Mutation Research, DNA Repair, 273 (1992) 1–28

MUTDNA 06477

Workshop on DNA repair

A.R. Lehmann, J.H.J. Hoeijmakers, A.A. van Zeeland, C.M.P. Backendorf, B.A. Bridges, A. Collins, R.P.D. Fuchs, G.P. Margison, R. Montesano, E. Moustacchi, A.T. Natarajan, M. Radman, A. Sarasin, E. Seeberg, C.A. Smith, M. Stefanini, L.H. Thompson, G.P. van der Schans, C.A. Weber and M.Z. Zdzienicka

(Great Britain and The Netherlands)

(Received 12 August 1991)
(Accepted 13 August 1991)

Keywords: Workshop on DNA repair; DNA repair, workshop; Repair genes; Repair proteins; Chromatin structure; Damage and repair

Contents

Correspondence: Dr. A.R. Lehmann, MRC Cell Mutation Unit, University of Sussex, Falmer, Brighton, BN1 9RR (Great Britain).

A workshop on DNA repair with emphasis on eukaryotic systems was held, under the auspices of the EC Concerted Action on DNA Repair and Cancer, at Noordwijkerhout (The Netherlands) 14–19 April 1991. The local organization of the meeting was done under the auspices of the Medical Genetic Centre South-West, The Netherlands (MGC), c/o Department of Radiation Genetics and Chemical Mutagenesis, University of Leiden (The Netherlands). Local organizers were: D. Bootsma (chairman), W. Ferro, J.H.J. Hoeijmakers, A.R. Lehmann, P.H.M. Lohman, L. Mullenders, and A.A. van Zeeland (secretarial assistance: Mrs. C. Escher-van Heerden and Mrs. R. Bontre). Over 190 scientists participated, and the format of the meeting followed that of the 1987 workshop on the 'Molecular Aspects of DNA Repair' (Friedberg et al., 1987). Plenary review talks in the mornings were followed, in the afternoon, by poster viewing in three or four parallel sessions. Groups of 15–20 posters were discussed in detail, and later on, in plenary sessions, chairpersons of the poster discussions reviewed the afternoons' posters. The principal themes of the meeting were the isolation and characterisation of repair genes and proteins, repair in specific sequences, consequences of defective DNA repair, and new methods for detecting DNA damage and repair. Remarkable progress has been made recently in all of these areas, and many exciting new results were presented. It is impossible to summarize all contributions to this (intensive) one-week meeting. Therefore, and for the sake of coherence, presentations that did not fit easily into any of the general themes of the meetings have not been included.

1. DNA repair genes and proteins

In order to understand the mechanisms of DNA repair and its consequences for cells and organisms, it is necessary to dissect the various processes into their component parts, and subsequently to reassemble them in a controlled manner. For any particular gene and its product, we need to clone the gene and understand the control of its expression, to purify the gene product

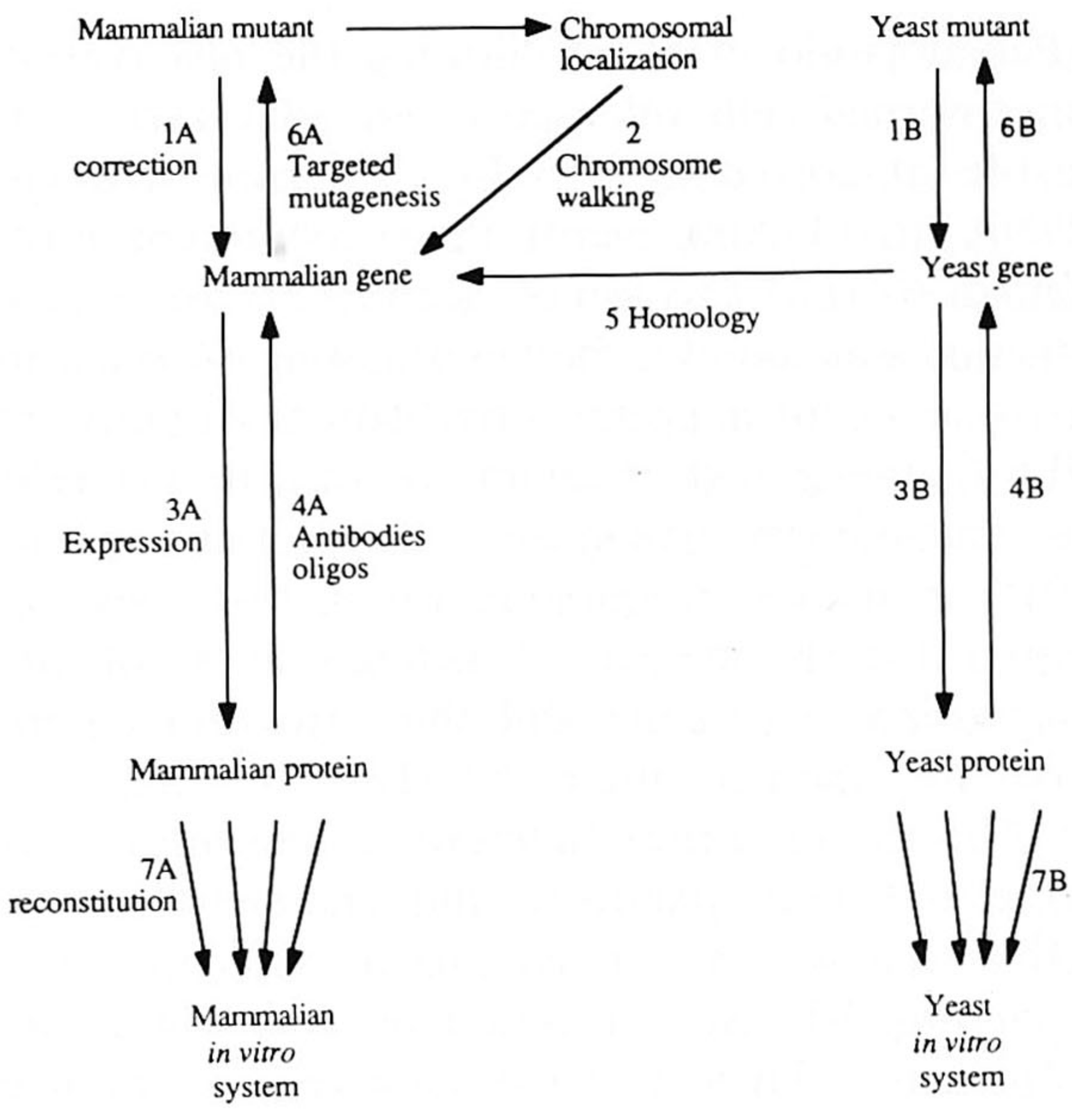

Fig. 1. Cloning of mammalian DNA repair genes.

and determine its mechanism of action, and to examine the effect of different mutations in the gene on the activity of its product and on the phenotype of the cell harbouring the mutant gene. Fig. 1 illustrates the different ways in which these goals are being achieved. Starting with a particular mutant cell which is deficient in DNA repair, the gene which is deficient in the mutant can be cloned from normal DNA by its ability to correct the mutant phenotype (Steps 1A, 1B). Alternatively if the chromosomal location of a gene is known from linkage analysis, it is possible to 'walk' to the gene using various molecular biological techniques (Step 2). Following characterisation of the gene, it can be inserted into an expression vector and used to generate the protein product (Steps 3A, 3B). Alternatively, the procedures can be used in reverse. In the few instances in which a DNA-repair protein has been purified, monoclonal antibodies or oligonucleotides can be used to isolate the gene from an appropriate library (Steps 4A, 4B). Finally, since there is now overwhelming evidence that most, if not all, DNA-repair systems are conserved throughout the eukaryotic kingdom, genes cloned from simple eukaryotes, such as the yeasts, can be used to isolate the homologous genes from higher eukaryotes (Step 5). After cloning of the gene, new mutations can be generated by targeted mutagenesis and their effects can be determined using 'reverse genetics' (Steps 6A, 6B). In vitro DNA-repair systems can be generated by reconstitution of purified gene products (Steps 7A, 7B). Alternatively a complex functional in vitro system can be dissected in order to identify the constituent proteins. Great progress has been made in all these areas in the field of DNA repair in the past two years and much of this work was reported at the meeting.

1.1. Isolation and characterisation of DNA-repair mutants

1.1.1. Xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy

The 7 complementation groups of xeroderma pigmentosum (XP) have been studied in detail over the last two decades. The relationship between XP and two other genetic disorders, Cockayne's syndrome (CS) and trichothiodystrophy (TTD) is becoming more complex. Within the last two years several patients with the clinical features of both XP and CS have been identified. The repair defects in these cells have been assigned to XP-B, XP-D or XP-G (Vermeulen et al., Rotterdam).

Many patients with TTD, a rare autosomal recessive disorder characterized by sulphur-deficient brittle hair, mental and physical retardation, have a DNA-repair defect which appears to be identical to that in XP-D. Using the probe characterized by Arrand, which partially complements XP-D (see below), no deletions or gross rearrangements were found in Italian TTD families, nor did a polymorphism detected by this probe segregate with the disease (Mondello et al., Pavia). Cancer proneness, a characteristic feature of XP has not been found for either CS or TTD. Since it has now been shown that XP-D is complemented by the ERCC-2 gene (see below), the tools for further analysis of the association with the other syndromes (possibly different domains at the protein complex level or overlapping genes) are partly available. The catalase content of the cells differentiates TTD from XP. Catalase activity is decreased by a factor of 5 in XP cell lines, whereas TTD cells exhibit normal levels of cata-

lase activity. This may be related to the difference in phenotype of these two diseases (Sarasin et al., Villejuif).

The repairability of two 4NQO adducts (C8 and N2 of guanosine) differ between members of the same XP complementation group which have the same UV-repair characteristics, demonstrating a further level of heterogeneity in XP (Jones, Swansea).

Enhanced sensitivity of CS cells to deoxyguanosine and camptothecin (an inhibitor of the rejoining step of topoisomerase I) was reported by Squires et al. (Cambridge). A correlation was found between the sensitivity to UV and camptothecin and the frequency of double-strand breaks (DSBs) induced following treatment by either agent. This led to the suggestion that disruption of replication in CS cells might generate lethal DSBs.

Paterson et al. (Edmonton) presented further data to support their earlier findings that prior to incision of DNA after UV-irradiation, the intradimer phosphodiester bond is cleaved. Such cleaved dimers accumulate in XP-D cells and appear transiently in normal cells. An enzyme capable of carrying out this cleavage reaction has been purified from human cell lines. It is a Mg^{2+}-activated 52-kD protein. It was proposed that the function of the cleavage reaction is to relieve conformational stress at the dimer, perhaps permitting transcription across the dimers to occur.

1.1.2. Fanconi's anemia

Fanconi's anemia (FA) is a chromosomal instability syndrome, characterised by cellular hypersensitivity to DNA-crosslinking agents. This disorder has been studied in detail by the group of Moustacchi (Institut Curie, Paris). FA cells have an antioxidant defence system functionally as efficient as that of normal cells in processing oxygen damage, as demonstrated by cellular responses to hematoporphyrin (Nocentini). The response of FA cells to DNA cross-linking agents was examined and hypomutability at two independent loci was observed. Moreover, in contrast to normal cells which predominantly demonstrated point mutations at the *hprt* locus, deletion mutants constituted the major class in FA (Papadopoulo et al.). Following the observation that normal cells released a diffusible factor capable of correcting the FA hypersensitivity to DNA cross-linking agents, the regulation of interleukin 6 (IL6) and tumor necrosis factor α production was found to be abnormal in FA cells. In vivo data confirmed this observation. Addition of IL6 to the growth medium restored to FA cells an almost normal response to cross-linking agents. The anomalies in cytokine production may account for the defect in differentiation of the hematopoietic system and the chromosomal instability, characteristic of FA (Roselli et al.).

The use of viruses (adenovirus and herpes) as efficient vector systems to study the expression of DNA repair genes in various mammalian cells, including XP, was proposed by Rainbow et al. (Toronto). This system does not require the use of permanent cell lines, and may therefore prove to be of great value.

The involvement of DNA repair genes in early development, particularly of the neurological system, is suggested by the clinical features of XP, A-T etc. The possible role of programmed DNA rearrangements during development was examined by the molecular characterization of small extrachromosomal circular DNA. This approach provides a new and interesting means to examine this question (Hubank et al., Sussex).

1.1.3. Rodent mutants

The series of *xrs* mutants generated by Jeggo has been used to study induction of chromosomal aberrations by X-rays. The frequency of X-ray induced aberrations in *xrs*-5 and *xrs*-6 mutant cells was found to be directly correlated to the relative ability of these cell to repair DSBs. Though these two mutants belong to the same complementation group there are marked differences in their response to genotoxic agents, e.g., *xrs*-6 cells are defective in incision of UV-induced lesions and respond with a dose-dependent increase in SCEs. The premature chromosome condensation (PCC) technique was found to be a simple and quick way to establish complementation between different X-ray sensitive mutants (Darroudi et al., Leiden). A strange inexplicable observation, that following irradiation of *xrs*-5 cells in G1 stage, the frequency of chromosome

type of aberrations saturates after 1 Gy and these frequencies were dependent on the culture medium, was presented by Virsik-Peuckert and Volkmer (Göttingen). When DSBs induced by restriction endonucleases (particularly those giving blunt termini) in CHO cells were measured over a period of 12 h the frequency continued to increase with time, indicating that the enzyme is active inside the cell and that there is a competition between the incision step and sealing of the breaks. This phenomenon was found to be very pronounced in *xrs*-5 cells supporting the competition hypothesis (Bryant and Costa, St. Andrews).

Several new mutants derived from either V79 or CHO hamster cells were described. The number of genetic complementation groups for the processing of UV damage is nine, and two possible new groups have been identified by complementation analysis. The genes defective in these mutants have in some cases been localised on human chromosomes (Numata et al., Sendai; Botta et al., Pavia). New mutants demonstrating hypersensitivity to DNA cross-linking agents have also been independently isolated and characterized (Zdzienicka et al., Leiden; Robson et al., Oxford), leading to at least nine complementation groups for repair, of this type of damage. Sensitivities of rodent cell mutants to other agents including drugs which interact specifically with AT rich sequences via the minor groove of the DNA were reported by Smith (Cambridge).

The successful cloning of *ERCC*-genes homologous to some of the XP genes via the complementation of rodent mutants (see below) will certainly stimulate the search for and characterization of more rodent mutants sensitive to different types of DNA-damaging agents.

1.1.4. Yeast mutants

Kiefer (Giessen) has studied the response to UV-irradiation of the *rad54* mutant of yeast. This mutant is known to be deficient in DSB repair and is sensitive to ionizing radiation. However, the mutant is also sensitive to UV which does not cause strand breaks directly. Nevertheless, DSBs do accumulate in the mutant following UV. Evidence was presented that such breaks accumulated even in the absence of nucleotide excision repair. These results parallel to some extent those of Squires et al. with human cells (see above).

23 complementation groups have been identified as being involved in DNA repair in the fission yeast *Schizosaccharomyces pombe*. Up till now they have not been characterised in great detail and epistasis groups have not yet been defined. The mutants can be classified in various ways, for example by whether they are defective in a caffeine-resistant pathway (putatively excision repair) or in a caffeine-sensitive pathway, which had previously been thought to involve recombinational repair. The work of Carr et al. (MRC, Sussex) provided an alternative means of classification, based on the behaviour at high temperature of double mutants carrying, in addition to the repair mutation, a temperature-sensitive mutation in the DNA ligase (*cdc17*) gene. One group of mutants died rapidly because they failed to arrest the cell cycle and entered a 'catastrophic mitosis'. Other mutants were unaffected, whilst a third group showed intermediate behaviour. Further work led to the suggestion that the UV sensitivity of the first group could be attributed to the failure of the cells to undergo G2 delay following DNA damage. Premature entry into mitosis prior to repair of damage in G2 led to UV hypersensitivity.

1.2. Isolation and characterization of repair genes and proteins (see Table 1)

1.2.1. Nucleotide excision repair

1.2.1.1. Mammals. Significant advances in the study of the human nucleotide excision repair pathway have been made in the last few years. Five of the human genes correcting rodent UV complementation groups have now been cloned, and efforts to characterize these genes have proceeded well. Interspecies comparisons have shown remarkable levels of conservation, revealing insights into the biochemistry of the repair process and identifying previously unknown repair genes. The homologous genes in yeast, rodent, and human repair systems are being identified. Significant progress has been made towards identifying the defective genes in the human genetic disorders XP and CS.

Table 1

Cloned Human DNA repair genes

Gene	Chromosomal location	Size of gene (Kb)	Number of aa in predicted protein	*S. cerevisiae* homolog	*S. pombe* homolog	Activity of gene product
ERCC-1	19q13	15–17	297	*RAD10*		?
ERCC-2 (XP-D)	19q13	20	760	*RAD3*	*rad15*	DNA helicase [b]
ERCC-3 (XP-B)	2q21	45	782	*ERCC-3*^{SC}	*ERCC-3*^{SP}	DNA helicase [b]
ERCC-5	13q	32				
ERCC-6	10q11	100	> 1500			DNA helicase [b]
XP-A	9q34	25	273	*RAD14*		
DNA ligase I	19q13		919	*CDC9*	*cdc17*	DNA ligase
HHR6A	Xq24	15	152	*RAD6*	*rhp6* $^{+}$	Ubiquitin-conjugating enzyme
HHR6B	5q23		152			
PolyADP-ribose polymerase	1q42	43	1043			
MGMT	10		207			O^6-Methylguanine–DNA methyltransferase
BAP1 [a]			323			APendonuclease
ANPG			230			Alkyl-*N*-purine–DNA glycosylase
UNG15	12		304			Uracil DNA glycosylase
XRCC-1	19q13	33	633			
KIN17	10p		200			

[a] Bovine gene.

[b] Presumed from predicted amino acid sequence.

Hoeijmakers (Rotterdam) discussed the *ERCC-1, 3*, and *6* genes, and the protein correcting XP-A (of which the gene was recently cloned and characterised by Tanaka and co-workers, Osaka). Functional analysis of the human *ERCC-1* protein was reported by van den Berg et al. (Rotterdam), who constructed cDNAs with specific mutations and assayed for correction of rodent group 1 mutants. The N-terminal 92 and C-terminal 4 amino acids could be deleted without loss of function, while mutations within the putative DNA-binding domain resulted in loss of function. Although full-length *ERCC-1* protein was rapidly degraded when overexpressed in *E. coli*, overproduction of a ubiquitin–*ERCC-1* fusion protein, from which full-length *ERCC-1* protein could be released by ubiquitin lyase, has been successful. *ERCC-1* shows homology to the *S. cerevisiae RAD10* gene and to parts of the *E. coli* UvrA and C proteins. In preliminary transfection experiments with an *ERCC-1/uvrC* hybrid gene a substantial but not complete correction of the mitomycin C (MMC) sensitivity, but no correction of the UV-sensitivity of rodent group 1 mutants was observed. If confirmed for other cross-linking agents, this finding may indicate that different protein domains are involved in the repair of damage in these two pathways.

Weber and co-workers (Livermore) reported that the human *ERCC-2* gene corrected the nucleotide excision-repair defect in XP group D. A differential cytotoxicity assay was used following electroporation with a complete cDNA clone in a modified pcD2 vector or with a control plasmid. Cells from the patient originally reported as XP group H were also corrected by *ERCC-2*, con-

firming that this patient belongs in group D. *ERCC-2* is the human homolog of the *S. cerevisiae RAD3* gene, which codes for an ATP-dependent helicase. Partial sequence determination of the *ERCC-2* gene from one XP-D cell line has revealed a Leu → Val substitution at position 461 within helicase domain III in one allele (the highly homologous yeast *RAD3* protein has Ile at the analogous residue).

The promoter of the human *ERCC-3 / XP-B* gene was studied by Ma and co-workers (Leiden, Rotterdam). The 5′-flanking region is GC-rich, lacks clearly identifiable TATA and CAAT elements, and has three sequence motifs, that are conserved between mouse and man. One of these, including a pyrimidine-rich stretch, is also found in the *ERCC-1* promoter region. The lack of TATA and CAAT boxes is similar to *ERCC-1*, but *ERCC-2* has both of these classical promoter elements. The 5′-flanking region of *ERCC-3* was linked to the chloramphenicol acetyltransferase gene, and studies indicated that deletion of the conserved regions (−369 to −358 and −357 to −339, relative to the ATG translation initiation codon) had no effect on basal promoter activity and that −129 to −2 were the minimum required. Weeda and co-workers (Leiden, Rotterdam) have identified two new XP group B patients using the *ERCC-3 / XP-B* gene. These patients show severe excision repair deficiency, but, unlike the original XP-B patient, they have not developed skin cancer, even at an advanced age. Thus, additional factors may be required for cancer-proneness. The yeast (*S. cerevisiae* and *S. pombe*) homologues to *ERCC-3* have been cloned and characterized. These homologues show ~ 50% amino acid identity with *ERCC-3* and have sequences consistent with the putative helicase functional domains postulated for the human protein. Results obtained in collaboration with S. and L. Prakash (Rochester) suggest that *ERCC-3*, like *ERCC-2*, may be essential for viability. There are also other parallels between *ERCC-2* and *ERCC-3*, including the fact that both seem to be involved in XP complementation groups which include patients exhibiting XP as well as CS clinical symptoms. This may be explained by assuming that both proteins have a similar role in the same step of the excision-repair reaction.

The *ERCC-5* cDNA was cloned and characterized by MacInnes and co-workers (Los Alamos). Northern analysis indicated an mRNA of ~ 4.6 kb. None of the partial cDNA clones from the pcD2 library conferred UV-resistance to the group 5 mutant UV135, but cotransfer with a cosmid containing the 5′-end of the gene allowed in vitro reconstruction of a functional minigene conferring UV-resistance by recombination. An incomplete 450 aa ORF is highly acidic and does not show homology to any of the characterized yeast-repair proteins. The gene maps to human chromosome 13q32-33. A second site of hybridization to chromosome 10 raised the question of similarity to *ERCC-6*.

ERCC-6 was found to have two low level mRNAs of 6.5 and 8.5 kb resulting from differential polyadenylation (Troelstra et al., Rotterdam). A partial cDNA clone (~ 5 kb) corrected UV61 and has an ORF encoding a protein of 1493 aa with a putative chromatin binding domain and a serine phosphorylation site. The presence of the 8 putative helicase functional domains suggests that *ERCC-6* might be a third helicase involved in nucleotide excision repair (in addition to *ERCC-2* and *ERCC-3*). Preliminary data suggest that *ERCC-6* (localized to chromosome 10q11-21.1) is located in a visible deletion which has been found in one chromosome of a putative CS patient.

Arrand and co-workers (CRC Gray Laboratory, Northwood) presented further characterization of the hamster genomic DNA region that confers partial correction of UV-sensitivity to XP-D cells, but not XP-A cells. This region detects 2 mRNA species in human cells. The human homologue of a gene adjacent to the correcting gene in the hamster region maps to chromosome 15. It remains to be determined if the human gene corresponding to the hamster gene conferring partial correction also resides on chromosome 15. Since *ERCC-2* now appears to be 'the' XP-D gene and maps to a highly syntenic region of chromosome 19, it is unlikely that the cloned hamster region is related to ERCC-2. The basis for the partial correction of XP-D by this hamster region is not understood, but the gene is not casein kinase, which has been previously reported to enhance survival of XP-C and D cells.

The XP-A protein has been partially purified

using microinjection into XP-A cells and UDS to assay for activity (Eker et al., Rotterdam). The protein has an apparent MW of 40–45 kD, withstands heat inactivation in the presence of SDS and exhibits DNA binding activity, the affinity for ssDNA being higher than for (UV) dsDNA. Results from analysis of the gene, presented by Hoeijmakers on behalf of Tanaka and co-workers (Osaka) indicated that the protein contains 273 aa with a very acidic region, a zinc finger, and a putative nuclear location signal. The N-terminal 53 aa are not essential. Analysis of XP-A patients revealed 4 positions with mutations resulting in stop codons. The most C-terminal mutation, at position 228, results in mild symptoms, while the others result in severe symptoms. Three sites are found among Japanese patients and the other is found in Caucasian and black patients. The gene is ~25 kb in size and maps to 9q34. 1. The major mRNA is 1.4 kb (Tanaka, Osaka).

1.2.1.2. Yeast. S. Prakash (Rochester) discussed the nucleotide excision repair genes of the yeast *S. cerevisiae*. The *RAD1* gene — like *RAD10* — is involved in a *RAD52* independent recombination pathway. Site-directed mutagenesis has revealed domains in the protein specifically implicated in repair or in recombination. The *RAD3* gene in yeast is essential for viability even in the absence of DNA damage and encodes an ATP-dependent $5' \rightarrow 3'$ helicase. The *S. pombe* homolog of *RAD3* is also essential and, as also found by Lehmann, Carr and co-workers (MRC, Sussex), it shows 65% aa identity with *RAD3* and 55% aa identity with *ERCC-2*. An *ERCC-2* yeast expression plasmid with the strong constitutive ADH promoter was shown to express the protein in yeast, specifically cross-reacting with *ERCC-2* and *RAD3* antibodies. Purification of the *ERCC-2* protein is in progress.

While *rad14*-1 and *rad14*-2 (missense point mutations) are only slightly UV-sensitive, a deletion mutant is highly UV-sensitive (Prakash). The gene has been cloned and encodes a 247 aa protein with 2 zinc finger domains (aa 67–92 and aa 211–238). *RAD14* shows significant homology to the XP-A gene (33% identity, 60% conservation over a 131 aa region) (Prakash). Thus, there are now 3 sets of genes for which homologs have been identified among the *S. cerevisiae* and XP complementation groups (*RAD3/XP-D-ERCC-2*, *ERCC-3SC/XP-B-ERCC-3*, and *RAD14/XP-A*). A rodent mutant is yet to be identified with a defect in the *XP-A* gene.

RAD2, *7*, and *23* all show regulated expression, increasing ~5-fold 30–60 min post-UV, increasing up to 15-fold in meiosis by 6 h, but remaining constant in mitosis. Induction of *RAD2* was also discussed by Siede and Friedberg (Dallas), who have identified multiple regulatory elements. These include four AT-rich tracts and two other regulatory sequences DRE1 and DRE2, which are also found in certain other UV-inducible genes. UV-inducible protein binding to DRE1 was demonstrated by band-shift assays. Deletion of the DRE1 element affected UV-sensitivity in synchronized cells in the G1/S phase of the cell cycle. It was suggested that DRE1 enhanced repair just prior to onset of DNA replication.

A similar type of complex regulation was also reported for the yeast *REV2* gene by Ahne et al. (Neuherberg). The *REV2* gene, involved in repair and mutagenesis, has an open reading frame for a polypetide of 473 aa which is predicted to be a membrane protein. The gene is DNA damage-inducible, heat-inducible and cell cycle regulated (maximal expression in mitosis).

12 genes have been cloned from the fission yeast, *S. pombe*, an organism evolutionarily very distant from *S. cerevisiae* (Lehmann, Carr and co-workers, MRC, Sussex). Most of these genes were cloned by complementation of UV or γ-radiation-sensitive mutants. 3 of these genes (*rad16*, *rad13* and *rad15*) are respectively homologous to the *S. cerevisiae RAD1, RAD2* and *RAD3* genes. The other cloned *S. pombe* genes, which are probably not involved in excision repair, do not show homology to any of the genes cloned thus far from other organisms. This raised the possibility that they may represent as yet uncharacterized repair or recovery pathways.

The strong sequence conservation from mammals to yeast found in many of the repair proteins leads to the question of just how complete are the nucleotide excision repair mutant collections from mammals and yeast, given the recent identification of *ERCC-3* homologs in both yeasts, and the apparently lacking *ERCC-5* and *-6* ho-

mologs. The extensive amino acid sequence conservation from man to yeast and lack of homology to the *E. coli* proteins (except for small domain homology between parts of *ERCC-1* and parts of *uvrA* and *C*) suggest that the nucleotide excision repair process in *E. coli* may be quite different from that in eukaryotic systems.

The stage is now set for studies to determine the genetic basis of the association between XP and CS and between XP-D and TTD. The next few years are anticipated to yield many insights into these relationships and into the phenotypic variation in the nature and extent of the repair defect within a given rodent or XP complementation group. As the repair proteins are isolated, studies of the biochemical roles they play in nucleotide excision repair and other DNA metabolic pathways will provide new levels of understanding of these vital cellular processes.

1.2.2. Other DNA-repair (associated) genes

1.2.2.1. Mammals. Apart from the 6 cloned human nucleotide excision-repair genes, cloning of a further 10 human-repair genes was reported, and progress has been made with several other genes.

(*a*) *DNA ligase*

The characterization of DNA ligase I and its relationship to Bloom's syndrome (BS) was reported by Tomkinson and co-workers (ICRF, South Mimms). This nuclear, replicative enzyme is a phosphoprotein, whose activity may be modulated by phosphorylation. Human cDNAs were cloned both by complementation of a yeast *cdc9* mutant in L. Johnston's laboratory and by hybridization with oligonucleotides corresponding to partial amino acid sequences of the bovine protein (Barnes et al., South Mimms). The gene was localized to human chromosome 19q. The protein's active site, which forms the enzyme–adenylate intermediate, was identified, and the sequence surrounding it was found to contain a conserved motif. Point mutations in conserved regions of ligase I cDNA were identified in the cell line 46BR, which was derived from an immunodeficient patient having some of the features of BS. The K_m for the formation of the enzyme–adenylate complex was much higher in 46BR than in normal cells. Mutations in ligase I in (classical) BS lines were not seen even though biochemical data suggest that BS cells have an abnormal DNA ligase activity (Barnes, South Mimms; Chu et al., Cornell). The defect in BS cells may involve altered post-translational modification of ligase I. Two additional mammalian ligases have been identified, and they each have properties distinct from ligase I.

(*b*) *RAD6 homologs*

The *RAD6* gene of *S. cerevisiae* codes for a ubiquitin-conjugating enzyme which is involved in post-replication repair, mutagenesis and sporulation. Two human homologs *HHR6A* and *HHR6B*, were cloned by evolutionary walking using the previously isolated homologous genes of *S. pombe* and *D. melanogaster* (Koken et al., Rotterdam). The two genes are 95% identical to each other and have 68% identity to *RAD6*. The cys residue in the active site (aa88) is conserved throughout. The genes are located in the human X-chromosome band q24-25 (*HHR6A*) and chromosome 5q23-31 (*HHR6B*). Other mammals also show the presence of two genes whereas *D. melanogaster* has only one gene homologous to *RAD6* (thus a duplication occurred $\sim 200 \times 10^6$ years ago). None of the homologs contains the acidic C-terminus characteristic of *RAD6*, which in *S. cerevisiae* is essential for sporulation.

(*c*) *Ataxia telangiectasia*

Four complementation groups for A-T have been previously identified on the basis of a functional complementation assay, and the genes for groups AB and C have been previously localised to chromosome 11q22.3. Using the technique of microcell mediated chromosome transfer, Lambert et al. (Stanford, Baltimore, Irvine and Dallas) have mapped the AT-D gene to the same region (11q22.3–23.1). This indicates that there may be only a single wild-type A-T gene with several functional domains, or that there are multiple closely linked genes.

James and colleagues (CEPH, Paris; UCSF, San Francisco) have used genetic linkage in 19 muliplex A-T families combined with genetic, in situ, and radiation hybrid mapping to localise the

A-T gene(s) more precisely within an approximately 5-cM region of chromosome 11q22.3–23.1. This high resolution map can now be used to obtain all the DNA which must contain the A-T genes(s) in the form of cosmid and YAC contigs for systematic screening for the complementing gene. Using an alternative approach to isolate the AT-D gene, Fritz et al. (Karlsruhe) have transfected immortalized A-T fibroblasts with two different cDNA expression libraries and two clones with an enhanced resistance to X-ray-irradiation are under further investigation.

(*d*) *xrs*

The procedure of microcell-mediated chromosome transfer was also used by Jeggo et al. (MRC, Sussex) in an attempt to localize the gene defective in the Chinese hamster X-ray-sensitive *xrs* mutants. So far, it has been shown that the human gene complementing this defect does not reside on chromosomes 5, 6, 12, 13, 15 and 21.

(*e*) *Fanconi's anemia*

In order to understand the molecular basis of FA, two different approaches have been used in attempts to clone the FA genes. Diatloff-Zito et al. (Paris) transfected FA cells with mouse genomic DNA and transformants displaying cellular and chromosomal resistance to different cross-links were isolated. A lambda phage library was constructed from FA(B) transformants and 3 phage recombinants were found to correct to various extents the sensitivity of FA cells to mitomycin C. An alternative approach to clone the FA(A) gene was presented by Telleman et al. (Leiden). The Chinese hamster V79 cell mutant (VH4) which is homologous to FA(A) has been described previously and this mutant served as a cellular model for cloning of the FA(A) gene and also for analysis of the FA(A) defect. The VH4 mutant was used as recipient for transfection with genomic human DNA. Twelve independent mitomycin-C (MMC) resistant primary transformants were isolated and most showed an almost wild-type level of resistance to this agent. Using Southern blot analysis of genomic DNA from these transformants, two transformants with common *EcoR*I (and *Pst* I) restriction fragments were detected when probed with human repetitive sequences.

(*f*) *SVM*

The complex phenotype of SVM, an Indian muntjac mutant cell line, has been resolved into a post-replicative repair (PRR) defect after UV and *N*-methyl-*N*-nitrosourea (MNU) treatments and a defect in the repair of SSBs and DSBs after dimethyl sulphate (DMS) treatment. These defects can be corrected by introducing mouse or human genes. Correction of the PRR defect is associated with a 9-kb fragment of a rescued cosmid and it results in error-prone replication after MNU or UV. Correction of the DSB defect is not associated with improved PRR activity. Both genes are being characterized further by Bouffler et al. and Ryan et al. (Cambridge).

(*g*) *Poly ADP-ribose polymerase* (De Murcia and co-workers, Strasbourg and Heidelberg)

Cloning of the poly (ADP-ribose) polymerase gene has resulted in the identification and characterization of a number of sequence motifs: (i) A zinc finger domain in the *N*-terminal region which is involved in the specific recognition of DNA strand breaks. (ii) A nuclear location signal responsible for the transport of the polymerase into the nucleus. These two properties have been exploited to inhibit the resident polymerase activity by over expressing its DNA binding domain. This situation confers a so-called 'dominant-negative' phenotype to the transfected cells. (iii) An autonomous catalytic domain in the C-terminal region exhibiting both the polymerizing and the branching basal activities.

(*h*) *Methyltransferases*

The cloning of the O^6-methylguanine-DNA methyltransferase (*MGMT*) gene was recently accomplished in several laboratories, and the structure and expression of the gene has been investigated. The *MGMT* gene was studied by Cairns-Smith and co-workers (South Mimms and Cambridge) in human cell lines. Coordinate regulation of *MGMT* and the enzymes galactokinase (GalK) and thymidine kinase (TK) was observed. For example, in Raji cells selection for the TK^- or $GalK^-$ phenotypes results in cells that are

Mex$^-$/TK$^-$/GalK$^-$. The simultaneous loss of expression of these genes is associated with characteristic hypermethylation patterns in the *MGMT* gene, which seem to be part of coordinated, interchromosomal changes in gene expression.

Chromosomal mapping of the human *MGMT* gene was presented by Zunino and co-workers (Genova), who measured enzyme activity in extracts of hamster/human cell hybrids. Human chromosome 10 correlated with the restoration of *MGMT* activity in the hybrids although in some hybrids the presence of this chromosome was insufficient to confer activity. These results suggest that another locus can repress the expression of the *MGMT* gene.

(*i*) *AP endonuclease*

An oxidative damage repair enzyme was isolated from bovine thymus by Robson et al. (Oxford). *N*-Terminal amino acid data were used to obtain a PCR-generated probe, which resulted in the isolation of a full-length cDNA clone designated *BAP1*. The translated amino acid sequence encoding a polypeptide of 35.5 kD is ~25% identical to the exonuclease III protein of *E. coli* although the bovine protein lacks 3′ → 5′ exonuclease activity. A cDNA of the human homolog of *BAP1* was isolated and its sequence found to be very similar to the bovine sequence.

(*j*) *Glycosylases*

O'Connor and Laval (Villejuif) reported the cloning of the human cDNA for 3-methyladenine DNA-glycosylase. The cDNA was isolated by expression and phenotypic correction of *E. coli* glycosylase mutants that are hypersensitive to methylation damage. In extracts, the ANPG enzyme (alkyl-*N*-purine–DNA glycosylase) causes release of 3-meAde and 7-meGua from radiolabeled DNA. The deduced amino acid sequence of human ANPG is ~85% identical to the rat homolog and has regions of homology with that of *E. coli* 3-methyladenine–DNA glycosylase II.

Olsen, Krokan, and co-workers (Bergen, Trondheim) described the cloning of the gene encoding a human uracil–DNA glycosylase, UNG, a very highly conserved protein of 304 amino acids. The cDNA (*UNG15*) was obtained by using a probe based on the *N*-terminal aa sequence. It shows high conservation at the aa level particularly with the *E. coli* enzyme (~55% identity) and — remarkably — to a lower degree with the yeast homolog, and it maps to human chromosome 12. No abnormalities were seen in Bloom's syndrome cells for the expression of *UNG15*. *UNG15* complements *E. coli ung* mutants for cell killing and mutagenesis induced by uracil. In vitro hybrid arrest analysis of the *UNG* transcript indicates that *UNG15* encodes a major form of uracil glycosylase. *UNG15* transcript levels were measured using in vitro transcription of total fibroblast mRNA. A ~7-fold increase in transcript level in late G_1/early S preceded a 2–3-fold increase in enzyme activity. The induction required transcription but not DNA replication. At mitosis the *UNG* level declined to the G_1 level. The *N*-terminal 'signal-like' sequence can be removed, whereas the *C*-terminal part is essential for function.

(*k*) *XRCC1*

Characterization of the human *XRCC1* gene, which corrects CHO mutant EM9, was reported by Thompson and co-workers (Livermore). The nucleotide sequence determination of cDNA and selected genomic regions suggests a 633 aa protein, which is required for the efficient repair of strand breaks produced by ionizing radiation or simple alkylating agents. No homology with any protein or translated cDNA sequence was found in database searches. For the purpose of performing gene targeting in mouse embryonic stem cells, the functional mouse *XRCC1* gene and cDNA clones spanning the entire ORF were isolated. This protein also seems to be highly conserved in evolution.

(*l*) *KIN17*

Angulo and co-workers (Gif-sur-Yvette, Marseille, and Paris) used monoclonal antibodies against *E. coli* RecA protein to screen mouse cDNA libraries. A strongly reacting clone *KIN17* was isolated encoding a putative protein of 44.6 kDa, with an 8-residue RecA motif, a putative zinc-binding domain, and potential nuclear location signals. *KIN17* maps to mouse chromosome 2 and human chromosome 10p. *KIN17* mRNA

was weakly expressed in mouse embryo and more readily detected in transformed rodent cell lines.

1.2.2.2. Drosophila. Pastink and co-workers (Leiden) reported the isolation of DNA-repair genes from Drosophila. Three mutations conferring sensitivity to methyl methanesulfonate were produced by P element insertion; two of these represented alleles of *mus*201 and *mus*205. Revertants of the *mus*205 mutant were obtained, and restriction fragments containing the insertion were cloned and mapped. The Drosophila homolog of human *ERCC-3* was cloned and found to be 71% identical to *ERCC-3* at the amino acid level.

These studies show the successful application of a broad range of approaches to the cloning of repair genes from mammalian cells and Drosophila. In many cases there is high conservation of sequences and motifs across great evolutionary distances. In the case of DNA ligase I, the cell line 46BR represents the first instance in which a clinical disorder is associated with identified mutations in a known repair protein.

1.3. Mechanisms of action of DNA-repair proteins

1.3.1. The UvrABCD system of E. coli

The availability of the components of the UvrABCD system of *E. coli* in a pure form has made possible detailed mechanistic studies of the reaction mechanisms including investigations on the physical interactions between the subunits of the system. Van Houten (Burlington, Vermont) reviewed the reaction mechanisms of the UvrABC excision nuclease and referred to deletion analysis of the UvrA protein made in the laboratory of Grossman. It appears that the *C*-terminal and middle regions are essential for DNA binding and UvrA dimerization respectively, and the *N*-terminus specifies the UvrA/UvrB interaction. He also described work from his own laboratory on the effect of damage size on recognition by the UvrA and UvrB proteins. The substrate was an oligonucleotide in which uracil was incorporated chemically and then removed by uracil DNA glycosylase to produce an AP-site. The AP-site was further modified with methoxy or benzoxyamine to produce DNA with residues of various sizes as substrate for band shift assays with either UvrA or UvrAB binding complexes. It appears that the UvrA protein alone has a relatively high affintity for the small lesion (i.e. the AP-site) whereas the UvrAB interaction favours binding to the larger benzoxy modified residue. He also reviewed the anchored search reaction of Grossman for DNA damage recognition; ie. UvrAB catalyzed unwinding of the DNA from a fixed position to scan impaired base pairing in a stepwise fashion during the search reaction.

Visse et al. (Leiden) have constructed DNA fragments site-specifically modified with cis-Pt to contain either a GG or a GCG intrastrand crosslink. The GG adduct is excised more rapidly than the GCG adduct although UvrAB binding complexes are formed rapidly in both cases, as judged from footprint experiments. They suggest that binding without cutting contributes to the formation of mutations in cis-Pt treated cells. The GG modified DNA was also used for band shift assays to demonstrate the existence of three different binding complexes, one with UvrA alone, one with UvrAB and the third with UvrB. These different binding complexes produced footprints of various sizes and are formed to a different extent depending on the relative amounts of UvrA and UvrB proteins added to the reaction.

Thiagalingam et al. (Johns Hopkins, Baltimore) have studied the mechanism of the DNA binding and ATPase activities of the UvrA protein by site directed mutagenesis in the two different nucleotide binding domains of the protein. The lysine residue in the gly–lys–thr sites was replaced with alanine, glutamine or arginine. It was concluded that both sites are required for damage specific DNA binding of the UvrA protein. Double mutant proteins are defective in ATP hydrolysis, whereas both single mutant proteins can hydrolyze ATP. However both single and double mutant proteins bind strongly to DNA, but in a damage-independent fashion. Studies of this type are of major importance in understanding how DNA damage is recognized by the UvrABC repair enzymes.

1.3.2. Other DNA repair proteins

The pyrimidine dimer specific endonuclease V from bacteriphage T4 (product of the *denV* gene)

was investigated by chemical modification (methylation), by site-specific mutagenesis and by studying the effects of metals on enzyme activity (Lloyd et al., Nashville). By chemical modification of lysine residues with radioactive methyl it was concluded that the active site is located very close to the *N*-terminus of the enzyme. Replacement of nonpolar aminoacids with postively charged residues in alpha helical domains increases binding to nontarget DNA, but it does not affect the ability to nick at AP sites or dimer-nicking activities to any great extent. Site-specific modification of a cysteine residue made the enzyme resistant to metal inhibition, implicating this residue in metal binding inactivation.

Yasui et al. (Sendai) have cloned the photolyase gene from *Neurospora crassa* and studied the importance of the *N*-terminal end for enzyme activity. Both yeast and Neurospora enzymes have protruding *N*-terminal ends not found in the enzymes from procaryotes. These regions are not homologous, but nevertheless appear essential both for DNA binding and for transport into the nucleus and mitochondria. They have also carried out site-directed mutagenesis of about 30 different aa's from regions conserved between the bacterial and eucaryotic sequences known so far. The active site of the enzyme was tentatively identified as a region where three different replacements abolished DNA binding.

The formamido pyrimidine DNA glycosylase from *E. coli* has been studied in great detail by Laval and collaborators (Villejuif). It appears to be a versatile enzyme with a substrate specificity much broader than previously assumed. The enzyme participates in repair of both alkylation damage as well as oxidative damage to DNA. The latter includes 8-hydroxyguanine, which is thought to be a major spontaneously formed DNA lesion caused by naturally occurring oxidizing agents present in cells. It was also shown that the UvrABC protein was able to remove this product in vitro. *E. coli* strains carrying both *fpg* and *uvrA* mutations were shown to be extremely sensitive to oxidizing agents.

The *MAG* gene from yeast is an inducible gene homologous to the *alkA* gene of *E. coli*, encoding a DNA glycosylase for removal of alkylated purines (Bjørås et al., Kjeller). The enzyme has been purified to homogeneity and has properties similar to the AlkA glycosylase with the notable difference of a much lower affinity for 3-methylguanine, which was thought to be the main substrate for the *alkA* function in *E. coli*. Interestingly, the sequences of this function are much more conserved between yeast and bacteria than between yeast and mammalian cells as judged from comparison with the sequence from rat cDNA reported recently by O'Connor and Laval.

We have now experienced a rapid development in the elucidation of lower eucaryotic repair systems, in particularly in the cloning of genes and analysis of sequences. The detailed protein characterization is lagging behind and is mostly confined to bacterial systems, with the exception of simpler functions like DNA glycosylases. Repair enzyme studies in bacteria have now reached the protein engineering stage with elaborate structure–function analysis.

1.4. In vitro repair systems

An alternative approach for studying DNA repair involves the development of in vitro systems, and subsequently dissecting them into their components. Subcellular systems for studying DNA repair in eukaryotes are of several kinds, ranging from the cell extract at one extreme to the microinjected whole cell at the other. The aim in all cases is to be able to manipulate and dissect the biochemistry of repair — ultimately, to reconstruct DNA repair from its component parts in the test tube. At the moment, the nearest approach to a truly in vitro system is the method developed by Wood and colleagues (ICRF, South Mimms), in which a very simple plasmid DNA molecule containing damage of a particular kind is incubated with protein extracts of mammalian cell origin and repair is detected as the incorporation of labelled nucleotides into the plasmid DNA. The assay has been used in the purification of repair proteins, testing their ability to complement the activity of extracts from repair-defective cells.

1.4.1. Excision repair synthesis in cell-free extracts

Coverley et al. (ICRF, South Mimms) reported their recent evidence that human single-stranded

DNA-binding protein (HSSB) is involved in excision repair. Antibodies to this protein suppress repair in the in vitro system; and addition of HSSB stimulates it. The stimulation is due to an increase in number of repair sites (rather than an increased patch size), and HSSB appears to have a role prior to incision. Aphidicolin, the inhibitor of DNA polymerases α, δ and ϵ, inhibits DNA-repair synthesis by cell extracts, but anti-polymerase α or β do not, confirming that, as is already known for other systems, DNA polymerase δ and/or ϵ is involved in UV-induced excision repair.

Topoisomerases have long been thought of as likely candidates for a role in DNA repair in view of their ability to modulate DNA topology and to stabilise DNA breaks (the intermediates in the reactions they catalyse). But specific inhibitors of topoI and topoII do not affect DNA repair in whole cells and Frosina et al. (Genova) have now found only a slight inhibition of in vitro repair of UV-irradiated plasmid DNA by CHO cell extracts when camptothecin (a topoI inhibitor) and/or VM26 (which blocks topoII) are present. However, it seems that camptothecin does not inhibit relaxation of plasmid DNA by topoI under these conditions, so a definite statement awaits further experiments.

The same in vitro plasmid DNA-repair system has been used by Calsou et al. (Toulouse) to look at the possibility that increased repair of the adducts produced in DNA by the chemotherapeutic agent cis-platin could account for resistance to this drug and failure of chemotherapy in some patients. They tested protein extracts of the murine leukaemic L1210 cell line and derivatives that are resistant either to cis-platin or to *N,N'*-bis [2-chloroethyl]-*N*-nitrosourea. When the plasmid DNA substrate was damaged with cis-platin, the extract from the cis-platin-resistant cell line carried out no more repair synthesis than the extracts of the other cell lines.

1.4.2. Rejoining of strand breaks in extracts

The rejoining of DSBs in plasmid DNA can also be achieved in vitro using human cell extracts. Thacker and co-workers (MRC, Chilton) introduced DSBs at specific sites in the *lacZ* gene of plasmid DNA using restriction endonucleases. After incubation with cell extracts the efficiency of rejoining was monitored by separating rejoined and broken plasmid DNA by gel electrophoresis; the fidelity of rejoining could also be assessed, by transforming bacteria with the rejoined plasmid and checking for *lacZ* expression. Rejoining efficiency was higher the more overhang there was at the restriction site. No difference was seen in rejoining efficiency between extracts of normal human and A-T cells, but the fidelity of rejoining was much lower for an A-T cell extract. The commonest feature of misrepaired DSBs was a deletion occurring between short direct repeats — a pattern that is known to occur with some deletions in human genes in vivo. Possibly one repeat becomes misaligned with the complementary sequence of the other repeat, with concomitant degradation of the intervening DNA.

1.4.3. Permeabilised cells

Though the in vitro repair systems based on cell extracts and plasmid DNA have already yielded much valuable information, their simplicity may be a limitation. The damaged eukaryotic chromosome is, after all, very different — in size, composition and topology — from naked plasmid DNA. With this in mind, various groups are looking at the alternative of permeabilised whole cells in which the damaged DNA resembles more closely the natural substrate, and some at least of the proteins involved in repair may still be found in their normal configuration and associated with chromatin. Ahnström and Håkansson (Stockholm), for instance, have permeabilised monolayer cultures of mammalian cells and extracted them with a high concentration of NaCl, which removes the structural chromatin proteins and leaves the DNA as supercoiled loops in nucleoids — still attached as monolayers to the culture surface. Surprisingly, if the cells were UV-irradiated before permeabilisation and salt extraction, DNA breaks accumulated on subsequent incubation, implying that UV endonuclease activity was retained — perhaps bound to dimers? — through the extraction process. If UV-irradiation was applied later, after preparation of the nucleoids, the endogenous incision activity was much lower. The aim is to develop a system for investigating the

effects of damage-specific endonucleases on the nucleoid DNA.

The method of Jackson and Cook has been adopted by van Hoffen and co-workers (Leiden). They embedded human fibroblasts in agarose microbeads and extracted isotonically until what remains is intact chromatin on the nuclear matrix. This material is capable of DNA replication and transcription, and replication is inhibited by UV-irradiation, as in whole cells. A similar isolation applied to confluent human fibroblasts gives microbead-embedded chromatin with a low capacity for replicative DNA synthesis (since the cells were in G_0), and in this case, UV-irradiation caused a stimulation of DNA synthesis relative to that seen in chromatin from unirradiated cells. It appears that the enzymes required for damage recognition, incision and repair synthesis are retained under these mild extraction conditions.

Collins et al. (Aberdeen) described a simple method for assaying the later stages of repair — resynthesis and ligation — in permeable cells. This method measures the rejoining of incomplete repair sites accumulated by incubating UV-irradiated cells with aphidicolin and hydroxyurea before permeabilisation. The rejoining after removal of inhibitors is virtually as rapid as in whole cells. It requires the presence of deoxyribonucleotides and is inhibited by aphidicolin, so it is clear that ligation occurs only after repair synthesis has filled in the gaps in the DNA. A novel method for assaying breaks in permeable cells is single cell gel electrophoresis (SCGE), in which — at high pH after embedding in agarose — broken DNA migrates from the cell as a 'comet', visualised by fluorescence microscopy. The action of exogenous endonuclease on UV-irradiated, permeablised, salt extracted cells is clearly seen by SCGE, and so this system, too, may be suitable for reconstructing repair in vitro.

1.4.4. Microinjection systems

Instead of permeabilising cells to allow the experimenter to introduce enzymes, antibodies etc., it is possible to microinject foreign substances into cells. An impressive demonstration of the repair capacity of Xenopus oocytes was described by Ackerman et al. (NIH). Plasmids containing UV-induced pyrimidine dimers were injected into oocytes and recovered at different times for analysis of the number of dimers repaired. This was done by incubating with T4 UV endonuclease to introduce breaks at residual dimers and analysing the distribution of breaks by alkaline agarose gel electrophoresis. More than 10^{10} dimers could be repaired in a single oocyte in 4–6 h. From the rapid appearance of completely repaired plasmid molecules while others were still unrepaired, repair appeared to be processive rather than distributive. In contrast to the results obtained with human cell extracts in vitro, the repair in oocytes was inhibited by aphidicolin, as well as by antibodies to DNA polymerase α. Finally, when M13 DNA containing a single AAF adduct at a specific site was injected, repair synthesis was localised to a patch of < 27 nucleotides around the site.

1.5. Mismatch repair

An important repair process, which was under-represented at the meeting was mismatch repair, which is involved in correcting errors introduced into DNA during replication. Two types of mismatch repair have been identified and partially characterised.

1.5.1. General mismatch repair

A general mismatch repair system has been found in bacteria, yeast, insects, plants and vertebrates (Xenopus and human cells). This process acts with a long-patch excision resynthesis mode of action (as demonstrated in *E. coli* and Xenopus) (Brooks et al., Paris). Its genetic effects indicate that it is a multi-faceted DNA-editing system: its deficiency causes a high increase in point mutagenesis, chromosomal rearrangements and interspecies recombination. Its DNA mismatch recognition coupled with helicase and nuclease activities are responsible for editing DNA templates against secondary structures, which prevents deletions, and for correction of DNA replication errors as well as for prevention of recombination between diverged homologous sequences.

1.5.2. Specialized mismatch repair

Specialized mismatch repair systems act by removing only one base from a specific mismatch.

An example is the G:T → G:C mismatch repair that may prevent 5-meC → T mutagenesis in bacteria and in mammalian cells (Nedderman and Jiricny, Rome). Such very short patch DNA repair systems are typical for *N*-glycosylase-initiated DNA repair (e.g. uracil repair, Dianov and Lindahl, ICRF, South Mimms). Indeed, the mammalian G:T → G:C repair appears to act via an *N*-glycosylase mechanism and a single (or very few) nucleotide replacement by DNA polymerase β (Jiricny, Rome).

2. Effect of chromatin structure and gene activity on DNA repair

2.1. Effect of chromatin structure

It has long been realised that both damage and repair of DNA are likely to be influenced by the macromolecular associations of DNA and protein in the nucleus. Distribution of damage can vary; for example, 6–4 photoproducts (6–4PP) and some types of chemical adducts occur more in linker (or nucleosome-free) DNA than within the nucleosomes, whereas cyclobutane pyrimidine dimers (CPD) are distributed equally between linker and nucleosome but show a 10.3 base periodicity imposed by the helical turns of DNA in contact with the nucleosomal core (Smerdon, Pullman).

A poly ADP-ribosylation shuttle mechanism, proposed by Althaus et al. (Zurich) may be responsible for the chromatin rearrangements that accompany DNA repair. Poly (ADP-ribose) polymerase activity is stimulated by strand breaks. A very strong interaction occurs between an ADP-ribose homopolymer and histones, and this regulates the association/dissociation of histones from DNA; the histone shuttle depends on poly (ADP-ribose) polymerase and its counterpart, the glycohydrolase, acting in concert. Only when the shuttle is operating do certain enzymes (e.g. a mammalian DNA helicase implicated in DNA repair) have access to the DNA, and it is very likely that the shuttle plays a role in the unfolding of chromatin domains during repair.

The poly (ADP-ribose) turnover system has been reconstructed in vitro from the purified enzymes i.e. the polymerase and the glycohydrolase, by Poirier et al. (Quebec). In a model system with the enzymes at concentrations representing a 1:1 ratio of the two activities, the product of the first enzyme serves as substrate for the second, and this system has been characterised in terms of NAD consumption, ADP-ribose production, and the steady-state level of the polymer. The intention is now to examine various repair enzymes for their activity in vitro in conditions of poly (ADP-ribose) turnover like those in vivo.

An involvement of poly (ADP-ribose) in DNA repair is suggested by a study of UV-resistant (LY-S) and UV-sensitive (LY-R) derivatives of the L5178Y mouse lymphoma cell line (Kleczkowska et al., Warsaw and Zurich). The excision repair-proficient LY-S cells contain 3 times higher levels of ADP-ribose polymers than do LY-R cells and at the same time the poly (ADP-ribose) in LY-S has a much shorter half-life; thus a great deal of ADP-ribose turnover occurs in LY-S cells. UV-irradiation with fairly high doses reduces the half life of poly (ADP-ribose) in both LY-S and LY-R cells. Whether the difference in poly (ADP-ribose) kinetics is causally related to the difference in UV-sensitivity is not clear.

2.2. Gene-specificity and strand-specificity of DNA repair

2.2.1. UV-lesions

2.2.1.1. Mammals. Hanawalt (Stanford) reviewed both the methods for measuring repair in specific sequences and data concerning preferential repair of CPDs in transcribed strands of genes. In several systems, DNA is repaired more rapidly in actively transcribed genes than in inactive genes, and the transcribed strand is often repaired more rapidly than the non-transcribed strand. As more systems are studied, however, the situation is becoming more complex. In two differentiating rat systems repair was slower than in the cultured rodent cells previously studied, and although some correlation of repair with transcription was noted, strand specificity was not found. This is consistent with the existence of multiple hierarchies of repair efficiencies within cells, a notion invoked time and again at this meeting. Hanawalt also presented data on repair in two mutant cell types which repair CPD and

6–4PPs differentially. The 'revertant' XP-129, previously shown to be deficient in removing CPDs from bulk DNA, in fact exhibits proficient repair of CPD in the *dhfr* gene. Thus the nearly normal UV resistance of these cells may not be a consequence solely of proficient repair of 6–4PPs. In addition, CHO UV61 cells, which also exhibit proficient removal of 6–4PP but are considerably UV-sensitive, showed a low but significant (30–40%) removal of CPD from the transcribed strand of the *dhfr* gene.

Mullenders (Leiden) discussed the removal of CPD from the human *ada* gene in various cell types. In normal cells, more rapid repair was observed in the transcribed strand of a fragment in the 5′ portion of the gene, similar to previously described results for *dhfr*. Two inactive sequences on the single X chromosome in these cells exhibited slower and less complete repair than that noted for the non-transcribed strand of active genes in these cells. In a mutant cell that does not transcribe the *ada* gene because of a deletion in the promoter, preferential repair of the transcribed strand was lost: both strands of the fragment in the 5′ end were repaired at the rate and extent observed for the non-transcribed strand in normal cells. The fact that this repair is still considerably more efficient than that observed for 'naturally' repressed sequences on the X chromosome was taken to indicate that even in the absence of transcription, chromatin structure/accessibility still affects repair.

In XP-C cells, repair of the transcribed strands proceeds as in the normal cells, but the repair of non-transcribed strands and inactive X-chromosome sequences is diminished to near zero. In CS cells, repair in both strands of *ada* resembled that in the inactive X chromosome sequences, rather than that of the non-transcribed strand in normal cells. Thus the defect is more than a simple loss of the ability to repair transcribing strands; the entire gene is repaired in the inefficient manner characteristic of repressed sequences. These conclusions about the levels of repair hierarchy in human cells and the deficiencies in XP-C and CS rest heavily on the significance of the efficiency of repair in inactive genes, and more data concerning such sequences is clearly needed.

Leadon (Berkeley) had previously reported that at early times after damaging treatment, fragments containing active human metallothionein genes acquired repair patches more rapidly than those containing unexpressed genes, and that increased expression of the active genes further increased the repair rate. In these studies, DNA fragments are separated according to content of repair patches and probed for specific sequences. Here he showed that these increases are specific to the transcribed strands of the genes, and are sensitive to alpha-amanitin. This was true for repair resulting from treatment with aflatoxin B1 or UV-irradiation and provides evidence that strand-specific repair is dependent on transcription, and can occur for aflatoxin adducts. Since most of the the repair synthesis occurring early after irradiation in human cells reflects repair of 6–4PPs, these results could be interpreted as evidence for strand-specific repair of this lesion. Kalle et al. (Leiden) reported development of an improved method for using antibody to isolate DNA containing BrdUrd repair patches, using biotinylated goat secondary antibodies and magnetic beads coated with streptavidin. This method is potentially useful for a variety of antibodies, and does not require the use of precipitation or adsorption to nitrocellulose for the separation of bound and free DNA fragments.

Two groups presented data from mouse cells in situ. Ruven et al. (Leiden) compared repair in the active *hprt* and inactive *c-mos* genes in mouse skin, finding proficient repair in the active gene but almost none in the inactive one. Bohr et al. (NIH) showed that in mouse B cells removed from the animal just prior to the experiment, repair of CPD in the *c-myc* gene was efficient on the transcribed strand, but nearly absent on the other strand. However, both strands in a fragment just 5′ to *c-myc* were repaired at high efficiency. No transcription of this fragment was detected by Northern analysis, but the possibility of non-productive transcription in this region remains. These reports suggest that the peculiar deficiency of cultured rodent cells in removing CPDs may in fact reflect a similar phenomenon in vivo.

Rasko et al. (Szeged, Hungary) reported attempts to study gene-specific repair in mouse

teratocarcinoma cells, which showed greatly reduced UV-induced nicking of genomic DNA when differentiated. Efficient repair was not observed in 24 h in the differentiated cells, consistent with results reported by Hanawalt that repair in differentiated rat cells is quite slow.

Considerable attention was paid to the problem of induction and repair of 6–4PPs. Studies with UV are confounded by the possibilities of differential induction and repair of these lesions, which are always induced in DNA along with the more frequent CPDs, and by the lack of a reliable, simple assay. Meschini et al. (Leiden), using photoreactivation of CPD and UvrABC nuclease, found comparable frequencies of 6–4PPs (i.e. non-CPD lesions) induced in the active mouse *hprt* and inactive *c-mos* genes, and equally proficient repair after 40 J/m^2. These results are similar to those published for the CHO *dhfr* locus by Thomas et al. However, Jen et al. (San Fransisco and Texas), using alkaline nicking of 6–4PPs photoconverted to their Dewar isomers, found a much higher frequency of 6–4PPs in the active *dfhr* gene in human cells than in a fragment containing the inactive delta-globin gene. It is not yet clear which sequence (if either) resembles the genome overall in this case. These results may be related to the recent finding of Mitchell et al. and Gale and Smerdon that 6–4PP formation is repressed in nucleosome core DNA compared to nucleosome free (predominately linker) DNA, and to results presented by Holmquist (Duarte) and by Pfeifer et al. (Duarte). The latter authors used ligation-mediated PCR to map the sites of hot-alkaline or T4 endonuclease V cleavage sites in specific sequences. Among several notable findings was the result that photoproducts (including CPD) in DNA sequences containing binding sites for regulatory proteins could be increased in yield by up to 18-fold. This suggests that although the average CPD yield for the large DNA sequences examined in several laboratories seems to be invariant, at a finer level of detail this may not be the case.

Such a non-random induction of photoproducts may account for the results reported by Murad et al. (Pullman). They studied repair and transcription of a construct that contains the small herpes *tk* gene under control of an LTR, integrated into the mouse cell genome. Although *tk*-RNA synthesis decreased after UV and then recovered, this recovery did not correlate with a high degree of repair in this DNA sequence. They suggested a new level of preferential repair, namely that of photoproducts in locations that disrupt promoter function. However, an unanticipated increased formation of lesions precisely in such regions might account in part for these results.

Walker and Baig (London and Ontario) reported development of a plasmid system for studying repair in mammalian cells. They transfected irradiated plasmids carrying the *E. coli lacZ* gene into CHO cells and measured the loss of T4 endonuclease V sensitive sites from various restriction fragments. Although some technical difficulties remain with controlling the introduction of plasmid, they observed significant repair in sequences carrying the induced gene but no repair in a fragment carrying silent sequences.

2.2.1.2. Drosophila. All the above pertains to repair in mammalian cells. De Cock et al. (Leiden) examined repair of CPD in genes in two cultured Drosophila cell lines. They found similar repair rates and extents for active (*gart* and *notch*) and inactive (*white*) genes and the genome overall. Induction of the beta-tubulin gene had no effect on its repair, and strand-specific repair was not observed in any gene examined. The lack of preferential repair of active genes in these cells may be related to the fact that all of their DNA appears to be in the 'potentially active' or 'open' configuration, in contrast to the situation in mammalian cells, where a large portion of the genome is in a highly repressed, condensed state.

2.2.1.3. Yeast. Terleth et al. (Leiden) had previously reported differential repair between the active *MATα* and repressed *HMLα* mating type loci in *S. cerevisiae* and that the repair in the latter was very slow except in the G2 phase of the cell cycle. Recent work has shown that strand-specific repair does not occur in the *MATα* locus in non-G2 cells, and that in fact the differential repair first observed is due to a repression of repair activity in *HMLα* compared to the genome average, and this is probably mediated by chro-

matin structure. Deletion mutations that abolish *MATα* transcription had no effect on its repair. However, *rad7* and *rad16* mutants appear to be unable to effect repair of the repressed *HMLα*. It appears that only a small fraction of the yeast genome is held in a condensed state during most of the cell cycle, but that those sequences require some system to allow their repair. In contrast to these results, Leadon (Berkeley) observed that removal of thymine glycols from the *GAL7* gene was enhanced when the gene was induced, using antibodies to the lesion as a means of separating repaired from unrepaired fragments. This enhancement of repair appeared to be dependent on actual transcription, rather than on induced changes in chromatin structure, because RNA polymerase II temperature sensitive mutants failed to show it at the non-permissive temperature.

Smerdon (Pullman) presented the work that he and Thoma (Zurich) had carried out with a yeast plasmid in which the minichromosome appears to have the repair characteristics of mammalian cells: non-transcribed sequences (the non-transcribed strand of *URA3* and sequences near the replication origin) had very slow repair, while fast repair was exhibited by the transcribed strand of the *URA3* gene and by other sequences which were transcribed non-productively. An important question is how these repair rates compare to rates in the genome under similar conditions.

The yeast data can perhaps be forced to fit a model in which most sequences in the genome are so well repaired owing to an open type of chromatin structure that transcription state does not markedly influence the repair rate. The small fraction of repressed sequences in these cells exhibit slower repair except when induced or opened in preparation for mitosis. Sequences on plasmids in a heavily irradiated cell may gain access to repair activity much more readily when transcription is blocked by a lesion. More data are clearly needed to help refine these ideas.

2.2.2. Non-UV-lesions

The degree to which repair of lesions other than UV photoproducts, particularly non-bulky lesions, is heterogeneous in the genome remains unclear. Bohr and coworkers (NIH) have compared repair of a number of lesions in the CHO *dhfr* gene to that in a non-transcribed sequence downstream from it. Preferential repair appears to be absent for methylated purines, cisplatin interstrand crosslinks and aminofluorene adducts. More efficient repair in the gene than in the silent sequence was found for nitrogen mustard adducts, cisplatin intrastrand adducts and 6–4PP, although not to the extreme degree as observed for CPD. Strong evidence for strand-specific repair has only been obtained for CPD.

Thomale (Essen) discussed general strategies for using antibodies to measure lesion frequency in specific sequences, and described in detail results for O^6-ethylguanine, presented in a poster by Hochleitner et al. By separating DNA fragments that bound antibody to the lesion from those that did not, and using PCR to measure the content of specific sequences in the two fractions, they were able to measure adduct formation in the β-actin and IgE heavy chain genes in rat hepatoma cells. Although it appeared that repair kinetics were similar for active and inactive sequences, the 'initial' absolute adduct frequency of the active gene was only about half that of the inactive one. It seems plausible that the initial supply of methyltransferase may have significantly reduced the adduct frequency in the active gene during the time of treatment, suggesting heterogeneity in repair under conditions of limited repair protein. Additional experiments are needed to examine this, but it appears that methods for using antibodies for lesions to examine repair in sequences are finally becoming practical.

Van Houten et al. (Burlington) measured repair in a 14-kb fragment of mitochondrial DNA of mouse L1210 cells. They used either the UvrABC nuclease to cleave DNA at sites of cisplatin adducts or UV photoproducts or T4 endonuclease V to cleave at CPD. A subline of L1210 resistant to cisplatin showed about a 3-fold reduction in cisplatin adduct induction, but neither the resistant nor normal cells removed the adducts from mitochondrial DNA. CPD were also not removed, a result reported many years ago, but — nevertheless — 60% of the UvrABC-sensitive sites were removed from mtDNA of UV-

irradiated cells. It was suggested that a relatively large fraction of lesions induced in mtDNA might be oxidation products like pyrimidine hydrates recognized and removed by UvrABC, which might be repaired in mitochondria by specific UV-endonucleases.

3. Consequences of DNA damage and repair

3.1. DNA damage and replication in vitro

Two posters dealt with the influence of DNA damage on replication, studied in in vitro systems. Strauss et al. (Chicago) found that the presence of guanine-aminofluorene on a M13mp2 DNA template blocks replication by the T7 DNA polymerase, but these lesions are bypassed by modified T7 polymerase, which lacks the 3′–5′ proofreading exonuclease activity (Sequenase 2). However, guanine-aminofluorene can block the action of HIV and AMV reverse transcriptases which lack 3′–5′ exonuclease activity. Sequenase 2 cannot bypass the slightly different lesion, guanine acetylaminofluorene, and it is not understood what determines whether a particular lesion is a block to replication. The spectrum of mutations produced by replication of M13mp2 DNA damaged by γ-radiation was also investigated. DNA synthesis (using Sequenase) on this template provides the possibility of mutations arising from misincorporation opposite damaged bases, and such mutations were analysed after transfection into bacteria. A high frequency of mutations resulted from changes at pyrimidines; most mutations at T were deletions rather than base substitutions. The low frequency of the latter is explained by the general tendency for A to be inserted at sites where bypass by the polymerase is possible but pairing is ambiguous.

The SV40 in vitro system is regarded as a model for mammalian replication. Bignami et al. (ICRF, South Mimms, and Rome) constructed a double stranded 109-mer corresponding to the SV40 replication origin with two binding sites for the viral T antigen, containing O^6-methylguanine at specific sites in one strand. With 10 methylated bases in the two binding sites, T antigen bound with reduced efficiency. Methylation in binding site II alone (at 7 sites) did not affect T antigen binding, but only 1 methylation in binding site I was inhibitory. The helicase activity of T antigen was also investigated. A single O^6-methylguanine in binding site I inhibited unwinding, but methylation at 3 bases in binding site I seems to destabilise the helix and enhances the T antigen helicase activity. Binding site I contains a sequence that caused curvature of DNA, and it seems that O^6-methylation of guanine may be most disruptive when it occurs at 'bends' in DNA.

3.2. Molecular mechanisms of mutagenesis

The detailed molecular mechanisms of mutagenesis are still largely unknown even in bacteria. Recently developed methodologies that allow the introduction of single adducts into well defined positions in a genome have made it possible to ask fundamental questions, most of which cannot be answered with randomly modified genomes:

(i) are mutations targeted at the site of the adduct?

(ii) at what frequency is an adduct converted into a mutation and as a corollary how frequently is the 'correct' base inserted across an adduct?

(iii) what are the different 'intrinsic' mutagenicities among different lesions?

(iv) to what extent does the local DNA sequence context modulate the mutagenicity of a given lesion?

(v) what is the quantitative effect of repair in the avoidance of mutations?

These issues were addressed by several participants. In a comparative study involving *E. coli* and yeast, Lawrence et al. (Rochester) determined the efficiency of translesion synthesis at specific UV-induced lesions located on single stranded vectors. Error frequencies varied from 6% to nearly 100% depending upon the conditions and the specific lesions that were bypassed. In this respect it was found that 6–4PPs are much more mutagenic than CPDs. A similar conclusion was reached by Zdzienicka et al. (see below), in a UV-induced mutagenesis study involving the *hprt* gene in mammalian cells. Essigmann (MIT) reviewed the work of his group and that of Fuchs (Strasbourg) on the biological effects of different cis-platin adducts in DNA. When single adducts were constructed in M13 phage the major adduct, the G–G intrastrand cross-link was toxic and, in an SOS^+ background produced mutations at a

low level. In contrast a minor adduct, the A–G intrastrand cross-link, appeared to be 5–10-fold more mutagenic. An 80–90 kD basic protein binding to cis-platin adducts has been purified, and cDNA clones expressing this protein have been isolated. Using single adducted double stranded plasmids, Lambert et al. (Strasbourg) demonstrated a strong positional effect of a single *N*-2-acetylaminofluorene (AAF) adduct on the induction of −1 frameshift mutations in a continuous run of guanine residues. Although 90% of the −1 frameshifts were targeted at the site of the AAF adduct, about 10% of the mutations occur 5′ to the adduct showing clearly the occurrence of 'semi-targeted' mutations. The authors discussed mechanistic implications in terms of slippage-mediated frameshift mutagenesis. Bintz et al. (Brussels and Strasbourg), investigated the genetic control of a new frameshift mutation pathway involved in the response to AAF adducts. This pathway differs from the classical UV-mutagenesis pathway in that it requires an SOS-gene that is not *umuD* or *C* and that the only role of RecA is to disrupt the SOS-regulon by cleavage of LexA. RecA is not needed directly or to process proteolytically any proteins other than LexA. The mutagenic properties of abasic sites in mammalian cells were investigated by Gentil et al. (Villejuif), who showed that in contrast to bacteria, mammalian cells incorporate adenine, cytosine and thymine with similar frequencies opposite an abasic site. Menck et al. (Sao Paulo) discussed the induction of mutations by singlet oxygen and found that in single-stranded mammalian shuttle vectors guanine residues are the major target for singlet oxygen lesions leading predominantly to the induction of G → T transversions.

3.3. Mechanisms of selectivity in repair and mutagenesis

Although the general finding discussed in Section 2.2 is that there is selective repair of some lesions in active genes, many studies involving different systems tend to suggest that transcription might not always be the only function modulating repair. Several posters addressed the question of selectivity in repair by investigating its consequence for mutagenesis. In addition to the role of transcription, the potential role of the asymmetric nature of replication and of factors such as the precise chromosomal location of a gene were discussed.

Several studies investigated the mutation spectrum, strand specificity and effect of DNA repair on mutagenesis in the *hprt* locus. Following UV-irradiation of repair-deficient hamster cells (Vrieling, Leiden), of XP-A cells (Steingrimsdottir et al., MRC, Sussex; Maher, East Lansing), or of normal human fibroblasts irradiated in S so that there was little time to repair DNA before replication (Maher et al.), the majority of mutations could be ascribed to damage at dipyrimidine sites located on the transcribed strands. In the hamster cells the mutations were exclusively G : C → A : T transitions whereas in the human cells 30% were transversions. In UV-exposed repair-proficient hamster (Vrieling) or G1 phase human cells (Maher), the strand bias was reversed, with most of the mutations being attributable to damage on the non-transcribed strand. These findings were consistent with the occurrence of preferential repair of UV-induced damage from the transcribed strand. Tasseron-de Jong and co-workers (Leiden) introduced a *hprt* cDNA minigene into five different locations in the hamster chromosome. In contrast to all the findings with endogenous genes, no strand bias was found for UV-induced mutations in either a repair-proficient or -deficient background. These findings are difficult to reconcile with any of the models proposed to account for the strand bias in the endogenous gene. A further interesting finding of the mutation spectrum in endogenous genes is that a large proportion of the mutations found in the *hprt* gene, whether induced in cultured cells, or occurring as background mutations in human circulating lymphocytes result in aberrant splicing of the *hprt* mRNA (Steingrimsdottir).

Using an EBV-derived shuttle vector containing the *gpt* gene, the groups of Nehls (Ulm) and of Dogliotti (Rome) have shown that MNU induces an asymmetric distribution of G → A transitions with > 90% of the mutations occurring at G residues in the non-transcribed strand. That this asymmetry was not related to transcription of the target gene was shown by using an inducible

vector: strand asymmetry was maintained irrespective of transcription.

An in vivo mutation assay involving transgenic mice, in which several copies of lambda sequences with the *lacZ* gene had been integrated, has been developed. Several strains have been generated, out of which one strain (35.5) with the insert in the X-chromosome exhibited a much higher frequency of spontaneously occurring mutations in comparison to another strain of mice (40.6) which harboured the insert in one of the autosomes. The basis for this hypersensitivity is under investigation (Gossen et al., Rijswijk).

3.4. Effects of alkylating agents

3.4.1. E. coli

In the hypermutable *E. coli* strain UC 1101, the induced frequency of mutations by ethylating agents was found to be very high in comparison to the induction by methylating agents, though the survival curves following treatment with both these two types of agents were similar. When the *ada* background was introduced into this strain, ethylating agents were equally mutagenic in ada^+ and ada^- strains, whereas methylating agents were more mutagenic in ada^- background. From these data it was concluded that the hypermutable strain is defective in alkyltransferase encoded in the *ogt* gene (Abril et al., Cordoba).

An oligonucleotide probing/colony hybridisation method was used by Zielenska et al. (Toronto) to monitor rapidly 174 mutations in 85 sites in a 180 bp target of the *lacI* gene of *E. coli*. The predominance of GC → AT transition mutations following EMS mutagenesis was consistent with O^6-ethylguanine production. EMS-induced mutation in a $uvrB^-$ strain was no different from wild-type, indicating the lack of repair of O^6-ethylguanine by the *uvr* system. However, longer-chain alkylating agents were more mutagenic in a uvr^- background than wild-type, suggesting that higher O^6-alkylguanines are substrates of excision-repair systems.

3.4.2. Drosophila

The mutational specificity of several monofuctional and cross-linking alkylating agents was examined in the *vermillion* gene of excision repair-deficient and -proficient *D. melanogaster* by Nivard et al. (Leiden). GC → AT transitions predominated with *N*-ethyl-*N*-nitrosourea and ethyl methanesulphonate but were almost absent after methylating agents, possibly a consequence of O^6-alkylguanine–DNA-alklytranferase activity. It was proposed that AP-sites were the cause of AT → TA, GC → TA and deletion mutations. These mutations were more frequent in the excision-repair-deficient strains.

3.4.3. Mammals

In a Friend murine erythroleukemia cell line which is deficient in MGMT, a TK^- mutant was found to be hypersensitive to UV and γ-rays which was attributed to error-prone repair mediated through an imbalance in the nucleoside triphosphate pools caused by the TK-deficiency. An $APRT^-$ Friend cell line exhibited an increased sensitivity to alkylating agents, which was thought to be due to reduced levels of ATP in these cells affecting ATP-dependent DNA repair (McKenna et al., Coleraine).

The relationship between O^6-methylguanine, its repair and cytotoxicity has for several years proved to be somewhat puzzling. Several posters addressed this question. The importance of MGMT in protecting cells against alkylating agents was illustrated by Kaina et al. (Karlsruhe, Oak Ridge) who transfected human *MGMT* cDNA under SV40-promoter control into CHO cells lacking MGMT activity. There was a dramatic increase in resistance to the lethal, mutagenic, SCE-inducing and, to a lesser degree, clastogenic effects of simple alkylating agents. Interestingly, protection was also afforded against ethylating agents, in contrast to the generally expressed view that mammalian MGMT is specific for methyl groups. In contrast to this type of experiment, which clearly indicates the importance of MGMT for protecting cells, MNNG-resistant cells selected from MGMT-deficient lines, in general acquire resistance by an alternative mechanism. There are several reports of this in the literature and two further presentations at the meeting. Aquilina et al. (Rome) isolated a CHO line which was 15-fold more resistant than the parent cell line to MNU, MNNG and 6-thioguanine but had very little increase in MGMT

activity. It was proposed that the resistant cells have the ability to 'tolerate' O^6-methylguanine and 6-thioguanine in DNA. Hall and coworkers (Lyon) isolated a similar series of MNNG-resistant hamster cell lines which were also resistant to MNU, MMS and 6-thioguanine despite having no detectable MGMT. Evidence for enhanced activity of DNA polymerase-β was presented, but the relationship of this finding to the tolerance to alkylation damage needs to be elucidated.

Klungland et al. (Oslo, Kjeller and Manchester) showed that Chinese hamster fibroblasts transfected with the bacterial *tag* gene (coding for 3-methyladenine DNA glycosylase I) are more resistant to the toxic effect of MMS, indicating that 3-methyladenine, as in *E. coli*, is a toxic DNA lesion.

3.5. Inducible effects of DNA damage

The growing number of DNA damage inducible (DIN) genes isolated during the last few years, as well as the characterization of the molecular mechanisms underlying these responses in a few cases, have now clearly demonstrated the existence of inducible phenomena in mammalian cells. However, the physiological significance of these responses is far from being understood. One reason might be the fact that the precise nature of the factors which are responsible for the generation of this response are not yet known. In the case of UV-irradiation dose–response curves and action spectra strongly suggest that unrepaired DNA damage is the trigger for the induction process in a number of cases (Herrlich, Karlsruhe). The mechanism by which DNA damage triggers the inducible response is still poorly understood. *gadd* (growth arrest and DNA damage inducible) genes originally isolated by Fornace and co-workers (Bethesda) might be induced by stalled replication. Evidence for this was presented by Kerr et al. (Oxford) who showed that the human *gadd45* gene is induced by serum starvation and that DNA-damage inducibility is a relatively slow process. Furthermore, in A-T cells, deficient in X-ray induced G2 arrest, a reduced induction of *gadd45* is observed. As far as immediate early genes are concerned (e.g. *fos* and *jun*), the very fast induction kinetics (within minutes), even in confluent cell layers, argues against an involvement of stalled replication forks in the induction process. Steegenga et al. (Leiden) performed dose–response curves with XP-C and CS cells which suggested that activation of AP-1binding activity (*fos/jun*) was not the result of interference of DNA damage with transcription. Instead it was suggested that unrepaired DNA damage in inactive chromatin might play a major role in the induction process.

Most of the research on inducible effects of DNA damage, presented at the meeting, was aimed at establishing a correlation between the induced response and known cellular processes. The discussion was centred on two major questions: (a) is the inducible response involved in carcinogenesis and (b) does the inducible response have a protective function?

3.5.1. Involvement in carcinogenesis

Abrahams et al. (Leiden) used enhanced reactivation (ER) and enhanced mutagenesis (EM) of Herpes virus to study inducible phenomena. A correlation was found between the absence of ER in fibroblasts from several patients with XP and TTD with the absence of cancer in sunlight-exposed skin areas (EM was normal in all these cell lines). A further link between the ER^+ phenotype and carcinogenesis was found by analysing a large number of skin fibroblasts derived from patients with hereditary cancer-prone syndromes. In retinoblastoma, aniridia, polyposis coli, Von Hippel–Lindau syndrome, Von Recklinghausen neurofibromatosis, dysplastic nevus syndrome, multiple endocrine neoplasia type 2 and Bloom's syndrome much higher levels of ER (ER^{super+}) were registered than in normal human skin fibroblasts. The significance of these findings is, however, still difficult to assess because the molecular mechanisms which underlie the ER^+ phenotype are not yet understood. Abrahams et al. also found a correlation between the ER^+ phenotype in normal and XP ER^+ fibroblasts with UV-induced stabilization of the p53 cellular antigen. In XP ER^- and TTD ER^- cells, constitutive levels of p53 had a higher stability and no UV-induced stabilization was observed. Besides protein stabilization, it appears that UV-light might also induce mRNA stabilization. Hilgers

and co-workers (Brussels) presented evidence that transcripts induced by interferon-α, interleukin-α and polyI–polyC were significantly stabilized by UV-irradiation. Although the impact of extending the life-span of transiently induced transcripts on cellular physiology remains to be determined, the experiment indicates that higher transcript levels can be present in cells (at least for a given time) without the need for increased mRNA synthesis.

From the molecular point of view, the UV response is the best characterized damage inducible process described so far. Work from the Karlsruhe group has shown that in mammalian cells UV-inducible genes can be divided into at least 3 categories depending on the class of transcription factors involved in the regulation process: (a) AP-1(dimer of proto-oncogenes *fos* and *jun*)-regulated genes (e.g. collagenase, stromelysin); (b) NFκB (homologue of *rel* proto-oncogene)-regulated genes (e.g. HIV-LTR); (c) SRF(serum response factor)-regulated genes (e.g. c-*fos* gene). Herrlich (Karlsruhe) reported that these transcription factors can be activated within minutes, in general by post-translational modification (e.g. phosphorylation in the case of *fos* and *jun*). As these transcription regulators play important roles during normal proliferation, differentiation and development, it is likely that unscheduled activation of these factors might contribute to the process of carcinogenic transformation.

Molecular evidence for interference of the UV-response with gene regulation during the normal differentiation process of primary human keratinocytes was presented by Gibbs and coworkers (Leiden). The *spr2* gene family was previously shown to be induced by UV-light and regulated during keratinocyte differentiation. Here the promoter of the *spr2-1* allele was used as a molecular probe to identify the transcription factors involved in both regulatory processes. Regulation during differentiation is mediated via an octamer binding factor. After UV-irradiation, increased levels of AP-1 (*fos/jun*) activity were monitored. Evidence was presented which showed that increased expression of *fos/jun* abolished octamer mediated induction during differentiation. Simultaneously to the repression of the normal regulatory process by AP-1, a UV-responsive factor (URF) is induced which interacts with sequences in the 5′ promoter region and is responsible for UV-induction. The analysis shows that during the UV-response normal gene expression can be blocked and a new regulatory system can be installed. Such changes in transcriptional states are likely to be important during carcinogenic transformation. Krämer et al. (Karlsruhe) showed that besides the activation of transcription factors, mammalian cells respond to UV-induced DNA damage by the induced synthesis of growth factors. Indeed EPIF, a previously described secreted activity which can transduce the UV-response to unirradiated cells, contains several growth factors which are at least immunologically related to interleukin 1-α and basic fibroblast growth factor (bFGF). The release of these factors prolongs the UV response and might have organismic consequences.

Further evidence for the transcriptional activation of an oncogene by DNA damage was presented by Ben-Ishai and Sharon (Haifa). The chimeric oncogene *trk* contains at its 5′ end sequences of the ribosomal protein L7a fused to the receptor kinase domain of the *trk* proto-oncogene (the recombinant gene was formed during transfection of NIH-3T3 cells!). DNA damage inducibility of the chimeric oncogene is probably due to L7a sequences. Indeed transcription of the L7a and L30 (but not L7) ribosomal genes is induced by DNA damage. In *trk*-transformed NIH-3T3 cells several chimeric transcripts are observed which are differentially regulated by UV light, some but not all being enhanced.

The involvement of the DNA damage-inducible response in the induction of long-term changes, particularly mutations, was documented by Van den Berg et al. (Karlsruhe). It was shown that increased expression of the c-*fos* gene resulted in a 2-fold increase of chromosomal aberrations including both gross changes and point mutations. When in normal cells *fos* expression was blocked by the addition of antisense oligonucleotides to the culture medium, UV-induced mutagenesis was largely inhibited. Apparently, *fos* plays a decisive role in the control of mutagenesis in mammalian cells. In this respect, mammalian cells might resemble bacteria, in which

UV-induced mutagenesis is dependent on the induction of the bacterial SOS system and is mediated via the *umu* genes. Devoret et al. (Gif-sur-Yvette) presented evidence with a dominant mutant *recA* (1730) that there is an essential role for RecA protein in the mutagenic complex, along with *umuC*, *umuD'* and the α-subunit of DNA polymerase III. Bridges and Bates (MRC, Sussex) warned that there should be an awareness of possible subtle differences between the operation of the mutagenic complex in excision-proficient and -deficient bacteria. They reported on work with a mutant *umuC* protein (UmuC-36) which, when present along with overproduced levels of umuD/D' protein, permitted mutagenesis to occur in *uvrA* or *uvrB* strains but not in excision-proficient bacteria. Inducible error-prone mutagenesis in mammalian cells was also discussed by Boesen and Simons (Leiden). Mouse lymphoma cells were treated with PUVA under conditions where mainly DNA-interstrand crosslinks are formed. Fluctuation analysis showed that a substantial part of the mutational response was due to an enhanced mutation frequency per cell per generation which persisted up to the twelfth generation after treatment. As crosslinks are non-persistent lesions which will generate mutations only during the first cell division after treatment, it was concluded that an inducible error-prone mechanism must be responsible for mutation induction at later stages. Because it was relatively persistent, however, it did not at first sight appear to behave like the pulsed overexpression of *fos* described by Van den Berg et al.

3.5.2. Induction of a protective function

The system responsible for removing alkyl (particular methyl) groups from the O^6-position of guanine was the first to be well characterized biochemically as well as genetically in prokaryotes. Its involvement in the adaptation phenomenon provides a model for a simple inducible DNA-repair system. Strike and Baker (Liverpool) reported 100-fold inducibility of MGMT activity in *Aspergillus nidulans* exposed to MNNG. There were three protein species and, as in other eukaryotes, they specifically reacted only with O^6-methylguanine. A fourth protein species found in induced extracts appeared to have the novel activity of removing methyl groups from methylphosphotriesters.

In mammalian cells the existence of MGMT activity is well documented, but the inducibility of the system has not been clearly established. Two groups reported, however, that MGMT activity could be clearly induced in rat hepatoma cells. Fritz et al. (Karlsruhe, Oak Ridge) found up to 5-fold increase in *MGMT* mRNA following treatment with MNNG, MNU, MMS, X-rays and UV-irradiation. This was reflected in an increase in MGMT activity. Similar results were reported by Habraken et al. (Villejuif) who observed the peak in activity 48 h after treatment with a single dose of various physical and chemical DNA-damaging agents. There was, however, some disagreement as to the occurrence of inducibility in other cells, particularly human liver cells. This may be due to differences in the end-points measured, or to differences in cells or the way they are handled. Four things are clear from these studies in rat-hepatoma cells: (1) The increase in MGMT activity is due to an increase in transcription, i.e. it is a 'genuine' induction process. (2) The magnitude of the induction is much less than in *E. coli* or *A. nidulans*. (3) Induction is probably cell-type specific. (4) The induction signal is generalized DNA damage, rather than specifically methylation; in this respect it resembles the SOS system more than the adaptation response in *E. coli*. It will be interesting to see whether there are other similarities with the SOS control system. Habraken and her colleagues also reported induction of 3-methyladenine DNA glycosylase in rat-hepatoma cells.

Another system that appears to be genuinely inducible at the transcriptional level is heme oxygenase (HO), which is induced in mammalian cells by UV-A radiation, hydrogen peroxide, sodium arsenite, SH reagents and the tumour promoter TPA. Tyrrell et al. (Epalinges) reported that the proximal promoter region of the HO gene in human fibroblasts contains elements involved in the stimulation of transcriptional activity by a variety of agents including oxidants. They have also detected inducible changes in the interaction of binding proteins with the DNA sequence upstream. There is an important link with cellular glutathione levels in this system. Not only

does glutathione depletion enhance the induction of HO mRNA following treatment with UV-A or hydrogen peroxide, but it also affects the level of gene expression in the absence of any exogenous inducing treatment (Lautier et al., Epalinges). There is more than one way in which HO might protect against oxidative stress (Tyrrell et al.) and further studies will be needed to clarify both the mechanism of protection and the aspects of gene expression.

Like HO, metallothionein (episome borne human MTII-A) is induced by heavy metals and also by dexamethasone and shows 10–30-fold increased protein levels (Lohrer and Robson, Northwood). These workers were using it to obtain a state in which a stress protein is constitutively induced. In certain mutagen sensitive genetic backgrounds (varying CHO lines) it enhanced resistance to MNU and MNNG. The authors question whether it is affecting DNA repair, however, and suggest that it might allow more time for repair by causing a G2 delay before cell division.

4. Recognition and detection of DNA damage

In this session the detection of DNA damage induced by 3 types of agents, namely alkylating agents, UV and ionizing radiation, were discussed with emphasis on methods to identify various types of DNA damage and to assess the relative efficiencies of the different DNA-repair processes responsible for their removal.

4.1. Alkylation damage

Sensitive immunological and electrochemical methods have been developed to detect ethylation and methylation adducts resulting from exposure to environmental alkylating agents as well as from treatment with chemotherapeutic agents (Van Delft et al., Rijswijk; Montesano et al., Lyon). These methods combined with HPLC show a high degree of specificity enabling the detection of 1 alkylation adduct in 10^7–10^8 nucleotides, thereby permitting the detection of N-7 or O^6-methylation or ethylation adducts in humans treated with chemotherapeutic agents or suspected of being exposed to methylating agents environmentally.

The repair processes responsible for the repair of these DNA adducts have been relatively well characterized in prokaryotic cells and Rafferty et al. (Manchester) described experiments in which specific antibodies were used to detect MGMT at the single cell level in mammalian tissues. Transgenic mice were bred which contained the *MGMT* gene under control of the metallothionein promoter. These mice had much higher levels of MGMT than non-transgenics and the MGMT levels could be further increased by feeding the mice with zinc. Carcinogenic alkylating agents show a high degree of cell and tissue specificity in the induction of mutation and cancer. It is evident that these immunological methods to detect DNA-alkylation adducts and MGMT protein at the cellular level are powerful tools to examine the role of these DNA adducts and repair processes in carcinogenesis. The appreciation of these approaches to molecular epidemiological studies in cancer is just beginning and it is anticipated that in the future these methodologically oriented studies will be more directly related to biological events relevant to cancer or mutagenesis.

4.2. UV damage

The UV-endonuclease assay was used by Klaude and Collins (Aberdeen) in combination with the unwinding technique of Ahnstrom for the detection of incision breaks. With this method CPD could be detected at doses as low as 0.1 J/m^2. At this low dose they observed a rapid removal ($t_{1/2}$of about 4 h) not only in normal human cell lines, but also in cells from mouse and hamster origin, which is in contrast with conventional methods employing relatively high UV doses which suggest that human cells appear to remove dimers more rapidly than rodents. Although a dose of 0.1 J/m^2 is low in comparison with that applied in conventional assays, this dose still induces about 4 dimers per 10^9 dalton of DNA. This means that if all dimers are converted into incision breaks this corresponds to a γ-ray dose equivalent of 16 Gy. The unwinding technique is normally applied in a dose range of 0–10 Gy of γ-rays. This might indicate that not all dimers are recognized in this assay. Apparently,

the release of DNA from the nucleus is not complete.

Arlett et al. (MRC, Sussex) found a correlation between the UV-C hypersensitivity of unstimulated (G_0) T-lymphocytes (survival) and the data obtained with the 'comet'-assay. They measured the level of incision breaks which could be detected during a short period after UV-exposure. The level of incision breaks appeared to be higher in unstimulated lymphocytes than in stimulated lymphocytes, suggesting that it was not the incision step which was the rate limiting step in the excision repair but one of the following steps, e.g. the strand-rejoining step. In XP cells (group D) incision breaks were not detected, either in stimulated or unstimulated lymphocytes.

Tornaletti et al. (Pavia) studied the effect of UV on DNA structure in an in vitro system. They studied different DNA conformations with respect to the accessibility of DNA for ss-DNA specific endonucleases. In all these structures they found that after UV exposure the number of ss-regions was significantly lower than the number of pyrimidine dimers. The observed ss-regions appeared to be not photoreactivable suggesting that lesions other than CPD might be responsible for the ss-regions (e.g. by inducing non-B DNA structures). Sequences able to undergo structural transitions by UV are also thought to be implicated in biological processes. Therefore, Tornaletti et al., also studied different plasmid DNAs, containing cruciform structures. Using P1-endonuclease, it was found that; (1) the number of P1 sensitive sites was 10–70 × less than that of pyrimidine dimers (depends on the conformation of the topologically ds-domain). (2) P1 sensitive sites are at least in part localized at AT-rich regions. (3) UV inhibits the extrusion of the cruciforms. (4) At low UV doses dimers are mainly induced at long runs of thymines. In conclusion, UV induces not only local perturbations of DNA structure but also influences the stability and/or formation of alternative secondary structures.

4.3. Ionizing radiation damage

There are at the moment several sensitive methods available for the detection of SSBs in DNA. However, for most of these methods, either radioactively labeled DNA or large amounts of cells are required. Two contributions discussed the detection of SSBs and base damage after in vivo exposure to ionizing radiation. A sensitive method for detecting SSBs was described by Van der Schans et al. (Rijswijk) who used an unwinding assay together with immunochemical detection of single-stranded DNA from white blood cells after total body irradiation of leukemia patients. SSB, as well as base damage could also be detected in the same blood samples of these patients using 'alkaline elution'. Base damage can be quantified in a similar way if the alkaline elution is preceded by treatment of the DNA with damage-specific endonucleases (e.g. a *Micrococcus luteus* extract). DNA in the eluted fractions can be quantified fluorometrically after addition of a Hoechst dye enabling the procedure to be used with unlabelled cells. With the methods described SSB and base damage can be assayed after irradiation doses as low as 0.5 Gy and 1.5 Gy, respectively. Van Loon et al. (Rijswijk), applying the same techniques, showed that there are differences in repair kinetics of radiation-induced DNA lesions between man and mice. The proportion of DNA damage remaining in white blood cells of mice after in vivo irradiation and subsequent repair was larger than that in human white blood cells after in vivo or in vitro irradiation and repair. These results stress the need, as for UV-induced damage, to verify procedures when extrapolating conclusions from animal studies to man.

Repair of radiation-induced DSBs measured by pulsed-field gel electrophoresis (PFGE) was reported by Meyn and Story (Houston). Two approaches were used. In the first a colon cancer cell line containing double minute (DM) chromosomes containing amplified epidermal growth factor receptor (EGFR) gene copies was used. These DMs will not enter an agarose gel unless fragmented by radiation. Following electrophoresis, the DNA fragments were transferred to nylon membranes and hybridized with a labeled EGFR probe. The resulting autoradiogram showed two bands of 0.8 and 1.6 Mbp as a result of one DSB in the circular structure of the monomer or the dimers respectively. During repair incubation these bands lost intensity as a function of time

illustrating the rejoining of DSB. In the second approach, after a dose of 30 Gy [^{14}C]thymidine labeled CHO cells were subjected to PFGE in such a way that fragmented DNA moved out of the plug. By measuring the amount of fragmented DNA the repair of DSB in these cells was quantitated. The repair kinetics with both systems appeared to be the same, and were also comparable with those obtained earlier with 'neutral elution'. The frequency of DSB induced in DNA in cells appeared to be 50 times less than in naked DNA under the conditions applied by the authors.

Chromosomal aberrations induced by ionizing radiation in human lymphocytes are used as biological dosimeters in radiation accidents and therefore it is important to investigate the induction and repair of chromosome damage in these cells. Conventional metaphase analysis and premature chromosome condensation (PCC) techniques were employed to determine the frequency of dicentrics and chromosome fragments at different times following in vitro irradiation of human lymphocytes. The frequencies of dicentrics in metaphases and PCCs were found to be similar whereas the frequency of fragments was very high in PCCs and decayed with time indicating that most of the dicentrics are formed immediately after irradiation. The chromosome painting technique using chromosome specific DNA libraries and in situ hybridization was found to be an efficient method to detect translocations between chromosomes. The frequencies of translocations were 3–4 times higher than the frequencies of dicentrics in irradiated lymphocytes, an observation which suggests that by scoring translocations, one would be able to detect exposure to low doses of ionizing radiation (Vyas et al., Leiden).

Acknowledgements

The main sponsor of the meeting was the EC Concerted Action (contract No. MR4-0228-UK). In addition, the meeting was sponsored by the Royal Dutch Academy of Sciences and the Medical Genetics Centre South-West Netherlands (MGC).

References

Friedberg, E.C., C. Backendorf, J. Burke, A. Collins, L. Grossman, J.H.J. Hoeijmakers, A.R. Lehmann, E. Seeberg, G.P. Van Der Schans and A.A. Van Zeeland (1987) Molecular aspects of DNA repair, Mutation Res., 184, 67–86.