC* and *FANCG/XRCC9* have been identified, but their interrelationship and molecular functions are still obscure. Here we report that the protein encoded by the *FANCG* gene localizes to the cytoplasmic and nuclear compartment and assembles in a molecular complex with the product of *FANCA*. Complex formation was demonstrated by immunoprecipitation from cell lysates of transiently transfected cells and by immunoprecipitation of *in vitro* translated products. Endogenous FANCA/FANCG complex could readily be detected in non-FA cells and in cells from FA patients of complementation groups D and E. By contrast, the complex was nearly absent in FA-B and FA-H cells and reduced in FA-C and FA-F cells, wild type levels were restored in these cells after correction of the cellular FA phenotype by transfection or cell fusion. These results strongly suggest that the FANCA/FANCG complex has functional significance in the FA pathway guarding chromosomal stability.

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