Nucleotide excision–repair in the test tube

The eukaryotic nucleotide excision–repair pathway has been reconstituted *in vitro*, an achievement that should hasten the full enzymological characterization of this highly complex DNA-repair pathway.

All living organisms have evolved mechanisms to cope with the problem of genomic instability. Cells have a number of different mechanisms for repairing the various types of damage — resulting from errors of internal metabolism or the action of external agents — that their DNA is prone to. Many of these mechanisms are relatively simple and specialized for the repair of strictly defined types of DNA lesions: those dealing with strand mismatches or small base adducts, for example. The nucleotide excision–repair (NER) pathway, however, can recognize a broad spectrum of helix-distorting lesions, mostly generated by environmental DNA-damaging agents, such as ultraviolet (UV) light. In eukaryotes, this versatility is accomplished by intricate interactions between many proteins, which mediate the excision and replacement of a single-strand patch substantially larger than the structurally affected DNA region itself. The basic features of NER have remained unchanged throughout eukaryotic evolution, from simple yeasts up to higher mammals, and many parallels extend even to prokaryotes such as *Escherichia coli*.

The complexity of eukaryotic NER is reminiscent of the other fundamental DNA metabolic processes — transcription, replication and recombination — and mutual interactions between these processes are now being uncovered. The classical biochemical approach to dissecting such processes involves the separation of the individual components and their reconstitution to a fully operative system. While simplified *in vitro* systems for basic transcription and replication have been available for some time, the reconstitution of NER has just recently been reported by Aboussekra *et al.* [1]. This delay is remarkable as, unlike replication and transcription, NER is only essential in the presence of environmental stress, so that mutants affected in this system can readily be identified and genetic analysis is possible. The well-known human inherited disorders xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and trichothiodystrophy are caused by mutations, defining at least ten complementation groups, affecting NER; many other, less well-known, NER mutants have been identified in yeast and cultured mammalian cells.

The first attempts to set up a mammalian cell-free NER system date back to 1976 [2]. However, this and other early systems lacked stability, reproducibility or specificity. Twelve years later, and twenty years after Cleaver's discovery that NER is defective in cells from XP patients, Wood *et al.* [3], followed independently by Sibghat-Ullah *et al.* [4], described how carefully isolated mammalian cell extracts can repair damage on simple plasmid DNAs. The conditions appeared to mimic the NER system rather closely, as most of the factors affected in the known mammalian NER mutants were required. Extracts from different mutants that complement each other *in vivo* — that is, after cell fusion — were generally also able to do so in the cell-free system.

The *in vitro* assay was soon successfully exploited for a number of enzymological advances. These included the isolation of the XPA, XPC and XPG proteins [5–7], the separation of the incision and excision/gap-filling steps, the discovery of the involvement of the replication factors PCNA (proliferating cell nuclear antigen) and RPA [8–11], the identification of the ERCC1/XPF enzyme complex [12,13] and the sizing of the excised patch to 27–29 nucleotides [14]. From 1984 on [15], most of the NER genes were gradually cloned by painstaking gene-transfer experiments carried out using the mutant cell cultures (reviewed in [16]). In addition, the human multiprotein TFIIH basal transcription factor complex was isolated and unexpectedly found to harbour at least three known repair proteins [17,18]. It is mainly the combination of these developments that has made the full *in vitro* reconstitution of NER possible.

The approach used by Aboussekra *et al.* [1] and, independently but worked out in less detail, by Mu *et al.* [19] was as classical as it was effective. First, a repair-competent mammalian extract was fractionated into at least eight parts using standard chromatographic procedures. Then, these fractions were tested one by one for the presence of known repair enzymes. Finally, the fractions were replaced by purified enzyme preparations, either derived from individual repair genes overexpressed in bacterial or insect cells, or from detailed classical purification protocols starting with HeLa nuclear extracts.

The initial stages of NER, comprising the damage recognition step and a dual incision around the lesion, were found to require the single proteins XPA and XPG, and the multi-protein complexes TFIIH, RPA (hSSB), ERCC1/XPF and XPC/HHR23B. The XPE-related protein UV-DDB acted as a dispensable stimulator. A new essential factor IF7 (incision factor 7), as yet only partially characterized, was also identified. Completion of the NER reaction, which involves patch-displacement, gap-filling and religation, could be accomplished by DNA polymerase ϵ and ligase I, in combination with the accessory replication factors PCNA, RPA and RF-C. Interestingly, this final stage could be performed just as

Table 1. Genes and proteins involved in nucleotide excision–repair.

Complex name	Human protein [chromosomal locus]	Protein type/activities	Yeast homolog	Additional involvement
		Lesion recognition		
	XPA [9q34]	42 kD zinc finger; binds UV-damaged-DNA, ERCC1, RPA, TFIIH	RAD14	NER only?
	IF7	Increases specificity		
UV-DDB	XPE [11] ?	127 kD; binds UV-damaged DNA 41 kD subunit?		
		Preincision patch demarcation		
TFIIH	XPB (ERCC3) [2q21] XPD (ERCC2) [19q13.3] P62 [11p14–15] $SSL1^{hs}$ [5q13] P41 P34 ...more? (such as CDK7, cyclin H..?)	89 kD; 3′→5′ helicase 80 kD; 5′→3′ helicase 62 kD 44 kD zinc finger; DNA-binding 41 kD 34 kD zinc finger	RAD25/SSL2 RAD3 TFB1 SSL1 TFB2 TFB3 ...(KIN28, CCL1?)	Basal transcription factor for RNA polymerase II Interaction with p53?
RPA/hSSB	RPA1 [17] RPA2 [1] RPA3 [7p22]	70 kD, DNA-binding subunit 32 kD subunit 14 kD subunit	RFA1 RFA2 RFA3	DNA replication
XPC/23B	XPC [3p25.1] HHR23B [3p25.1]	125 kD; ssDNA-binding 58 kD; ubiquitin fusion protein	RAD4 RAD23	
		Incision		
	XPG (ERCC5) [13q32–33]	180 kD endonuclease; 3′ incision	RAD2	Transcription?
ERCC1/XPF	ERCC1 [19q13.3] ERCC4 (XPF?) [16p13] ERCC11	33 kD (p39) 110 kD? ERCC1/XPF catalyzes 5′ incision	RAD10 RAD1	Mitotic recombination
		Gap filling and ligation		
	LIG1 [19q13]	100 kD	CDC9	DNA replication
	PCNA [20p12-ter]	28 kD trimer; interacts with polymerase ϵ and RPC	POL30	DNA replication
Polymerase ϵ	POLE [12q24.3] ?	250 kD catalytic subunit A 55 kD subunit B	POL2	DNA replication
RF-C		37, 38, 40, 140, 145 kD subunits	RFC1, RFC2, CDC44?	DNA replication

Most of the specific functions for the various NER components suggested in this table are so far based only on circumstantial evidence, and await formal enzymological proof. The table summarizes the results of numerous authors who cannot be properly acknowledged within the scope of this article; we would refer readers to [16] for a more detailed recent review.

well by a combination of the *E. coli* DNA polymerase I Klenow fragment and ligase.

A total of between 25 and 30 proteins (summarized in Table 1) are minimally required to reconstitute the full NER reaction; this is, however, just an estimate, as some uncertainties remain. For instance, the TFIIH preparation used in the experiments contained at least six polypeptides, known as the 'core complex', which are also required to support *in vitro* transcription. It is, however, very likely that (minor) accessory factors present in this preparation, such as cyclin-dependent kinase CDK7 [20], are also involved in NER. As a second example, the composition of the ERCC1/XPF complex is still uncertain, as it could not be purified to homogeneity using classical chromatography, a failure also experienced by two other groups [19,21]. Besides ERCC1 and an unknown 110 kD protein, identified by South-western blotting [1] and immunoprecipitation [21], the complex carries the complementing activities of the XPF and

ERCC11 mutants [12,13]. Finally, the newly identified factor IF7 also remains uncharacterized.

The *in vitro* reconstitution of NER clearly opens the door to further dissection of its individual steps, their order and the protein–protein interactions involved. In the years to come, our knowledge of mammalian NER can be expected to become at least as detailed as that of the much simpler uvrABCD excinuclease system of *E. coli*. Despite all these promises for the future, adapting the basic NER reaction to simplified *in vitro* conditions has its price. Cells repair the transcribed strand of their active genes much faster than the remainder of the genome, by an NER subpathway called transcription-coupled repair (TCR). In patients with CS, TCR is impaired, but the global NER facility is unaffected [22]. The gene products CSA and CSB are not required for the reconstituted *in vitro* NER reaction, and extracts of CS mutant cells (both human and hamster) have normal NER activity. This implies that the test-tube conditions selectively reflect the global repair subpathway, which is not entirely unexpected as the damaged plasmid used as the substrate is not transcribed in the experimental conditions. It also fits with the need for the XPC/HHR23B complex, which is known to be essential for the global NER subpathway only [22].

Recent attempts to set up a repair assay with a substrate carrying an active transcription unit and ribonucleotides added in the reaction mixture have not yet resulted in a clear-cut CSB-dependence or CSA-dependence. It appears that much more is needed to reproduce the TCR subpathway *in vitro*; presumably the chromatin structure of the repair substrate has to be taken into account as well. SV40 minichromosomes with a partially intact chromatin structure can be repaired *in vitro* [6], but this repair activity also appears largely to reflect the global subpathway, as it is strongly dependent on XPC/HHR23B. As a matter of fact, the human homologs of the yeast global repair proteins RAD7 and RAD16 could not be traced in the *in vitro* system yet.

Most of the NER proteins exist as stable complexes, but recent work has suggested that a minor fraction may come in the form of a multi-protein supercomplex, tentatively called the 'repairosome' [23]. The reconstitution studies however, indicate that such a structure, if present in the *in vitro* conditions, can be readily assembled from and separated into its individual components. In the near future, detailed work with the reconstituted system will undoubtedly reveal the significance of the appealing repairosome concept.

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