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REVIEW

Nucleotide Excision Repair: DNA Damage Recognition and Preincision Complex Assembly

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Abstract—Nucleotide excision repair (NER) is one of the major DNA repair pathways in eukaryotic cells counteracting genetic changes caused by DNA damage. NER removes a wide set of structurally diverse lesions such as pyrimidine dimers arising upon UV irradiation and bulky chemical adducts arising upon exposure to carcinogens or chemotherapeutic drugs. NER defects lead to severe diseases including some forms of cancer. In view of the broad substrate specificity of NER, it is of interest to understand how a certain set of proteins recognizes various DNA lesions in the context of a large excess of intact DNA. This review focuses on DNA damage recognition and following stages resulting in preincision complex assembly, the key and still most unclear steps of NER. The major models of primary damage recognition and preincision complex assembly are considered. The contribution of affinity labeling techniques in study of this process is discussed.

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Key words: nucleotide excision repair, repair factors, damage recognition, preincision complex, photoaffinity labeling

Endogenous reactive metabolites and exogenous factors lead to various damages to DNA in cells. The genetic stability of organisms is achieved by a broad spectrum of repair mechanisms [1-4], among which nucleotide excision repair is of high significance. This process removes a wide range of lesions damaging the double helix such as pyrimidine dimers arising upon UV irradiation and bulky chemical adducts arising upon environmental factors or chemotherapeutic drugs. Nucleotide excision repair (NER) as a complex process involves not less than 30 polypeptides [5, 6]. There are two ways of damage repair: global genome repair (GG-NER) removing lesions in the whole genome DNA and transcription coupled repair (TC-NER) removing lesions from the transcribing DNA strand. TC-NER is associated with the functioning of RNA-polymerase II [7]; its arrest at the damage point is a signal for the assembly of repair protein complex. Despite intensive study using different methods including *in vivo* methods, the mechanism of damage recognition during GG-NER has long remained unclear. Only within recent years the majority of researchers have agreed that heterodimer XPC–Rad23B–Cen2 is the first among NER factors to recognize a lesion. The subsequent assembly of NER factors on the damaged DNA, the composition and the lifetime of intermediate structures, and the precise mechanism of the reaction are not yet fully described. In this review we analyze current views on the mechanism of damage recognition and the assembly of the protein complex in the process of global genome repair in higher eukaryotic cells.

ADVANTAGES OF SUCCESSIVE GG-NER COMPLEX ASSEMBLY: DYNAMICS AND FUNCTIONAL FLEXIBILITY OF THE SYSTEM

The molecular mechanism of GG-NER is rather well studied. The elimination of damage is successive and includes the following stages (Scheme 1): damage recognition, DNA unwinding around the damage (formation of open complex), damage excision as a part of the oligonucleotide complex, and single-strand gap filling (DNA resynthesis). The GG-NER process has been reconstructed in an *in vitro* system [8-10]. It was shown that only six core repair factors (XPC–hHR23B, XPA, RPA, TFIIH, XPG, and ERCC1–XPF) are necessary

Abbreviations: a.a., amino acid residue; Cen2, protein centrin-2; NER, nucleotide excision repair; nt, nucleotide residue; RPA, replication protein A; XP, *xeroderma pigmentosum*.

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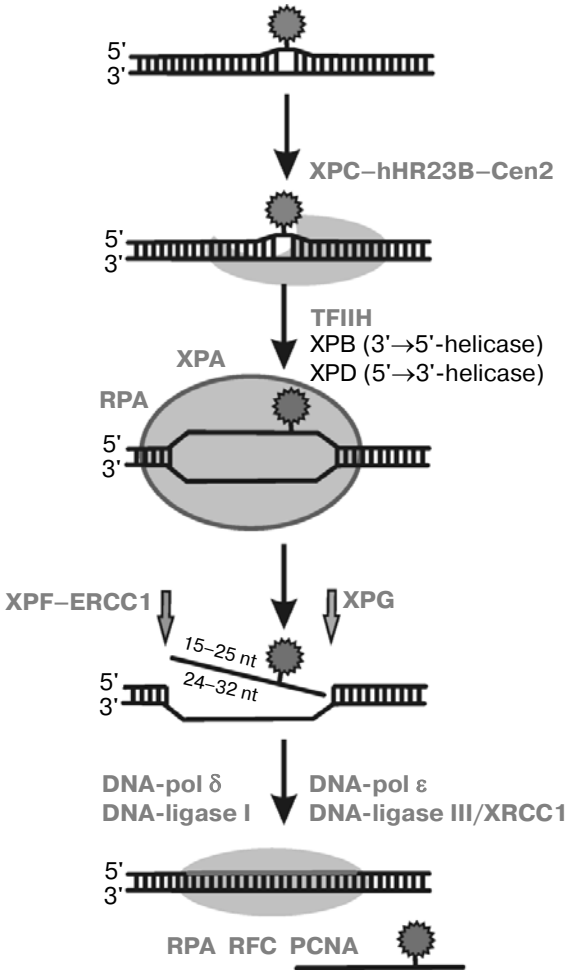
Core proteins of GG-NER

Protein	Subunit composition	Properties and activities	Functions in GG-NER
XPC–hHR23B	XPC (p125) HR23B (p58)	DNA binding heterodimer stabilization	primary damage recognition
XPA	XPA (p36)	contains Zn^{2+}-finger, DNA binding	complex assembly, recognition, and/or damage verification (?)
RPA	RPA70 (p70) RPA32 (p32) RPA14 (p14)	contains Zn^{2+}-finger, DNA binding DNA binding heterotrimer assembly, DNA binding	stabilization of single-stranded DNA regions, damage recognition (?)
TFIIH	XPB (p89)	DNA-dependent ATPase, 3′→5′-helicase	DNA duplex unwinding, complex assembly, damage detection
	XPD (p80)	DNA-dependent ATPase, 5′→3′-helicase	
	p62 p52 p44 p34 CDK7 (p40) Cyclin H (p36) MAT1 (p32) p8	core subunits core subunits contains Zn^{2+}-finger contains “ring finger” domain protein kinase protein kinase regulatory subunits complex stabilization	
XPG	XPG (p133)	structure-specific endonuclease	DNA cleavage from 3′-direction of damage
ERCC1–XPF	ERCC1 (p40) XPF (p115)	heterodimer stabilization structure-specific endonuclease	DNA cleavage from 5′-direction of damage

and sufficient for the development of the excision stage to repair most damages *in vitro*. The subunit composition and basic properties of these factors are shown in the table.

Three models of the assembly of NER complex on damaged DNA have been proposed during the study of the GG-NER system: the reparosome model, the model of coordinate binding, and the model of successive binding. The reparosome model proposes the presence of the “reparosome” in cells – a complex of six or more proteins that constantly scans the genome for damage. The model of coordinate binding proposes that proteins with moderate affinity to damaged DNA (XPC–hHR23B, XPA, and RPA) are able to form a triple complex whose affinity to the DNA damage is increased by virtue of additive effect. Still the most reliable data favor the successive binding model implicating the existence of a specific order of binding of repair factors to DNA damage. These data are a result of a set of *in vivo* experiments using fluorescence-labeled proteins. Green fluorescent protein (GFP) covalently bound to the experimental repair factor was used as a fluorescent label [11-13]. It was shown that GFP–ERCC1, GFP–XPB, and GFP–XPA are freely moving in the nuclei of non-irradiated cells, but after UV-irradiation they migrate to the irradiation site and are temporally immobilized on the DNA damage. The protein diffusion coefficients were proportional to their molecular weight, which does not agree with the presence of a large “reparosome” in the cell. Individual proteins may form larger complexes already at the DNA damage site. These complexes may be dynamic and not stable so that they cannot be detected by currently used methods.

Prior assembly of the proteins in a “reparosome” would be useful because it would allow effective beginning of repair at any time, although dynamic protein dissociation/association increases the number of possible combinations and makes the system more flexible and multifunctional. It allows the proteins that are involved in different DNA metabolic pathways to quickly switch from one process to another. It is known that all NER factors are also involved in other metabolic processes involving DNA. XPC–hHR23B complex interacts with some DNA-glycosylases (the enzymes of base excision repair) and may work as a “switch” between the processes of excision repair of bases and nucleotides [14, 15]. The small subunit of HR23B complex takes part in proteolytic degradation of proteins on 26S proteasomes [16]. Multisubunit complex TFIIH, originally discovered as a



Nucleotide excision repair

Scheme 1

transcription factor [17], also takes part in cell cycle control [18]. Excision nuclease ERCC1–XPF participates in recombinant repair [19], and XPG takes part in base excision repair [20]. For a long time XPA was thought to be involved only in the NER process, but recently data on its involvement in cell signal transmission in respond to DNA damage has been obtained [21-23]. RPA, one of the most abundant nuclear proteins, is involved at least in replication, repair, and homologous recombination of DNA [24-26] and contains binding domains to many proteins involved in different repair pathways.

The model of successive binding suggests the presence of proteins responsible for damage recognition and following involvement of other repair factors to the damage site. There is still no consensus about which NER factors are the first to recognize the lesion. Supposedly, this role may belong to proteins that have higher affinity to damaged DNA compared to native DNA: XPC–hHR23B, XPA, and RPA. UV-damaged DNA-binding protein (UV-DDB) is involved in the recognition of cyclobutane-pyrimidine dimers (CPD) formed in DNA after UV-irradiation; it provides binding of XPC–hHR23B to the DNA damage [27-30]. The GG-NER system recognizes the damage against the background of intact DNA. However, none of the mentioned factors has a preferential affinity to the lesion [31]. Probably the necessary specificity of the repair complex is attained by cooperative effects during assembly. Most known data proposes XPC–hHR23B being the primary factor recognizing the lesion [32-34]. In experiments to define the order of repair factor assembly on the DNA damage, it was found that a plasmid with multiple lesions preincubated with XPC–hHR23B is repaired by cell extracts faster than a plasmid preincubated with XPA–RPA complex [32]. These results agree, according to the authors, with the proposal that XPA–RPA complex is involved not in the primary damage recognition but in the following steps assisting TFIIH at the stage of DNA duplex unwinding. In subsequent studies it was shown that the recruiting of XPC–hHR23B and TFIIH to the lesion site does not depend on ATP. However, it was found that ATP is necessary for the recruiting of XPA [35], which means that the incorporation of XPA in the preincision complex takes place only after the duplex unwinding performed by TFIIH helicases using ATP.

Later, using the method of local UV-irradiation of a cell combined with fluorescent antibody labeling, it was shown that the damage-recognizing XPC–hHR23B complex is probably very important for the recruiting of other NER factors to the repair process [36]. XPC–hHR23B is accumulated in cells on DNA damage irrespectively to the presence of XPA. However, the recruiting of XPA to the damage site takes place only in the presence of XPC–hHR23B. XPC–hHR23B is also necessary for TFIIH binding to the photolesion, whereas XPA protein is not needed for the binding of TFIIH to the lesion site. The incorporation of RPA and XPG in the repair complex precedes incorporation of XPA and is independent of XPA [12].

Despite the existence of data favoring the idea that XPC–hHR23B–Cen2 complex is the main factor for primary damage recognition in GG-NER, some experimental data suggest the model of cooperative (random) binding as an alternative. According to this model the lesion is primarily detected by any of these three proteins (XPA, RPA, or XPC). Cooperative action of XPA (which is able to bind RPA and TFIIH), RPA, and XPC (able to bind TFIIH) leads to the formation of a four-component complex on the DNA damage. The second stage is the kinetic control of the specificity of the developed complex by TFIIH helicase activity and the arrest of the reaction in the case of the formation of nonspecific complex, or the stimulation of the process in the case of specific complex formation. This type of DNA lesion scanning is universal and allows formation of an effective repair complex for any type of DNA damage [37-40].

XPC–hHR23B COMPLEX IS A SENSOR OF DISRUPTIONS OF REGULAR STRUCTURE OF DNA DUPLEX

XPC protein (125 kDa) forms a stable triple complex with hHR23B (the homolog of yeast protein Rad23) [41] and Cen2 proteins [42]. XPC interacts with hHR23B by the evolutionarily conservative C-terminal domain. The hHR23B protein stabilizes XPC [43], in particular it prevents its proteolytic degradation. The addition of hHR23B stimulates XPC activity in NER in an *in vitro* system [44], and furthermore only the XPC-binding domain of hHR23B (56 a.a.) was necessary for developing this effect [45]. Two Rad23 homologs were found in human cells—hHR23A (50 kDa) and hHR23B (58 kDa), whose identity is 57% and similarity is 76% [41]. Both proteins are able to form stable complexes with XPC and equally stimulate its repair activity in cell extracts and a reconstructed system [46]. Nevertheless, the whole cellular XPC is associated with hHR23B [41].

Both hHR23B and hHR23A homologs contain ubiquitin-like domain UbL at the N-terminus, and two ubiquitin association domains, UBA1 and UBA2 [41, 45]. Ubiquitin is a highly conservative polypeptide (76 a.a.) found in all eukaryotes. One or a few ubiquitin molecules covalently bind to the acceptor protein using the special ubiquitin ligase system and introduce the protein to different regulatory processes such as degradation, repair, translocation, and cell cycle control. It was shown recently that hHR23B could inhibit XPC ubiquitination, therefore protecting XPC from ubiquitin-triggered degradation [47].

Cen2, a small acidic Ca^{2+}-binding protein with molecular weight 18 kDa, is the third subunit of the recognizing complex. Cen2 is known to play a role in the cell cycle, in particular in basal body localization and separation of microtubules. This protein is not obligatory in the *in vitro* repair, though it increases the affinity of XPC–hHR23B dimer to the damaged DNA and stimulates damage excision in cell extracts [48].

XPC–hHR23B has higher affinity to specific damage such as thymidine dimers ((6-4)-photoproducts) and acetylaminofluorene DNA-adducts [32, 33, 49]. Purified XPC is able to bind to single- and double-stranded DNA and support damage excision in the absence of hHR23B, whereas the latter does not have DNA-binding activity [41, 44]. The key point in XPC–hHR23B binding to DNA is destabilization of DNA double helix triggered by the damage, but not the damage itself [50, 51]. XPC–hHR23B was shown to effectively interact with DNA incorporating short regions of non-paired bases, not depending on the presence of the damage [34]. Moreover, XPC more efficiently binds to single-stranded oligonucleotides than to double-stranded, which agrees with the presence of a motif similar to the structure of protein domains binding single-stranded DNA [52]. When this occurs, XPC more effectively binds to intact single-stranded oligonucleotides than to damaged ones. According to biochemical studies XPC–hHR23B interacts not with the damage but with the region of native strand opposite the lesion [51, 52]. This hypothesis was proved by X-ray analysis of the complex of yeast XPC ortholog (Rad4) with a fragment of damaged DNA [53].

A mechanism of two-stage damage recognition was proposed on the basis of experiments [54-56]. In the first stage XPC works as a sensor of double-stranded DNA fluctuations triggered by the damage but does not take part in direct recognition of bulky lesion [54-58]. It was shown earlier using scanning microscopy that XPC–hHR23B binding to DNA induces DNA bend [59]. The result of this process is the formation of a structure that may work as a signal for attracting other repair factors, for example, TFIIH. The interaction with TFIIH is performed by the C-terminal domain of XPC [60]. The second step, DNA scanning for the presence of modified nucleotide, is initiated after TFIIH binding. The scanning is performed on one strand of DNA duplex in $5'\rightarrow3'$ direction, evidencing the participation of XPD helicase as a part of TFIIH complex in this process. The strand being scanned is chosen by XPC binding [56].

XPA AND RPA PROTEINS AS BASIC STRUCTURAL ELEMENTS OF NER PREINCISION COMPLEX

XPA is a small (36 kDa) Zn-binding protein that is able to form a heterodimer in solution [61]. However, according to fluorescence microscopy data, after UV irradiation the protein migrates to the DNA lesion as a monomer [12]. XPA was shown to be able to interact with other repair factors: RPA [62], ERCC1 [63], and TFIIH [64]. RPA is a stable heterotrimer consisting of 70-, 32-, and 14-kDa subunits (RPA70, RPA32, and RPA14, correspondingly). The main function of RPA is the stabilization of single-stranded DNA. This is one of the most abundant human proteins binding single-stranded DNA. RPA is known to participate in all basic metabolic processes: replication, repair, and homologous recombination [24-26]. RPA in NER probably takes part in all major stages of the process: damage recognition, preincision complex assembly, and in subsequent repair synthesis.

Both proteins, XPA and RPA, have increased affinity to disruptions of DNA double strand such as non-canonical base pairs, “bubbles”, and small loops [65]. RPA supposedly participates in NER together with XPA. Indeed, the affinity of XPA–RPA complex to damaged DNA is higher than RPA affinity by more than an order of magnitude [66]. In turn, RPA stabilizes XPA binding to DNA [67]. However, the affinity of XPA–RPA complex to DNA damage is by an order of magnitude lower than

that of XPC–hHR23B [31, 68]. Originally these proteins were considered as candidate factors to accomplish primary damage recognition in DNA. However, they are not currently considered in this regard. XPA was shown to prefer artificially created DNA structures containing crossed DNA duplexes [65, 69] and have increased affinity to DNA structures containing transitions from single-to-double-stranded DNA including Y-structures [70] and DNA with 3′- or 5′-overhanging single-strand ends [38, 70]. RPA, in turn, detects and stabilizes DNA intermediates containing single-strand regions. Open DNA duplex, formed after DNA untwisting around the damage, contains both structural elements (DNA twists and single-strand regions) and thereby can be effectively recognized by RPA–XPA complex. In this respect RPA and XPA proteins do not participate in damage recognition but play a structural role in providing normal three-dimensional structure of DNA intermediate before the damage excision. It is known that XPA has direct contacts not only with RPA but also with ERCC1 protein [71], one of the subunits of ERCC1–XPF heterodimer—structure-specific endonuclease cleaving damaged DNA strand in the 5′-direction from the lesion. It was shown recently that these contacts are specifically important for NER process but not for the other repair pathways [72].

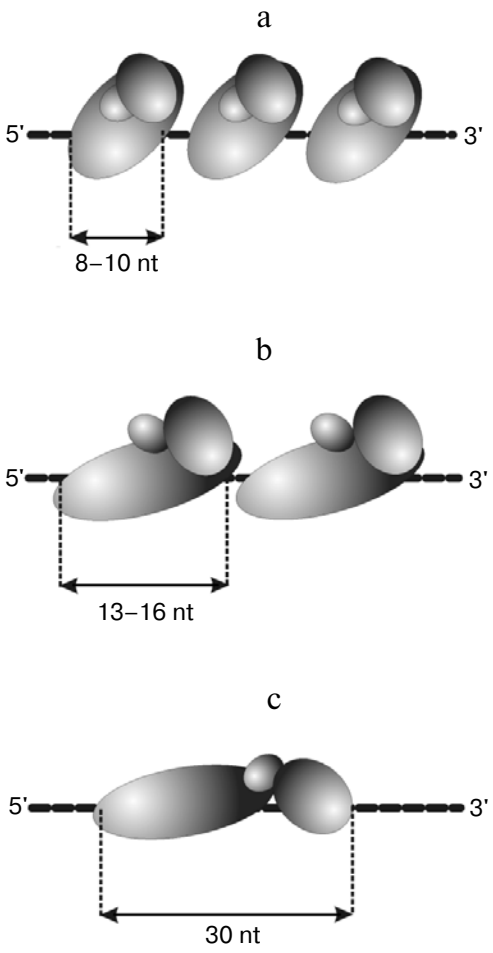


Fig. 1. Models of RPA binding to single-stranded DNA depending on the length of the bound region. a-c) RPA in globular, intermediate, and extended conformations, respectively.

RPA binding to single-stranded DNA may be exerted in different ways with the formation of complexes of different type depending on the length of the single-stranded DNA region (Fig. 1). The protein is in globular, transitional, or extended conformation [73, 74]. Note that in the latter case the size of the binding site approximately equals the size of a single-stranded region in open DNA duplex formed in the NER process. It is known that RPA binds to single-stranded DNA in a polar manner [75-77]. The polarity of RPA binding is expressed in subsequent binding of its DNA-binding domains (A, B, C, D) in 5′-3′-direction of single-stranded DNA. It was shown that the polarity of RPA binding determines the orientation of its subunits on single-stranded DNA. Subunit RPA70 is initially located on the 5′-end of the single-stranded DNA region and further locates its DNA-binding domains (A, B, and C) in the 3′-direction. The orientation of RPA32 subunit (D domain) close to the 3′-end is defined by the size of the single-stranded region that modulates RPA conformational changes [73, 77]. It was proposed that RPA polarity is important for the orientation on DNA and stimulation of repair endonucleases XPG and ERCC1–XPF, and RPA is probably bound to DNA in extended conformation [76]. However, later it was shown that a DNA region about 13 nt long opens as a result of TFIIH helicase activity, and further unwinding of the duplex, incorporating damage, occurs with the participation of RPA and XPA [78]. Therefore, we propose that RPA is incorporated in the preincision complex in transitional conformation corresponding to the size of partly opened region (~13 nt).

In open complex RPA preferentially interacts with intact strand protecting it from endonuclease attack [79]. In experiments on purified proteins binding to small DNA fragments it was shown that preferential RPA binding to native DNA is increased after XPA addition [80]. There is also data confirming that XPA inhibits RPA DNA-unwinding activity [65, 81]. After the damage excision RPA stays bound to DNA unlike the majority of other repair factors, probably stabilizing single-stranded gap and attracting replication factors RFC and PCNA [82] for the next NER stage – single-strand gap filling.

BULKY PHOTOREACTIVE dNMP ANALOGS IMITATE DAMAGES REMOVED BY THE NER SYSTEM

The NER process is characterized by multiple stages with the formation of unstable intermediate complexes enabled by protein–nucleic and protein–protein interac-

tions. For studying mechanisms of complex and dynamic processes including DNA replication and repair, new approaches revealing unstable interactions and defining the structure of intermediate complexes need to be developed. One such approach is the method of affinity modification using reactive DNA intermediates from different stages of the studied process [66, 83-85]. This method allows study of the architecture of DNA–protein intermediates originating during NER and discovering the role of specific proteins at the different stages of this process.

Wide NER substrate specificity suggested that bulky photoreactive arylazide groups incorporated in DNA may imitate damage being removed by NER. First of all it was shown that DNA incorporating such groups are recognized and processed by the bacterial NER system—UvrABC complex [83]. The mechanism of damaged DNA translocation from UvrA to UvrB preceding the excision of damaged region by UvrC protein was discovered using photoreactive DNA duplexes containing modified nucleotides and mutant UvrB forms. The data suggested that the damaged DNA is translocated from UvrA to UvrB in three stages: binding of damaged DNA region with UvrA and UvrB, UvrA being in direct contact with DNA; translocation of DNA to UvrB subunit mostly contacting intact DNA strand; locating of damage in UvrB recognition pocket accompanied by UvrA release. It was shown later that bulky photoreactive dNMP analogs imitate damage being removed by the eukaryotic NER system [86].

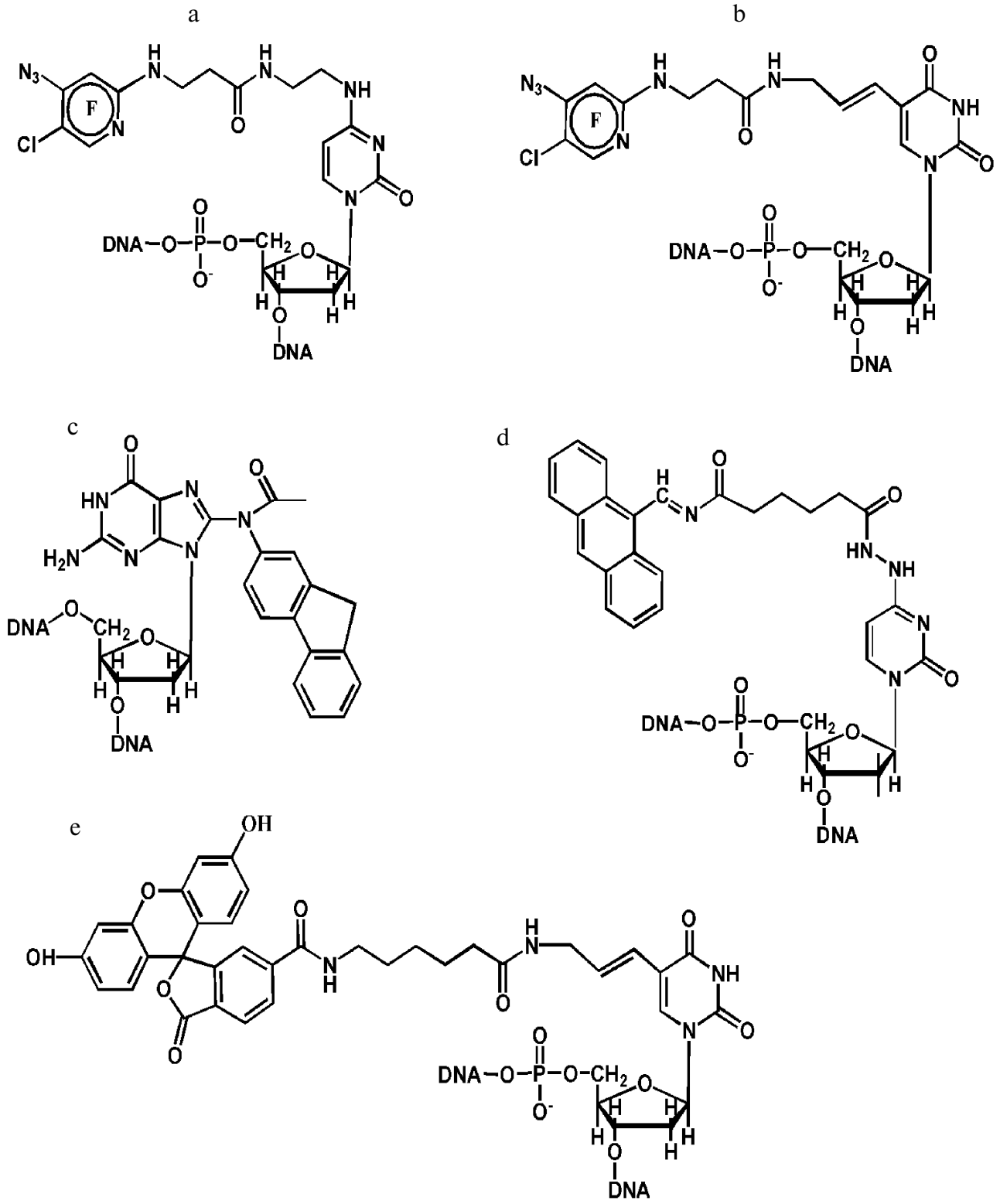
The substrate properties of two photoreactive nucleotide analogs, FAP-dCMP and FAP-dUMP, were studied (Scheme 2, a and b). Modified oligonucleotides containing experimental adducts were incorporated in circular double-stranded plasmids incubated in extract of HeLa cells containing the whole NER system. Acetylaminofluorene dG derivative (AAF-dG, Scheme 2c) was used as a standard lesion being repaired by NER [87]. Oligonucleotides incorporating FAP-dCMP, FAP-dUMP, or AAF-dG residues were annealed with single-stranded plasmid pBluescript II SK(+), and after total fill-in of the chain with subsequent ligation the derived double-stranded DNA was isolated [88]. After the incubation of DNA substrates in HeLa cell extract, oligonucleotides containing damage were detected using the method of polymerase fill-in in the presence of complement oligonucleotides and radioactively labeled $\alpha[^{32}P]$dNTP followed by electrophoretic separation [88, 89]. The detected excision products 24-32 nt long are common for the NER reaction. Therefore, both experimental analogs, FAP-dCMP and FAP-dUMP, are recognized and removed by the NER system, although less effectively than AAF-dG (Scheme 3).

The structural properties of DNA adducts probably play the key role in recognition and removal of the damage: the efficiency of repair of different damages varies by a few orders of magnitude and correlates with the disturbance introduced in the DNA-duplex structure by the particular damage [90-92]. The introduction of a bulky substituent at an exocyclic cytosine amino group (as in FAP-dCMP) prevents the formation of a canonic hydrogen bond and, therefore, leads to local destabilization of the duplex, unlike the substituents at the C5-position of cytosine or uracil (as in FAP-dUMP). On the basis of this assumption, we could expect that FAP-dCMP would be more effectively repaired than FAP-dUMP. However, the experiments revealed that DNA containing FAP-dUMP was repaired more effectively than DNA containing FAP-dCMP. The additional destabilization of the DNA duplex around the damage by the incorporation of few non-complementary nucleotide pairs led to increased efficiency of FAP-dCMP excision, but it had virtually no influence on the effectiveness of FAP-dUMP repair. It was shown previously that DNA containing small "bubbles" in the absence of damage is not processed by NER, although it is bound by XPC–hHR23B [51]. Therefore, FAP-dCMP and FAP-dUMP residues imitate damages processed by the NER complex.

XPA AND RPA INFLUENCE THE MODIFICATION OF XPC–hHR23B BY PHOTOREACTIVE DNA

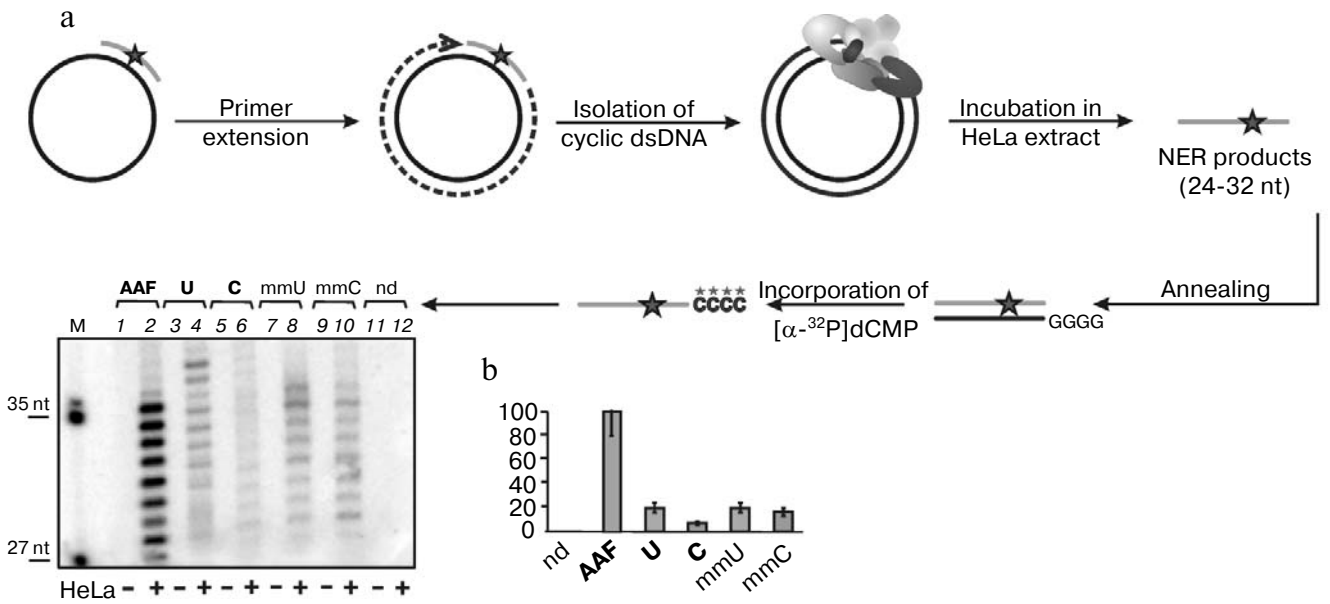
Photoreactive DNA containing FAP-dCMP or FAP-dUMP residues were used as model DNA structures to study the interaction of heterodimer XPC–hHR23B and other NER factors with damaged DNA [86]. Modified single-stranded oligonucleotides (ssC, ssU) were annealed with complementary oligonucleotides followed by the formation of intact DNA duplexes (dsC, dsU) or duplexes containing several non-complementary bases opposite modified nucleotide (mmC, mmU). After the incubation with XPC–hHR23B and subsequent UV-irradiation, the modification products were analyzed using electrophoresis. In all cases only XPC (p125), the large subunit of the complex, underwent modification. The efficiency of modification and protein DNA-binding activity were decreased in the presence of Mg^{2+}. The highest effectiveness of covalent cross-links formation was observed in case of single-stranded DNA. The structure of mmC demonstrated higher level of cross-links to XPC than dsC, whereas the yield of XPC modification products for mmU and dsU was almost the same. These results correlate with the effectiveness of repair of corresponding DNA structures in HeLa cell extract, conforming to the hypothesis that the rate of damage excision depends on the effectiveness of primary DNA damage recognition by XPC–hHR23B factor. Therefore, XPC interacts with bulky arylazide groups connected to the base by rather extended linkers. In this case the contact with the photoreactive group is possible even in the absence of direct XPC contact with the damaged nucleotide.

The damage recognition during NER is coordinated by multiple protein–protein and protein–nucleic acid



Nucleotide analogs imitating bulky damages removed by NER. a) Exo-N-{2-[N-(4-azido-2,5-difluoro-3-chloropyridin-6-yl)-3-aminopropionyl]-aminoethyl}-2′-deoxycytidine-5′-monophosphate (FAP-dCMP); b) 5-{N-[N-(4-azido-2,5-difluoro-3-chloropyridin-6-yl)-3-aminopropionyl]-trans-3-aminopropenyl-1}-2′-deoxyuridine-5′-monophosphate (FAP-dUMP); c) 8-[N-(2-acetylaminofluorenyl)]-2′-deoxyguanosine (AAF-dG); d) exo-N-[2-(4-anthracenyl)-ethyl]-2′-deoxycytidine-5′-monophosphate (Antr-dCMP); e) 5-{3-[6-(carboxyamido-fluoresceinyl)-amidocapromoyl]-allyl}-2′-deoxyuridine-5′-monophosphate (Flu-dUMP)

Scheme 2



Production of double-stranded plasmids containing dNMP photoreactive analogs and a set of damaged DNA fragments excised in NER system *in vitro* (a). Relative radioactivity of excision products normalized by dG-AAF (b)

Scheme 3

interactions. Experiments on XPC–hHR23B photoaffinity labeling in the presence of other NER proteins, XPA and RPA, showed that both factors influence to some extent the interaction of XPC with damaged DNA [40, 86]. Thus, XPA in the presence of Mg^{2+} stimulated XPC modification, whereas in the absence of Mg^{2+} no influence of XPA on XPC modification was observed. The influence of XPC and RPA was mutual and depended not only on the presence of Mg^{2+} but also on DNA structure. RPA on single-stranded DNA displaced XPC independently from Mg^{2+} as expected. However, despite low affinity to DNA duplexes, RPA stimulated XPC cross-links to such DNA, and in the absence of Mg^{2+} the effect was significantly higher—the effectiveness of XPC modification increased more than twofold. In the case of mmC DNA we observed mutual inhibiting of labeling of both proteins in the presence of Mg^{2+}, whereas without Mg^{2+} no mutual influence was observed. The ability of RPA to modulate the interaction of XPC with damaged DNA depending on its structure and the presence of Mg^{2+} may reflect different functions of RPA at the different NER stages, and these effects certainly need further investigation.

PHOTOREACTIVE DNA AS AN INSTRUMENT FOR STUDYING TOPOGRAPHY OF NER COMPLEXES

Photoinduced cross-links of NER proteins with photoreactive DNA structures imitating damaged DNA allow analysis of topography of DNA–protein complexes formed during NER. DNA duplexes containing photoreactive FAP-dCMP residue or 4-thiouridine (^{4}S-dUMP) in one strand and a bulky group (anthracene attached to dC, Antr-dCMP in Scheme 2d) in the opposite strand were used for the analysis of interaction of XPC–hHR23B, XPA, and RPA with DNA in case of damage in one or both strands of the DNA duplex [93]. For all experimental proteins the levels of photo cross-links to single-stranded DNA were higher than to DNA duplex with similar photoreactive group. In case of DNA containing ^{4}S-dUMP as a photoreagent, cross-links to DNA duplex were significantly weaker than to single-stranded DNA, whereas for FAP-dCMP-containing DNA the difference was insignificant. DNA duplex containing ^{4}S-dUMP in one strand probably imitates intact DNA as the thio-group causes minimal disruption in the structure of the DNA duplex. Indeed, the level of cross-links to the thio-group somewhat increased while adding the bulky substitute in the opposite strand of the DNA duplex, Antr-dCMP, and also non-complementary nucleotide opposite ^{4}S-dUMP. The anthracene residue causes distortion in the structure of DNA duplex, though in lesser degree than the FAP-group [94].

The yield of XPC modification products was drastically decreased when an anthracene residue was incorporated opposite FAP-dCMP, as both strands of DNA duplex contained bulky substitutes. Experiments on binding showed that the affinity of XPC–hHR23B to DNA was virtually unchanged. Therefore, the decrease in modification level is probably a consequence of geometric changes in DNA–protein complex during the introduc-

tion of bulky substituents in both strands of the DNA duplex. Analysis of the repair of DNA containing damage in both strands in the NER system of HeLa extracts conducted by Swiss researchers showed almost total inhibition of the repair process in regard to those DNA [51]. Later it was proposed by the same group that during binding to damaged DNA, XPC interacts with the region of the strand opposite the damage [52]. This assumption was directly confirmed by X-ray structural analysis of the complex of yeast XPC ortholog, Rad4, with a fragment of damaged DNA containing cyclobutane pyrimidine dimer as damage in the region of three non-paired nucleotides [53]. In the crystal structure Rad4 binds to DNA in two regions. The first contains N-terminal α/β-domain and BHD1-domain (beta-hairpin domain) and binds a region of double-stranded DNA 11 nt long. Two additional BHD-domains (BHD2/3) bind DNA around the damage. The BHD3-domain loop incorporates in the DNA helix through the major groove, displacing two nucleotides forming the damage. Rad4 does not proximately interact with the CPD residue, but it has direct contact with two nucleotides of intact strand located opposite the damage. This contact takes place in the protein cavity formed by the BHD2 and BHD3 domains.

It was assumed that Rad4 binds damaged DNA by the induced fit mechanism presupposing the scanning of DNA duplex for the presence of open regions where non-paired nucleotides may bind with BHD2/3 domains. This assumption is well confirmed with the model of multiple stage recognition in NER, according to which the damages that thermodynamically destabilize DNA duplex are the preferential NER substrates [91]. However, X-ray structural analysis of the Rad4–Rad23 complex with a DNA fragment shows only one, probably the most stable, configuration of this complex. However, the dynamic aspects of the mechanism of damage recognition by XPC–hHR23B complex in higher eukaryotes remain unclear and need alternative approaches for their study, including the method of photoaffinity labeling.

The use of photoreactive groups imitating bulky damage allows covalent fixing of polypeptides directly contacting this group. However, in the presence of rather extended linkers in these structures the proteins interacting with the opposite strand of the DNA duplex may also undergone modification. To define the contacts of NER factors with an intact DNA strand during the process of damage recognition, DNA structures containing 5-iodo-2′-deoxyuridine-5′-monophosphate (5I-dUMP) residues in various positions of the intact strand and dUMP fluorescein derivative (Flu-dUMP, Scheme 2e) as damage in the opposite strand were constructed [40]. Photoreactive groups with zero size linker, like 5I-dUMP, are supposed to react only with amino acid residues in direct contact with them [95]. The Flu-dUMP residue was used as damage, as it was shown that this analog is recognized and repaired in the NER system [96]. The non-complementary pair was incorporated in the position neighboring the damage to increase destabilization in the DNA double helix.

The evaluation of the affinity of NER factors to DNA structures according to the band-shift assay showed that none of the proteins—XPC–hHR23B, XPA, or RPA—reveal significant preference in binding damaged DNA, which is necessary for the discrimination of the damaged region in a huge massive of native DNA. These data agree with the assumption of cooperative interactions of several proteins at the stage of damage recognition. Indeed, on simultaneous addition of RPA and XPC–hHR23B, some increase in the affinity of the latter to different DNA structures was observed, more remarkable in case of damaged DNA [40], i.e. RPA stimulated XPC–hHR23B binding to damaged DNA in a greater degree than to intact. The simultaneous presence of XPA and XPC–hHR23B gave the effect of mutual stimulation in binding of these proteins to DNA. The interaction of XPC and XPA and of XPC and RPA during binding to UV-disrupted DNA was also shown using footprinting assay [97]. However, the mechanism of this interaction remains unclear.

RPA also stimulated modification of 5I-dUMP-containing DNA structures by XPC–hHR23B, and the maximal stimulation was observed for DNA duplex containing non-complementary nucleotide pair near the damage [40]. It was also shown that the stimulation effect involves protein–protein interactions: mutant RPA lacking domains responsible for protein–protein interactions did not affect XPC–hHR23B modification. On the addition of SSB protein from *Escherichia coli*, a prokaryotic analog of RPA, stimulation was not observed, evidencing the specificity of interaction between XPC–hHR23B and RPA. Notably, the dependence of XPC–hHR23B modification intensity on the position of a 5I-dUMP residue in the intact strand corresponded with the X-ray data for Rad4, i.e. the 5I-dUMP positions demonstrating maximum levels of XPC modification coincided with the places of Rad4 and DNA contact [53]. This correspondence probably allows accounting data on the mechanism of damage recognition obtained for the yeast protein in regard to the NER system of higher eukaryotes for whose proteins X-ray analysis is still missing.

As mentioned above, XPA and RPA proteins are important elements of the complex formed on the damaged DNA region at the stage preceding damage excision by endonucleases ERCC1–XPF and XPG, the so-called preincision complex [12, 36]. DNA structures containing non-complementary regions 15 nt long with bulky adduct (Flu-dUMP) in one strand may be considered as models imitating DNA intermediates formed in the primary stage of preincision complex assembly, after partial unwinding of DNA duplex in the damage region by TFIIH helicases [78]. The use of such structures containing 5I-dUMP in different DNA positions allowed studying XPA and RPA

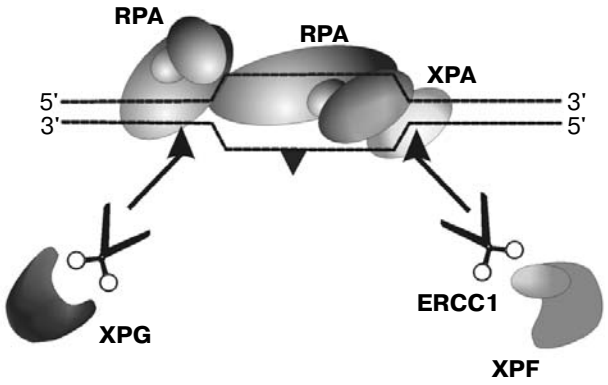


Fig. 2. Proposed model of preincision complex assembly with participation of XPA and RPA [98]. The specific orientation of XPA and RPA in partly open DNA duplex ensures the right seating of excision nucleases ERCC1–XPF and XPG.

localization against the damage in partly open DNA duplex. It was shown that XPA interacts basically with the transition of open region to the duplex part from the 5′-direction from the damage [98]. This localization of XPA corresponds to its function of attracting ERCC1–XPF heterodimer to the place of damaged strand incision at 5′-direction from the damage [72].

Therefore, the direct data on XPA localization on DNA at the stage of NER preincision complex assembly was obtained for the first time. Together with RPA, mostly interacting with non-damaged DNA strand, XPA participates in formation and maintaining of the normal structure of the DNA–protein complex necessary for precise seating and coordinate action of excision nucleases ERCC1–XPF and XPG (Fig. 2). The proposed model is based on the data on protein complex formation and fluorescence labeling and assumes the participation of two RPA molecules in the formation of the preincision complex [98]. It can be assumed that one molecule interacts with a single-stranded region of the intact strand in partially open DNA duplex and simultaneously with XPA, which is located on the transition of the open part into the duplex at the 5′-side from the damage, and the second RPA molecule is located near the opposite transition, at the 3′-side from the damage, and may interact with XPG. RPA might bind single-stranded DNA in this complex, being in transitional conformation [73]. The participation of more than one RPA molecule in preincision complex assembly is justified by the necessity to create contacts both with structurally different DNA regions in partly open duplex, and with other proteins attracted to the duplex. This assumption agrees with the domain structure of RPA and its conformation, and also with the "bubble" size. Moreover, the seating of several RPA molecules may be necessary for further unwinding of DNA duplex to the size of open region ~30 nt, preceding the excision of damaged DNA fragment [65, 99]. It should be noted that the right orientation of XPA and RPA on DNA is achieved in the absence of TFIIH, which is probably displaced from the complex with DNA by these proteins.

On the whole, the results of studying the interaction of NER proteins with model DNA structures imitating the substrates and intermediates of different stages of the process using fluorescent labeling demonstrate the descriptiveness of this approach in studying architecture and dynamics of DNA–protein complex formation, being sensitive to local structural changes in a DNA damage region.

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