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The eukaryotic nucleotide excision repair pathway

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Abstract

Nucleotide excision repair (NER) is the most versatile mechanism of DNA repair, recognizing and dealing with a variety of helix-distorting lesions, such as the UV-induced photoproducts cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4 PPs). In this review, we describe the main protein players and the different sequential steps of the eukaryotic NER mechanism in human cells, from lesion recognition to damage removal and DNA synthesis. Studies on the dynamics of protein access to the damaged site, and the kinetics of lesion removal contribute to the knowledge of how the cells respond to genetic insult. DNA lesions as well as NER factors themselves are also implicated in changes in cell metabolism, influencing cell cycle progression or arrest, apoptosis and genetic instability. These changes are related to increased mutagenesis and carcinogenesis. Finally, the recent collection of genomic data allows one to recognize the high conservation and the evolution of eukaryotic NER. The distribution of NER orthologues in different organisms, from archaea to the metazoa, displays challenging observations. Some of NER proteins are widespread in nature, probably representing ancient DNA repair proteins, which are candidates to participate in a primitive NER mechanism.

Keywords: Ultraviolet; DNA repair; Apoptosis; DNA lesions; Evolution

1. Introduction

Cells from all living organisms are constantly being threatened by the action of environmental agents or endogenous cellular metabolism byproducts, which can interact with and modify the DNA structure. These alterations in DNA can result in cellular dysfunctions, such as genetic instability, mutagenesis or cell death. Thus, the removal of lesions in this molecule is a vital process for every single cell. Nucleotide excision repair (NER) is the most versatile and flexible DNA repair pathway of living cells as it deals with a wide range of structurally unrelated DNA lesions. NER acts on the removal of lesions, that distort the DNA double helix, interfere in base pairing and block DNA duplication and transcription. The most common examples of these lesions are the cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs), the two major kinds of injuries induced by ultraviolet (UV) light, besides several kinds of bulky adducts induced by chemical agents. The basic NER mechanisms have been strongly conserved throughout evolution, although the enzymes involved differ from prokaryotes to eukaryotes (see discussion below). Basically, after DNA damage recognition, the sequential action of helicases and endonucleases open the double helix and cleaves the damaged strand few bases away from the lesion. This is followed by the removal of the DNA segment containing the lesion and gap polymerization using the intact strand as template.

In eukaryotes, most of NER studies were performed with cells mutated at different steps of the pathway, including cells from human patients with genetic syndromes directly related to DNA repair, such as xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and trichothiodystrophy (TTD). Although clinical manifestations of these syndromes differ considerably, varying from high cancer predisposition to aging symptoms, the common feature of the three syndromes is photosensitivity, manifested at different levels. The work

Abbreviations: CPDs, cyclobutane pyrimidine dimers; GGR, global genome repair; NER, nucleotide excision repair; 6-4 PPs, 6-4 photoproducts; TCR, transcription coupled repair; UV, ultraviolet.

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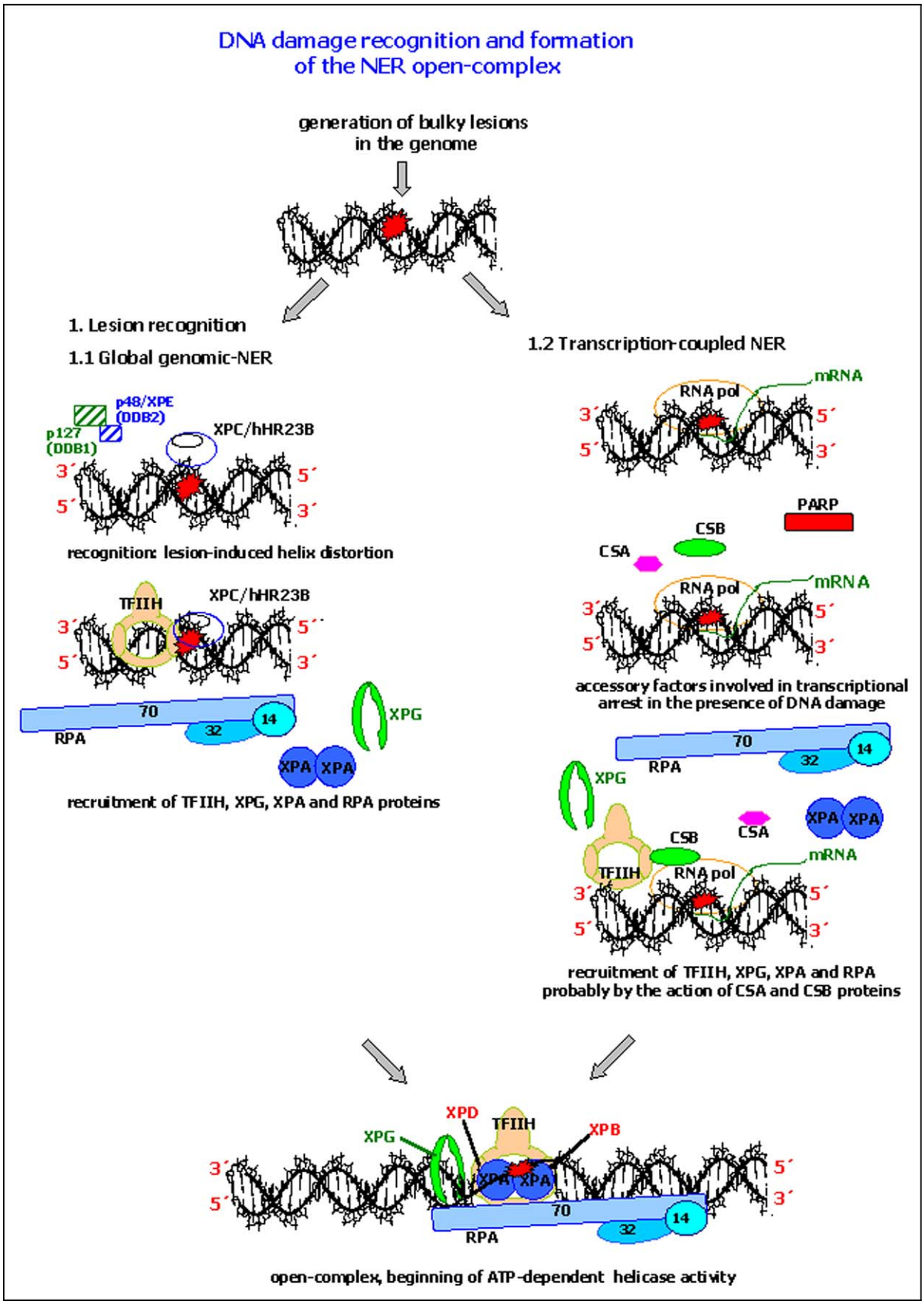

DNA damage recognition and formation of the NER open-complex
generation of bulky lesions in the genome
1. Lesion recognition
1.1 Global genomic-NER
p48/XPE (DDB2)
p127 (DDB1)
XPC/hHR23B
3´
5´
recognition: lesion-induced helix distortion
TFIIH
XPC/hHR23B
70
14
32
RPA
XPG
XPA XPA
recruitment of TFIIH, XPG, XPA and RPA proteins
1.2 Transcription-coupled NER
RNA pol
mRNA
PARP
CSB
CSA
accessory factors involved in transcriptional arrest in the presence of DNA damage
recruitment of TFIIH, XPG, XPA and RPA probably by the action of CSA and CSB proteins
XPD
XPB
open-complex, beginning of ATP-dependent helicase activity

with these NER defective syndromes indicated that they are very heterogeneous, with several complementation groups representing distinct repair genes. Mutated human and rodent cells contributed to cloning and identification of most of the genes encoding for the proteins involved in NER [1,2]. These proteins were named according to their specific complementation groups. Seven complementation groups were identified for XP syndrome (XPA–XPG) and two for CS (CSA and CSB). In this review, we will focus on the mechanism of NER in human cells and the concerted action of the different proteins involved. The association of this mechanism to the clinical manifestations in these syndromes is discussed in detail in the accompanying work [3].

All key NER factors were cloned and the reaction core has been reconstituted in vitro, requiring the collaborative engagement of more than 25 polypeptides [4]. These biochemical studies are very informative regarding the higher order association of NER proteins with implications on how this process functions within the cell. However, they do not provide clear evidence of whether the repair involves the sequential assembly of individual factors or the loading of a "repairosome" NER holo-complex onto a DNA lesion. Recently, the ability to damage specific portions of nuclei of mammalian cells, with the use of local UV irradiation methodology, finally confirmed that NER action involves the sequential and coordinated protein assembly in lesion-containing sites, instead of the pre-assembled complex [5].

For a long time, the dynamics of NER has been known to be dependent on damage recognition: the transcription coupled repair (TCR) selective for lesions present in the transcribed strand of expressed genes, and the global genome NER (GGR) that acts over the rest of the genome. These two subpathways reflect the temporal difference in which lesions are removed, as lesions on the DNA strand that is being transcribed by RNA polymerase II are repaired faster than those in other genome regions, including those present in the non-transcribed strand of active genes [6]. The repair rate for GGR also depends on the type of lesion. For example, the 6-4 PPs, which generate larger distortions in the double helix, are removed from the genome five times faster than the CPDs, although the latter are more abundant after UV irradiation [7]. In addition, genome condensation in chromatin may also interfere with the rate of lesion recognition and removal [8]. In the following sections, we will describe the main events that occur when DNA lesions are identified in the genome and removed by the NER pathway. The possible sequential participation of the different players will be represented step by step, considering the proteins of mammalian cells. The conservation of these proteins among eukaryotes, based on genome or EST DNA sequences, will be discussed at the end of this work. Some intriguing differences may contribute to understand how this process has been built up during evolution.

2. The NER pathway step by step

2.1. Recognizing DNA lesions in DNA

The first step of the NER reaction is the recognition of the DNA lesion, this differing considerably in TCR and GGR subpathways (Fig. 1). In GGR, the XPC–hHR23B complex is responsible for the crucial damage-sensing step. The specific role of the XPC–hHR23B complex was not evident for quite some time, but since the critical work of Sugasawa et al. [9], new studies demonstrated this complex as the first NER factor that senses damage [5,10]. Sugasawa et al. [9] used a DNA damage recognition–competition assay to show that XPC–hHR23B was the earliest lesion detector and that NER factors repaired only damaged plasmids preincubated with this complex. In addition, the authors showed through a DNase I footprinting assay that after XPC–hHR23B binding, the DNA conformation around the lesion changes considerably. Thus, besides initiating the GGR through sensing and binding on lesions, XPC–hHR23B distorts the DNA double helix. Experiments with human cells in culture using the local UV irradiation technique confirm this idea [5]. This clever technique consists of irradiating the cell monolayer covered with polycarbonate membranes containing pores of 3–8 μm, thereby generating UV photolesions in restricted areas of the nuclei. When cells were locally irradiated and immunolabeled with antibodies specific for repair enzymes, it was demonstrated that these proteins changed their pattern of distribution in vivo, migrating to the damaged sites and forming "foci" in the regions of pores [5,11]. This type of experiment, when performed with cells derived from XP patients, indicated that the XPC–hHR23B complex is the first component to arrive at the site of the damage, being also necessary for the recruitment of all subsequent NER factors [5]. A model for the binding of the XPC–hHR23B complex

Fig. 1. Model of DNA damage recognition by GGR and TCR and formation of the NER open-complex. (1) After the generation of bulky lesions in the cellular genome, their recognition is performed differently depending on the location: (1.1) GGR is responsible for recognizing lesions in the whole genome; the XPC–hHR23B complex is the major player in this pathway, sensing helix-distortions in DNA. The heterodimer DDB protein (composed by the subunits p48 and p127) may have an accessory role in lesions poorly recognized by XPC–hHR23B. XPC has a domain responsible for interaction and recruitment of TFIIH [19], the next factor to assemble in lesion-containing sites, which presents a ring-like structure [61]. The endonuclease XPG, the RPA and XPA proteins are also recruited, in order to form the pre-incision complex; (1.2) transcription-coupled repair, TCR, identifies bulky lesions in the transcribed strand of active genes. The transcriptional complex stalled in front of a lesion is the recognition signal; CSA and CSB proteins are probably the NER factors involved in coupling transcription to TCR action. CSB interacts with RNA polymerase complexes, as well as with the TFIIH transcription factor. PARP-1 enzyme may also play a role in transcriptional arrest in response to DNA damage. Thereafter, there is the recruitment of XPG, RPA and the XPA dimer [73], with formation of the pre-incision complex. The structures presented for these proteins, including XPA dimer, are hypothetical.

to lesions was recently reported, and also suggests that a single protein complex is already sufficient for NER initiation, at least for 6-4 PPs lesions [12].

In whole-cell extracts, most of the 125 kDa XPC protein [13] is found complexed with the 58 kDa hHR23B gene product [14]. However, a trace amount copurifies with hHR23A, the other human homologue of the yeast NER factor Rad23 [15], suggesting some functional redundancy. This redundancy is confirmed in the mHR23B-deficient mice, which show a severe phenotype including growth retardation, male sterility and facial dysmorphology, albeit NER proficiency [16]. hHR23 proteins contain a ubiquitin-like N-terminus and two ubiquitin-associated domains, thus suggesting a link to the ubiquitin/proteasome-mediated protein degradation pathway. Both proteins showed capability for binding and stimulating XPC in vitro [17]. The stimulatory activity of hHR23 seems to be due to the stabilization of XPC protein [15]. Analysis of *mHR23A/B* double-mutant cells showed that HR23 proteins function in NER by governing XPC stability via partial protection against proteasomal degradation [18]. Interestingly, another protein was identified that cooperatively stabilizes XPC, thereby stimulating NER in vitro. This third protein, a component of the XPC–hHR23B complex, is centrin 2 (CEN2), one of the three human isoforms of centrin that is found in the centrosomes [15].

The XPC protein domain involved in interaction with DNA was mapped to the C-terminal region, partially overlapping regions described for interaction with other NER factors, such as hHR23B and TFIIH ([19]; see Fig. 1). The XPC–hHR23B complex has affinity for a variety of lesions. However, in some cases this affinity is reduced, as in the case of CPDs [20]. One possible explanation is that the DNA helix distortions caused by CPDs are too subtle to be recognized by XPC–hHR23B. In fact, the introduction of one or two mismatched base(s) opposite to a CPD potentiates helix distortion and significantly increases the binding of XPC–hHR23B [20]. Based on these observations, it has been proposed that other factors, such as the XPE protein, can cooperate in lesion detection [20,21].

The specific role of the XPE protein is unclear, as it is dispensable for the NER reaction in vitro [4]. On the other hand, it is certainly required in vivo, as its deficiency results in clinical manifestations of XP. It has been suggested that the genetic defect of XP group E patients is due to mutations in the damaged-DNA binding (DDB) factor. The DDB factor is a heterodimeric protein complex, composed of the 127 (p127/DDB1) and 48 kDa (p48/DDB2) subunits [22], and the latter was found mutated in some XPE patients. Doubts concerning the XPE protein identity result from reports showing that not all XPE patients carry a mutation in the 48 kDa subunit [23]. However, a closer look at genetically unrelated XPE patients confirmed that the true XPE phenotype results from defects in DDB activity, specifically in the p48 subunit [24]. In addition, these authors have investigated four DDB proficient cell strains from patients previously assigned to XPE, which resulted in their re-classification into other XP complementation groups.

The DDB complex has increased affinity for certain types of lesions, suggesting that it facilitates the identification of lesions that are poorly recognized by XPC–hHR23B. Recently, it was demonstrated that XPC does not efficiently bind to CPDs in vivo [17]. However, it does so in cells overexpressing the XPE/p48 subunit of DDB. Thus, it seems that DDB recruits XPC to CPD-containing sites, stimulating the repair of these lesions [25]. This hypothesis is also supported by work on rodent cell lines that are DDB deficient. Transfection of the *p48* gene into hamster cells enhanced the removal of CPDs from genomic DNA and from the non-transcribed strand of an expressed gene [21]. Similar to rodent cells, p53-mutated human cell lines have very low endogenous levels of the *p48* transcript. The stable transfection of the *p48* gene into p53-mutated cells enhanced the GGR rate of CPDs significantly. These results confirm previous reports that suggested p53 control of the *p48* gene [26]. Furthermore, p48 protein was shown to be a substrate for the *Cul-4A*, a member of the cullin family of ubiquitin ligases, overexpressed in many tumor cells. This protein stimulates ubiquitination and degradation of p48 and this may help to regulate the steady-state levels of DDB in the cell [27].

The XPC–hHR23B and DDB complexes are dispensable from TCR. For this NER subpathway, the first signal for repair activity seems to be the blockage of transcription elongation by RNA polymerase II in front of DNA lesions. It is not clear whether the stalled RNA pol II is displaced and/or dissociated from DNA, in order to allow for accessibility of NER machinery to the damaged site. Two proteins are required at this stage: CSA and CSB. CSA is 44 kDa protein and contains five WD-40 repeats, important for protein–protein interaction [28]. CSB is a 168 kDa protein, a member of the DNA-dependent ATPase SWI/SNF family, involved in chromatin remodeling [29]. Although both gene products have already been biochemically characterized and are known to play essential roles in TCR, their exact functions remain to be elucidated. CSA protein seems to be rapidly translocated to the nuclear matrix, after DNA damage. This translocation is independent of XPC and XPA, but requires the CSB protein, which is not found at the nuclear matrix. Furthermore, CSA was shown to co-localize with the hyperphosphorylated form of RNA pol II at the nuclear matrix during the process of TCR [30].

On the other hand, the CSB protein appears to reside in the RNA polymerase II-containing elongation complex [31]. CSB has been shown to interact in vitro with RNA polymerase II engaged on elongation of templates containing pause sites [32], possibly involving the recruitment of TFIIH to the damaged site, during TCR [33]. Unlike CSB, CSA does not directly bind Pol II [32]. CSB has also been suggested to act as a non-essential elongation factor, required for bypass of pause sites and of structured RNA [34–36]. More recently, CSB was also found to be associated with TFIIH, XPG and RNA polymerase I in the nucleolus, and this com-

plex is necessary for efficient rRNA transcription [37,38]. These results are consistent with observations that, at least in yeast cells, TCR does occur during ribosomal RNA synthesis mediated by RNA pol I [39]. Thus, although initial data indicated that RNA pol I and III do not elicit TCR in mammalian cells [40,41], this conclusion may need to be re-evaluated.

As expected, CSB protein is able to alter DNA conformation and remodel chromatin in vitro in an ATP-dependent fashion, which links repair to chromatin remodeling [42]. A recent work performed in yeast cells gives rise to some ideas regarding the relationship of chromatin to the molecular mechanism of TCR: the CSB homologue, Rad26, forms a complex with the newly identified Def1 protein in chromatin [43], which appears to have a role in the DNA damage response, but it is not required for TCR. Def1 defective cells are unable to degrade RNA pol II in response to DNA damage and are compromised for transcript elongation. The authors suggest that stalled RNA pol II triggers a coordinated rescue mechanism requiring Rad26/Def1 complex. In the case of lesion persistence due to the inefficient action of Rad26, Def1 would be required for the ubiquitination and proteolysis of RNA pol II. This model cannot be fully applied to human cells, as no homologue for Def1 was found in mammals. However, ubiquitination of RNA pol II after DNA damage seems to be essential for the recovery of RNA synthesis, a property that fails to occur in CS cells [44]. Therefore, it is possible that this function might be carried out by a divergent protein analogue in these cells.

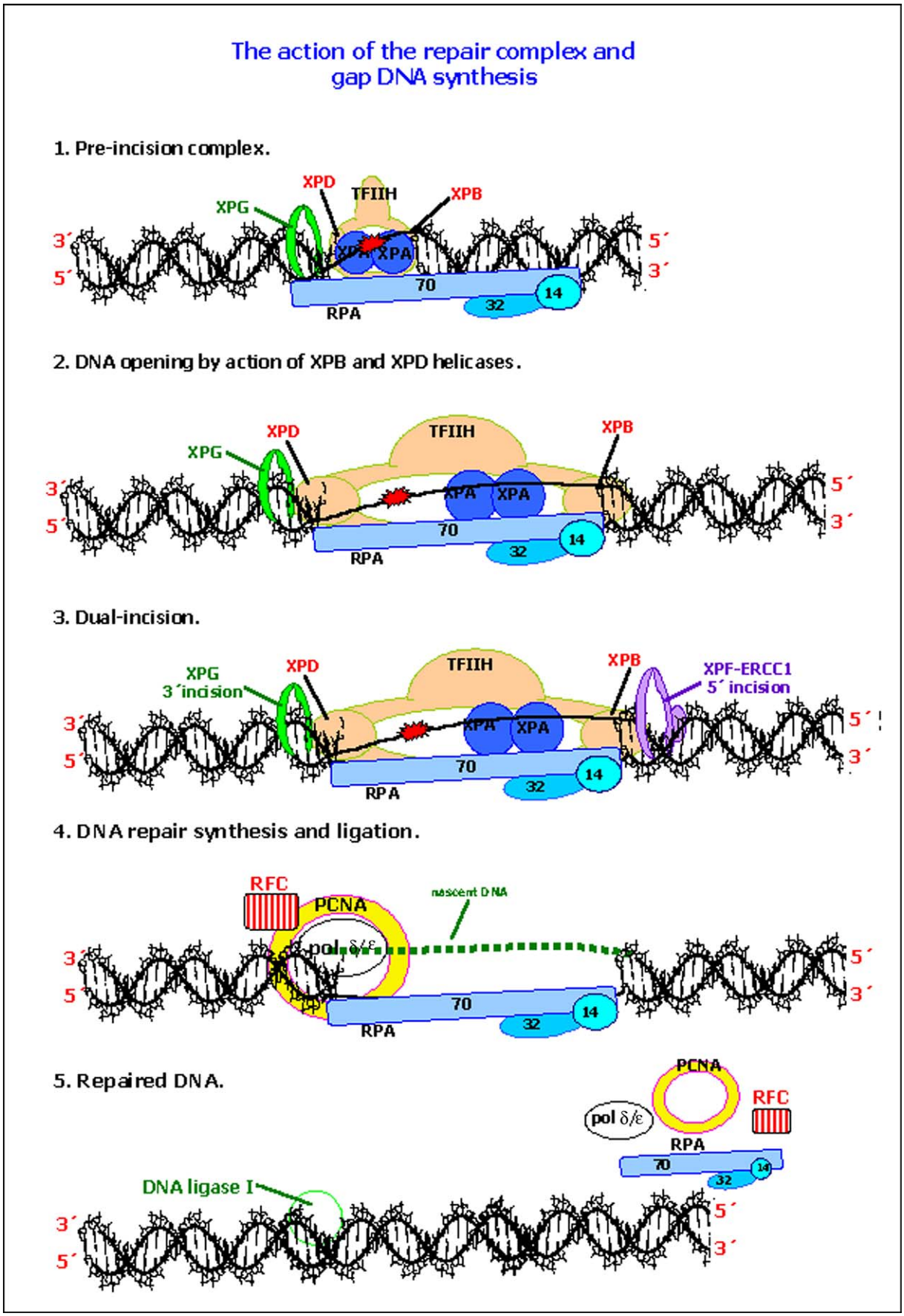
2.2. Recruiting NER factors to the open-complex

Once lesions have been traced by the GGR or TCR machinery, an open-complex structure will be formed in the vicinity of the lesion, requiring the coordinated action of XPC–hHR23B with other NER proteins, TFIIH, XPA, RPA and XPG [45,46]. The contribution of each protein or complex to the formation of such a structure is discussed below and illustrated in Fig. 1.

TFIIH is a nine-protein complex essential for NER activity, initiation of RNA polymerase II transcription and with a possible role in cell cycle regulation [47–50]. The nine subunits, cyclin-dependent kinase 7 (cdk7), cyclin H, MAT1, XPB, XPD, p34, p44, p52 and p62, can be associated in different subcomplex arrangements in the cell. The core complex is formed by p34, p44, p52, p62 and XPB, and is connected with the cdk-activating kinase (CAK) complex (cdk7, cyclin H, MAT1) through XPD association [51]. Cdk7 is a serine/threonine kinase, which is regulated by cyclin H and MAT1, and phosphorylates several substrates including the carboxy-terminal domain of the large subunit of RNA pol II [52]. This activity is not required for the NER activity in vitro [53]. The XPB and XPD proteins exhibit DNA-dependent ATPase and helicase functions [54,55] and have complementary activities: while the XPB protein unwinds the DNA in the $3' \rightarrow 5'$ direction, the XPD unwinds in the opposite direction [56,57]. Interestingly, a recent work with the XPD protein of *Drosophila melanogaster* demonstrated that this protein negatively regulates the cell cycle function of CAK [50]. Decreased amounts of XPD conferred elevated CAK activity and increased cell proliferation, while overexpression of this gene impaired CAK activity, generating a mitotic defect and cell death [50]. The specific roles for the other TFIIH subunities, p34, p44, p52 and p62, are still not clearly understood. p34 and p44 contain Zn^{2+}-finger motifs and a putative DNA-binding capacity [58]. The N-terminal part of p44 has been shown to positively regulate the helicase activity of XPD, whereas the C-terminal is involved in promoter escape [59]. The p52 subunit was shown to be essential for XPB anchoring within TFIIH, and deletion in its C-terminal region resulted in a dramatic reduction of NER and transcriptional TFIIH activities [60]. The molecular structure of the TFIIH complex, organized in a ring-like structure was recently revealed by electron microscopy [61].

The XPA gene product is a 32 kDa, Zn^{2+}-finger, DNA binding protein that shows affinity for UV-damaged DNA [62,63]. In general, this affinity is related to the extent of helical distortion in the DNA backbone. This characteristic had led to the proposal of a role for this protein in lesion recognition. However, as XPC–hHR23B has been shown to act first in damage detection, the function of XPA was reconsidered (see below). In addition, XPA showed to interact with two novel proteins, XAB1 and XAB2, whose functions in NER are not clear. The XPA binding protein 1 (XAB1) is a cytoplasmic GTPase protein that appears to be involved in nuclear localization of XPA [64]. The XPA binding protein 2 (XAB2) also interacts with CSA and CSB proteins, as well as RNA pol II [65]. Microinjection of antibodies against XAB2 into fibroblasts resulted in TCR and transcription inhibition, suggesting that this protein may be a new component in both processes [65].

The XPA protein interacts with other NER proteins, such as the TFIIH [66] and RPA [67]. Replication protein A (RPA) is a complex composed of three subunits (70, 32 and 14 kDa), with important roles in the three "Rs" of DNA metabolism: replication, recombination and repair [68,69]. During NER, this single-stranded DNA-binding protein complex is required for full double helix opening around the lesion, as it binds to the undamaged DNA strand [70]. The 70 kDa subunit of RPA contains three DNA-binding domains (DBD), and in vitro studies indicate that it covers around 30 nucleotides in the template strand of DNA during repair. Thus, only one RPA complex is supposed to be enough to assemble in the pre-incision complex, in order to protect the undamaged strand and recruit replication factors for DNA repair synthesis [71]. Recent work has demonstrated that RPA can recognize and bind DNA structures that present helical distortions, independent of the XPA protein [72]. Moreover, purified human XPA protein has been shown to form a homodimer in solution, and this dimer can interact with RPA, forming a XPA2–RPA complex [73]. Apparently, XPA and RPA are required for the formation of the pre-incision com-


The action of the repair complex and gap DNA synthesis
1. Pre-incision complex.
XPD
TFIIH
XPB
XPG
3´
5´
5´
3´
XPA
XPA
70
14
32
RPA
2. DNA opening by action of XPB and XPD helicases.
XPD
TFIIH
XPB
XPG
3´
5´
5´
3´
XPA
XPA
70
14
32
RPA
3. Dual-incision.
XPG
3´incision
XPD
TFIIH
XPB
XPF-ERCC1
5´ incision
3´
5´
5´
3´
XPA
XPA
70
14
32
RPA
4. DNA repair synthesis and ligation.
RFC
PCNA
nascent DNA
pol δ/ε
3´
5´
5´
3´
70
14
32
RPA
5. Repaired DNA.
PCNA
RFC
pol δ/ε
RPA
70
14
32
DNA ligase I
3´
5´
5´
3´

plex during NER. It has been proposed that these proteins are able to double check DNA bending and unwinding, and, as a consequence, they could serve as regulatory subunits that verify the damage-specific recruitment of NER factors [74]. Alternatively, they could control the correct three-dimensional assembly of NER intermediates prior to endonucleolytic cleavage [74].

The XPG gene product is a structure-specific 3′-endonuclease, a member of the Fen 1 protein family [75]. This endonuclease is essential for the incision step during NER, albeit, intriguingly, also being required during open-complex formation. Recently, the *Saccharomyces cerevisiae* counterpart of XPG, Rad2, was shown to be required for efficient RNA pol II transcription [76]. It will be interesting to observe whether this is also true for mammalian cells. The role of XPG during DNA incision will be discussed later.

The controversial sequential assembly of this multi-protein NER complex was solved recently. Previous biochemical experiments have generated conflicting models for the recruitment of TFIIH to the damaged DNA. Although the copurification of XPC with TFIIH in mammalian cells was reported [77,78], others failed to observe detectable amounts of a stable association between XPC–hHR23B and TFIIH in undamaged cells [79]. In contrast, XPA may also be required for TFIIH assembly [66]. Experiments performed using the local UV irradiation technique indicated that the recruitment of TFIIH to sites of DNA damage occurred in XPA, but not in XPC, deficient cells [5], confirming that, in vivo, the XPC–hHR23B complex is essential for the TFIIH assembly. However, the full opening of ~25 nucleotides (Fig. 2), mediated by the XPB and XPD helicases, is only achieved in the presence of XPA, RPA and XPG proteins [45,46].

The subsequent steps of NER assembly in this pre-incision complex remain unclear. The XPG endonuclease was observed to be associated to the repair complex without the presence of XPA [5], although at this stage endonuclease activity does not take place. Moreover, the assembly of XPG appears to be mediated by its interaction with TFIIH [80]. Once the opened complex is assembled and the lesion correctly positioned, the next step involves the dual incision of the DNA strand containing the damage.

2.3. Endonucleases-mediated removal of damaged oligonucleotide

The incisions occur at both sides and few nucleotides away from the lesion, require the action of the structure-specific endonucleases XPG and ERCC1–XPF (Fig. 2).

The ERCC1–XPF stable complex is formed by the 33 kDa ERCC1 and 103 kDa XPF proteins [81–83]. The stability of the individual components in the cell is dependent on heterodimer formation, which is obtained through the interaction of their C-terminal domains [84]. This complex is a structure-specific 5′-endonuclease that, besides acting in NER, seems to be involved in interstrand cross-linking repair and homologous recombination [85,86].

The assembly of both endonucleases does not occur synchronically. As discussed above, XPG endonuclease is recruited to the pre-incision complex without the presence of XPA, since cells mutated for this protein still accumulate the XPG protein at damaged spots [5]. However, the recruitment of XPG is not enough for activating its endonuclease activity, as no incision activity is detected in these XPA deficient cells. It has been suggested that XPA, in cooperation with RPA, triggers XPG endonuclease activity [83]. In contrast to XPG, the recruitment of ERCC1–XPF to the damaged DNA requires XPA protein, since its accumulation in the damaged spots was not observed in XPA deficient cells [5].

The correct positioning of both endonucleases is a crucial event for the correct incision around the damaged fragment. RPA bound to the undamaged strand confers strand specificity to ERCC1–XPF, and its interaction with XPA may facilitate or even stabilize its positioning onto the damaged strand [87]. In the case of XPG, RPA may contribute but is not sufficient to confer its strand specificity. TFIIH is the likely candidate for this specificity [88].

Once the incision complex is assembled, the catalytic activity of the endonucleases takes place. In vitro experiments have indicated that TFIIH inhibits the endonuclease activities of both XPG and ERCC1–XPF, in the absence of ATP, what is reversed upon addition of ATP. The requirement of DNA unwinding and regulation of endonuclease activities may help to prevent inadvertent incisions prior to full opening of the double helix [89]. The incisions are made asymmetrically around the lesion and consensus exists that the 3′-incision precedes the 5′-incision [53]. The XPG protein is responsible for the incision in one strand of duplex DNA, at 3′ of the border of the open DNA intermediate, approximately two to eight nucleotides away from the lesion [45]. On the other hand, ERCC1–XPF carries out the 5′-incision, approximately 15–24 nucleotides away from the lesion [45]. Precise incision locations may vary depending on the type of lesion [90], but the incisions are independent of each other. The 3′-endonuclease activity of XPG is detected in the ab-

Fig. 2. Model of action of the repair complex and gap DNA synthesis. (1) Once the pre-incision complex is assembled in the lesion site, (2) ATP-dependent helicase activities of the TFIIH's components, XPB (3′–5′ direction) and XPD (5′–3′ direction), are responsible for opening the double helix at the lesion site, generating a gap of ~25–30 nucleotides. (3) The XPG endonuclease nicks two to eight nucleotides downstream (3′) of the lesion, while the XPF–ERCC1 endonuclease is recruited to the complex; and makes the incision 15–24 nucleotides upstream (5′) of the lesion. The heterotrimer RPA complex shows a specific polarity when loading to the lesion site, with the 70 kDa large subunit protecting around 30 nucleotides [71]. RPA and XPA proteins help to assure strand specificity to XPF–ERCC1 activity, stabilizing the correct positioning of the repair-complex. (4) After incisions, the damaged oligonucleotide dissociates from the DNA molecule and DNA repair synthesis is performed by DNA polymerases; the permanence of RPA is required to protect the template strand from degradation and also to assemble replication factors, as PCNA and RFC. (5) The final step is performed by the ATP-dependent activity of DNA ligase I, resulting in completely restored DNA. The structures presented for these proteins are hypothetical.

sence of ERCC1–XPF, but, although the 5′-endonuclease activity of ERCC1–XPF does not depend on prior XPG cleavage, it requires the presence of this protein in the repair complex [91,92]. The damaged fragment seems to dissociate from the gapped DNA in vitro without the requirement for the DNA repair synthesis components [53].

2.4. DNA repair synthesis

The ERCC1–XPF 5′-incision completes the incision stage and leaves a hydroxyl(OH)-group at the 3′-terminus of the gap generated by the removal of the damaged oligonucleotide. As this terminus may act as a DNA primer for DNA polymerases, no additional modifications are required to start DNA synthesis at this side of the gap [82]. Probably, at this stage most NER proteins leave the damaged area and DNA repair synthesis machinery takes place. However, RPA is required for gap-filling DNA synthesis in order to protect the template strand against nucleases. RPA may also be important for promoting assembly of the DNA replication machinery.

Two DNA polymerases are implicated in the synthesis of the new DNA fragment. In vitro studies performed with antibodies and chemical inhibitors showed that both DNA Pol δ and Pol ε carry out NER DNA synthesis [93,94]. In agreement with this finding, PCNA is required for efficient DNA synthesis, as this protein acts as a processivity factor for both polymerases [95]. Another protein that also acts as a processivity factor in NER is the replication factor C (RFC). Both cofactors work as a complex that facilitates the assembly of the above polymerases. This complex is formed after the binding of RFC to 3′-termini of DNA primers, facilitating the loading of PCNA [96]. Confirming this model, repair synthesis was obtained in vitro with the presence of the five components, PCNA, RFC, RPA, and either Pol δ or Pol ε [97].

Finally, the last step of NER takes place: ligation of the 5′-end of the newly synthesized patch to the original sequence, a function that seems to be performed by the human DNA ligase I [98].

3. NER kinetics

The new approach of local UV irradiation has been extremely useful for the in vivo understanding of NER mechanisms and dynamics. As discussed above, with this new methodology it became clear that NER action involves the sequential and coordinated assembly of every protein in lesion-containing sites. In addition, new insights about kinetics of this pathway have been clarified by this technique. It was observed that most NER enzymes leave the damaged sites around 4 h after UV exposure, even with the persistence of unrepaired photoproducts [99]. Analysis of the dynamics of the XPG proteins revealed that they are found in aggregates within the nucleus, but 2 h after UV irradiation XPG disperses in the nucleus overall. The number of aggregates increases again 4–8 h after UV exposure, possibly also reflecting DNA repair protein mobility [100]. Previous biochemical studies have shown that less than 50% of CPDs are repaired in 4 h in the genome overall, in contrast with almost 100% of 6-4 PPs removal in the same period of time [101]. These results led to the interpretation that the kinetics of 6-4 PPs repair is dictating a first “round” of migrational response of NER proteins to the lesion sites. Interestingly, measurements of the removal of CPDs and lesions generated by the UV-mimetic agent, *N*-acetoxy-2-acetylaminofluorene (NA-AAF), in mammalian cells, showed a biphasic kinetics, where around 40–50% of these lesions were removed during the first 6–8 h after exposure to the agent [102,103]. The removal of the remaining lesions seems to occur at a much slower rate, and is not completed before 48 h after the treatment. Quantification of the contribution of NER in transcribed and non-transcribed strands of active genes also revealed distinct kinetics [104]; 8 h after UV irradiation, 80% of the CPDs were removed from the transcribed strand, in contrast to 15–30% of these in the non-transcribed strand of the same gene. Altogether, these reports indicate that, depending on the lesion type and location in the genome, NER recruitment and activity may occur differentially. These observations may suggest two distinct NER responses after induction of bulky lesions in the genome: (i) the immediate response, which removes the most helix-distorting lesions (such as 6-4 PPs) concerning the genome overall, and TCR of CPDs. The TCR process for CPD lesions, which occurs faster than GGR, repairs around 80% of CPDs in the transcribed strand of active genes, thereby allowing for recovery of RNA synthesis and cell survival; (ii) the secondary response, carried out at a much slower rate, is responsible for eliminating the remaining lesions in the genome. This response probably does not involve mass recruitment of NER proteins to the lesion sites, and, thus, does not interfere with the foci pattern exhibited early after cellular injury, which is most likely due to the initial immediate response.

4. The NER interplay with cell cycle progression and apoptosis

Parallel to the NER response, the sensing of these bulky lesions in the genome is directly connected to transcriptional and replication arrest (Fig. 3), which may involve cell cycle checkpoint mechanisms [105] and the induction of apoptosis [106]. Several signaling cascades can be triggered by DNA injury, and the ATM-related protein (ATR) is one of the central proteins induced as an upstream event after UV-damage. ATR is a member of the phosphoinositide 3-kinase-related family, readily activated after DNA damage. In contrast to Ataxia–Telangiectasia mutated protein (ATM; reviewed in [107]), which is primarily associated with ionizing radiation-damage response and double-strand breaks, ATR responds to UV-damage and stalled replication forks

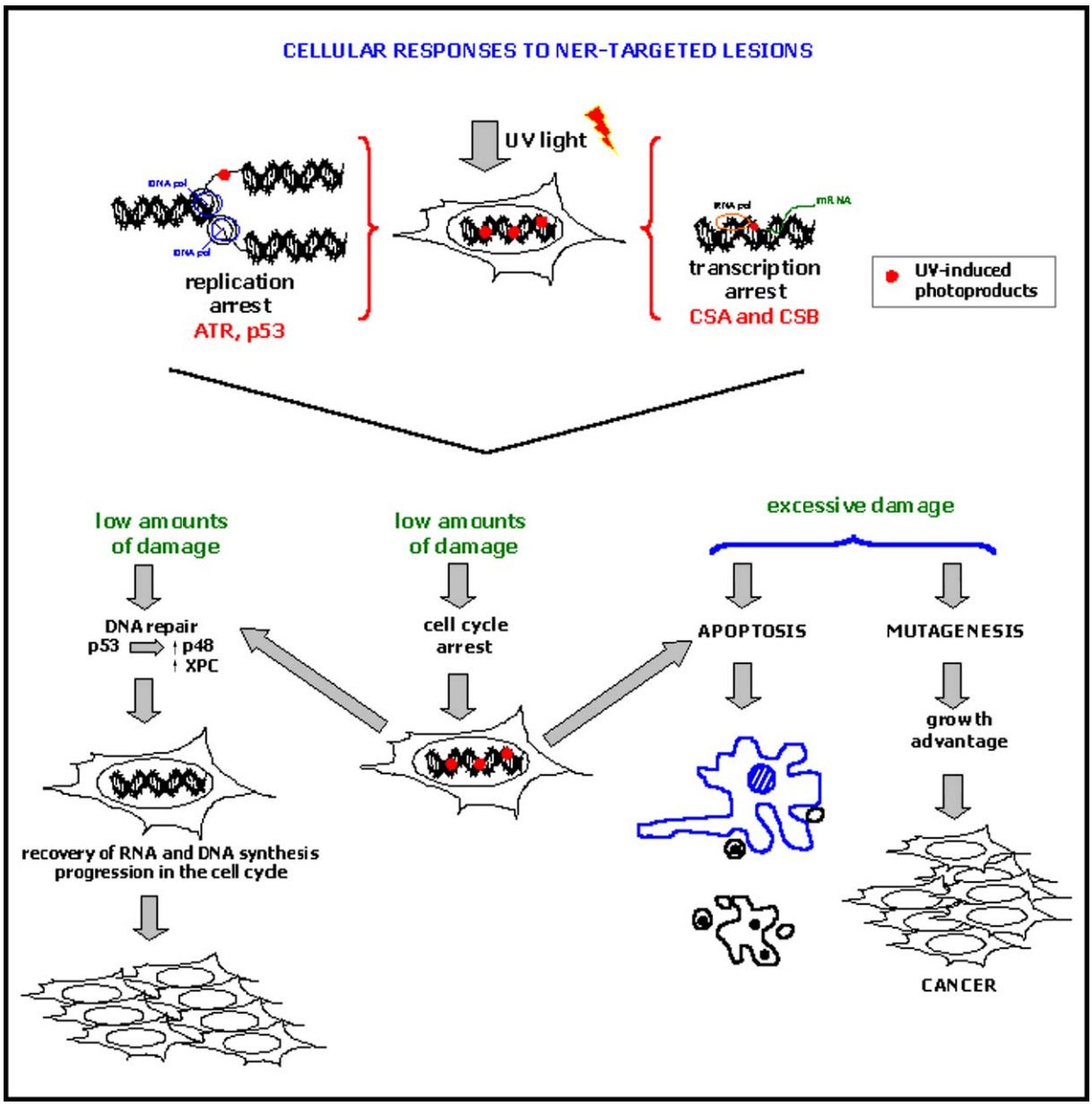


Fig. 3. Different cellular responses after detection of UV-induced photoproducts. Bulky lesions in DNA, such as CPDs and 6-4 PPs, represent a drastic physical hindrance to the progression of replication and transcription machineries. In blocked replication forks, sensors, such as ATR protein and p53, are involved in transmitting the signal of danger to other proteins in the cell. CSA and CSB proteins probably act in transcription stalled in front of lesions, recruiting TCR factors. DNA repair pathways can handle low amounts of DNA damage, what may involve p53-dependent induction of NER proteins as XPC and XPE/p48. Cell cycle arrest may provide additional time for the lesion removal. In the case of excessive genomic injury, a programmed cellular suicide (apoptosis) can protect the organism from genetic instability, leading to cell fragmentation, and phagocytosis by neighboring defense cells. When all these rescue mechanisms are not able to deal with DNA damage, persistence of lesions can be responsible for mutations, leading to genetic instability and carcinogenesis.

[108,109]. In fact, there is evidence suggesting that ATR directly binds to 6-4 PPs, activating its kinase activity [110]. Upon activation, ATR starts a cascade of checkpoint signaling events, which may result in G1 or G2 arrest, or in S phase delay [108]. ATR can phosphorylate a series of different substrates, including p53 and the checkpoint kinase Chk1. These events will end up in cell cycle arrest due to transcriptional inhibition of genes required for cell cycle progression, thus furnishing NER with an additional time frame to remove UV-photoproducts. As it will be discussed later, some NER proteins are targets for transcriptional induction by p53, an event that potentiates NER action.

The effects of photoproducts on DNA replication are related to the impairment of replication forks. This impairment is not only a passive consequence of the physical presence of a lesion, but an active response of temporal pauses in the replication progression in the presence of blocking lesions [111]. How a blocked replication fork is recognized and signals for S-phase checkpoint is still obscure, but checkpoint components may be able to recognize

DNA repair factors positioned at lesion sites and/or structures related to DNA repair intermediates [112]. It was demonstrated that inhibition of replicative polymerase activity prevents checkpoint proteins loading in chromatin, thus suggesting that ongoing replication is necessary to induce and establish this response [113]. One possibility is that checkpoint proteins are normally bound to the replication apparatus, remaining inactive unless the fork encounters problems [114]. Alternatively, checkpoint proteins, such as ATM and ATR, could directly recognize the blocked replication [112,114]. In fact, stalled replication forks, in front of DNA lesions, may activate ATR. This raises the possibility that it acts as a lesion recognition factor necessary for checkpoint signaling in response to lesions that block DNA polymerase movement [115].

Transcriptional blockage signals specifically to TCR and apoptosis induction after UV irradiation [106,116]. Other proteins than those involved in TCR can be recruited to this damage site, like PARP-1 [117,118]. When strand breaks are induced in DNA, this enzyme binds to the breaks initiating the (ADP-ribosyl)ation of many of the proteins in the nuclei, including PARP-1 itself and histones [119]. This process may directly affect the repair capability of the cell. PARP-1 has been shown to reduce the rate of transcription elongation by RNA polymerase II, which is reversed when the protein automodifies itself [120]. This is in agreement with observations that PARP-1 activity inhibits transcription factors preventing their binding to DNA, indicating that poly(ADP-ribosyl)ation negatively regulates transcriptional synthesis by the RNA polymerase II complex [117,118]. This effect may also be related to the reduction of the RNA transcription in UV-irradiated mammalian cells and to the induction of apoptosis. Considerable evidence supports the involvement of PARP responses in cells treated with alkylating agents, but its role after UV irradiation is still under debate [121,122].

Other cellular responses related to transcriptional arrest in front of bulky lesions were also reported, although whether they represent active signals to repair and cell death remains obscure. The RNA polymerase II complex loads to the promoter site in the hypophosphorylated form (form IIa). The TFIIH factor contains the kinase activity responsible for phosphorylation of the carboxy-terminal domain of the RNA polymerase II complex, which then leaves the promoter site and progresses in the elongation mode as a hyperphosphorylated form (IIo; [123]). Cells defective in recovery of RNA synthesis after UV-induced DNA damage, such as CSB mutated cells, display an accumulation of the IIo form of RNA polymerase II during several hours after UV exposure, a feature not observed for NER-proficient cells [124]. Further analysis strongly indicated that the CSB protein is involved in the ubiquitin-mediated proteasomal degradation of stalled transcriptional complexes, which would allow for lesion removal and the restart of transcription elongation [124]. These data reveal the multi-role feature of the NER factor CSB, coupling transcription to repair, chromatin remodeling and recycling of transcription complexes after damage detection in TCR, thus allowing for recovery of transcription and cell survival.

Although low doses of UV irradiation lead to the induction of protection mechanisms in the cells, including cell cycle arrest and DNA repair responses, high doses trigger an active type of cell death known as apoptosis. In fact, these responses result in different transcription patterns that follow low or high doses of UV irradiation of mammalian cells [125,126]. Apparently, the reduction of RNA transcription by DNA damage seems to be one of the first signals for the induction of apoptosis [106,116]. In fact, cells defective in the removal of lesions in the transcribed strand of active genes, such as CS cells, display increased rates of apoptotic death after exposure to lower UV-doses in comparison to TCR-proficient cells, indicating that the ability to remove damage from active genes is essential for the recovery of RNA synthesis and survival after cell injury [106,127]. This idea is further supported by the correlation of RNA transcription inhibition in the presence of CPDs and the UV-induction of apoptosis observed in rodent and human cells expressing the CPD-specific photolyase from the marsupial rat kangaroo. The removal of such damages by photoreactivation recovered RNA transcription, thus preventing apoptosis. This also suggests that the CPD-photolyase has access to the damage at stalled transcription complex [116,128].

The repair machinery itself may participate actively in apoptosis execution when the amount of lesions reaches a higher threshold, leading the cells to death [129,130]. NER is upregulated in early-stages of p53-induced apoptosis of DNA-damaged cells [129]. This upregulation appears to be the result of positive transcriptional control of *XPC* and *p48* (XPE-DDB2 subunit) genes by p53 [131,132]. Moreover, p53 seems to be essential for the assembly of repair machinery, since p53-deficient Li–Fraumeni syndrome cells are much less efficient in XPC and TFIIH recruitment to CPD sites [99]. In repair processes, p53 may also act as a chromatin accessibility factor, facilitating the access of NER proteins to the lesion site [99,133]. These results confirm previous observations of decreased repair capacity in cells with mutations in p53, this role seeming to be restricted to GGR, since these cells do not show any impairment for TCR [134,135]. However, interaction of p53 with XPB, XPD and CSB [136] suggests that p53 may also play a role in TCR. The UV wavelength used to induce DNA damage might explain why there is no defect in TCR in p53-deficient cells, since irradiation of cells with UVB showed that p53 might play a role in both GGR and TCR [137]. The interplay between p53 and NER proteins is even more complex when we consider that cells derived from XP patients, complementation groups XPB or XPD, were shown to be resistant to p53-induced apoptosis, after microinjection of a p53 expression vector into the cells, while cells deficient for XPA or XPC underwent normal apoptosis. This suggests a role for XPB and XPD proteins in p53-mediated apoptosis besides their role in transcription and DNA repair [138].

Table 1
Presence of NER orthologues in several organisms

Human protein	*C. elegans*	*D. melanogaster*	*S. cerevisiae*	*A. thaliana*	*P. falciparum*	Archaea [a]
XPA	Yes	Yes	Yes (Rad14)	No	No	No
XPB	Yes	Yes	Yes (Rad25)	Yes	Yes	+/–
XPC	Yes	Yes	Yes (Rad4)	Yes	No	No
XPD	Yes	Yes	Yes (Rad3)	Yes	Yes	+/–
XPE/DDB p48 subunit	No	No	No [b]	Yes	No	No
DDB p127 subunit	Yes	Yes	No [b]	Yes	?	No
XPF	Yes	Yes	Yes (Rad1)	Yes	Yes	+/–
XPG	Yes	Yes	Yes (Rad2)	Yes	Yes	+/–
CSA	?	?	Yes (Rad28)	Yes	?	?
CSB	Yes	?	Yes (Rad26)	Yes	?	No
ERCC1	Yes	Yes	Yes (Rad10)	Yes	Yes	No
hHR23A and hHR23B	Yes	Yes	Yes (Rad23)	Yes	Yes	No

Genomic databases were analyzed for the presence of protein sequences corresponding to orthologues of mammalian NER enzymes. (+/–) Similar proteins in archaeal genomes occur only in some of the species; (?) this indicates that a member of the same protein family is present, although it is not a clear orthologue of mammalian and fungal prototypes.

[a] Considering all full archaeal genome sequences.

[b] There are no homologues of DDB-complex in *S. cerevisiae*, but DDB2 exist in other fungi, such as *S. pombe* [165]. On the other hand, the unrelated Rad7–Rad16 DNA binding complex of *S. cerevisiae* may be functionally replacing mammalian DDB.

5. Eukaryotic NER evolution

The whole NER mechanism is well conserved in nature, with bacteria and eukaryotes showing analogous lesion recognition, DNA incision, fragment excision and repair synthesis. Although bacterial and eukaryotic NER enzymes are homologous within each kingdom, they lack sequence similarity when the two groups of organisms are compared. The presence of the global and transcription-coupled subpathways extends the overall resemblance of bacterial and eukaryotic NER mechanisms. Bacterial NER is accomplished by the UvrABC system, including the recently identified Cho protein, a divergent UvrC homologue, and the Mfd transcription–repair coupling factor (reviewed by van Houten et al. [139]), while the eukaryotic picture, derived mostly from studies with yeast and mammals, is composed of the myriad of proteins described above. Nevertheless, the universality of these mechanistic models is now being challenged by genomic information. In this section, we will briefly discuss the impact of the genomic data revolution on our current view of NER.

NER proteins are well conserved among eukaryotes, but some significant differences are found between more distantly related phylogenetic groups, as shown in Table 1. The prototypic mammalian proteins are present in the genomes of model organisms such as *D. melanogaster* and *Caenorhabditis elegans*, suggesting that NER is essentially equal in animals. Recently, the transcriptome of *Schistosome mansoni*, an acoelomate human parasite and an early divergent metazoa, was described [140], these eukaryotic NER genes also being present (data not shown). This indicates that probably all the metazoa share a homologous NER system. The *XPA* and *CSB* genes from *C. elegans* were identified and elegant RNAi experiments demonstrated that the products of these genes are involved in protecting the organism from UV irradiation, confirming they participate in DNA damage repair [141,142]. Similarly, *Drosophila* XPA, XPB, XPD, XPF, XPG and ERCC1 orthologues have already been functionally characterized [143–148], further supporting the functional conservation of NER in animals.

The most striking differences are found in plants and in protozoan, as deduced by the genome sequences from *Arabidopsis thaliana* and *Plasmodium falciparum*. In *A. thaliana*, orthologues of the XPB and XPD helicases [149–151] and of the XPF and XPG endonucleases [152–154] were identified. Plant lineages mutated for these genes show phenotypes consistent with deficiencies in DNA damage removal, demonstrating their impairment in NER. However, *A. thaliana* and *P. falciparum* apparently lack XPA homologues, although curiously both have the XAB1 orthologue, identified in human cells due to its XPA-binding properties. Both genomes are supposed to be completed and, although some DNA sequences from specific chromosome locations of these organisms may still be missing, the absence of this gene is astonishing. It is important to add that, up to now, no *XPA* orthologue, with significant similarity, was observed in any other plant DNA sequence. In *A. thaliana* and *P. falciparum*, the only gene products with some domain similarity to XPA correspond to proteins related to chromatin segregation, but that differ considerably in size and aminoacid sequence. XPA is thought to play a crucial role in lesion recognition in the mammalian and yeast models (*Rad14* orthologue), so it is tempting to speculate that its role in the recognition step has been overestimated, or simply these organisms have other divergent unknown proteins playing

this NER function. In *P. falciparum*, the global repair XPC protein is also surprisingly missing, suggesting that this organism may have a very different mechanism for DNA damage recognition for GGR. An alternative mechanism for repair of transcriptionally inactive DNA is known in *S. cerevisiae*. The Rad7–Rad16 complex functions as a NER factor stimulating damage-specific incision [155]. Orthologues of Rad7 are restricted to fungi, but Rad16 orthologues are found in *A. thaliana* and *P. falciparum*. Although it is not known whether Rad16 functions without the Rad7 partner in DNA repair, this protein is an obvious candidate to perform a role in DNA damage recognition in these organisms. NER research in these organisms, including the relative contribution of GGR and TCR, is promising and may lead to exciting discoveries.

The scenario of NER in archaea is even more challenging. Cell extracts from *Methanobacterium thermoautotrophicum* produce a repair patch typical of bacterial organisms (10–11 nt) after photoproduct excision [156]. This led to the interpretation that, in these organisms, NER is essentially equal to bacteria. To date, at least 16 complete genomes from archaea organisms are available (http://www.tigr.org). Only a selected group of these, particularly mesophilic methanogens (*M. thermoautotrophicum* and *Methanosarcina* sp.) and halophiles (*Halobacterium* sp.), have UvrABC orthologues. These organisms correspond to only few members of the Euryarchaeota phylum, and this has been suggested as a case of recent lateral gene transfer from bacteria to archaea [157,158]. Some archaea, that do not have UvrABC, do have detectable orthologues of the eukaryotic nucleases XPF and XPG, as well as the XPB and XPD helicases. The involvement of the archaeal XP genes in bona fide NER, if one such exists, awaits demonstration, but initial experiments begin to elucidate their function. The archaeal XPF protein is present in two different forms: a shorter one, present in Crenarchaeota, composed only by the nuclease domain, in an architecture similar to the Mus81 protein involved in recombination; and a euryarchaeal form, similar to the eukaryotic XPF, containing an additional N-terminal DEAH helicase family domain [158]. This latter form has shown to be a structure-specific nuclease [159]. The short XPF also has nuclease activity upon several DNA structures, including Holliday junctions, but is only active in the presence of the archaeal PCNA orthologue [160].

The situation is even more intriguing in some archaea that present a mixture of bacterial and eukaryotic NER components. It has been suggested that the NER pathway in archaea was eukaryotic in character, and has been supplanted in some species by the bacterial version [158]. Furthermore, complementing the puzzling of NER complex in archaea is the lack of any obvious orthologue of the eukaryote damage-recognition proteins, such as XPA, XPC and XPE. This picture is similar to that found in *Plasmodium*, suggesting that NER has undergone increasing refinement during evolution, with the acquisition of new proteins in higher eukaryotes.

6. Concluding remarks

The intricate NER mechanism, that senses the damaged DNA, recruiting the proteins that prepare and remove an oligonucleotide containing the lesion, and restoring the genetic information, has been gradually disclosed. New methods, such as the ability to damage DNA in specific regions of the nucleus (local UV), are providing means to monitor the dynamics of protein kinetics and traffic to the damaged area. The access order to the repair complex has been identified for several of the players, but certainly new information will help us to better understand how, when and under which conditions the different lesions are removed by NER. This mechanism occurs within the nuclei of living cells, affecting several DNA metabolic processes, such as replication and transcription. The interactions of these different processes with NER are still unclear, even though we have known for several years that many of the NER enzymes have other functions in the cell [161,162]. The TCR is a clear example of such connections in DNA metabolism processes. Several novel data indicated that other proteins may also be associated to NER, such as the participation of mismatch repair enzymes (MutS and MutL human orthologues) in TCR and apoptosis [163]. Moreover, TCR may also contribute to the removal of lesions promoted by oxidative stress and normally repaired by the base excision repair [161,164].

However, we are still far from understanding the sophisticated network of events that are triggered in the cells after lesions are introduced in DNA. To this end, the genomic revolution will certainly make a big difference in our capacity to identify the genes that are being expressed in damage stress situations, by transcriptome (microarray) or proteome analyses. Although in their infancy, these techniques have already contributed to the recognition of different patterns of responses in many circumstances of DNA damage in the cell, specially concerning the induction of repair and apoptotic related genes after UV irradiation. Direct genome sequence data is also an enormous source of information that may help us to understand how the organisms have evolved for their defenses against environmental and cellular aggression of DNA. In all these aspects, NER has been confirmed to be a fundamental process in the maintenance of the genetic stability, helping to keep the balance between life and evolution.

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