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Title

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Journal

Lawrence Berkeley National Laboratory

Authors

Tsutakawa, Susan
Cooper, Priscilla K.

Publication Date

2001-03-02

Transcription-Coupled Repair of Oxidative DNA Damage in Human Cells: Mechanisms and Consequences

Susan Tsutakawa and Priscilla K. Cooper*

Life Sciences Division, Lawrence Berkeley National Laboratory,
University of California, Berkeley, CA 94720

Running Title: Transcription-Coupled Repair of Oxidative Damage

* To whom correspondence should be addressed:

Priscilla K. Cooper
Life Sciences Division, Mail Stop 74-157
Lawrence Berkeley National Laboratory
1 Cyclotron Road
Berkeley, CA 94720

Tel: (510) 486-7346
Fax: (510) 486-6816
Email: pkcooper@lbl.gov

Recent advances described at this 65th Cold Spring Harbor Symposium have dramatically illustrated how our understanding of DNA repair processes has progressed well beyond the original concept of a simple progression of enzymatic steps organized into discrete pathways to encompass the emerging view of these processes as ensembles of dancers in an intricately choreographed network of cellular responses to DNA damage. Thus, there is increasing evidence for connections between repair pathways, not only through overlap in the lesions upon which they act but surprisingly even in the identities of their protein participants, and in addition it is now clear that many if not all repair processes are intimately connected to other vital DNA transactions such as replication, transcription, and recombination. The process of transcription-coupled repair (TCR) is illustrative of this complexity, evidently directing at least two different damage removal mechanisms and requiring novel functions of key proteins from other repair pathways.

DNA damage not only poses a threat to the genetic integrity of cells but more immediately can result in lethality through obstruction of normal DNA transactions. In particular, since RNA polymerases can be blocked by a variety of DNA lesions, transcription is a key target for cell killing by DNA damage. Accordingly, cells have developed a system to rapidly and preferentially repair DNA damage on transcribed strands. Transcription-coupled repair (reviewed in Citterio et al. 2000b; Hanawalt and Spivak 1999; Hanawalt 1994; Leadon 1999; Thompson 1998; van Gool et al. 1997b) is an evolutionarily conserved process that is operationally defined by the more rapid removal of lesions in transcribed strands (TS) of active genes compared to non-transcribed strands (NTS). The rate of NTS repair in general reflects that in the genome overall (global genome repair). The original model for TCR (Mellon et al. 1987) postulated that blockage of RNA polymerase elongation by a lesion in the DNA template provides a signal for rapid recruitment of the repair machinery to the offending lesion. A corollary to this model is that only those lesions that block RNAP will be subject to TCR. Because it was originally shown to occur for UV-induced lesions and then for certain bulky chemical carcinogen adducts, TCR has been presumed to be a sub-pathway of nucleotide excision repair (NER), an idea that was reinforced by the observation that N-methyl purine damage in DNA from exposure to alkylating agents, which is repaired not by NER but by base excision repair (BER), is evidently not preferentially repaired (Scicchitano and Hanawalt 1990). However, studies over the last several years of the repair of

oxidative DNA damage have allowed the redefinition of TCR as a discrete pathway for initiating rapid removal of transcription-blocking lesions by either NER, BER, or perhaps other repair mechanisms. These developments are reviewed and analyzed here, with particular emphasis on the multiple roles of the human XPG protein in all three of these processes.

Lesion Removal by Global DNA Excision Repair.

In global DNA excision repair, bulky DNA adducts and base lesions throughout the genome are repaired by two distinct pathways, NER and BER respectively (for review of both excision repair pathways see Lindahl et al. 1997; Lindahl and Wood 1999). The human proteins known to be required for each are listed in Fig. 1.

Nucleotide Excision Repair (NER). Much of our understanding of NER in humans (recently reviewed in de Boer and Hoeijmakers 2000; de Laat et al. 1999; Thompson 1998) has come from study of the highly sun-sensitive disease xeroderma pigmentosum (XP), which is caused by genetic defects in eight genes, *XPA* through *XPG*. NER results in excision of helix-distorting lesions such as those induced by UV as oligonucleotides between 25-32 nucleotides in length. A heterodimer of XPC/hHR23 is the first step in lesion recognition, and both it and XPA are required for initiation of NER. The transcription initiation factor TFIIH, a nine-subunit complex that includes two helicases XPB and XPD, catalyzes open complex formation around the lesion. The ssDNA binding protein RPA together with XPA stabilizes the open complex formation and positions the two structure-specific endonucleases that excise the damage. The first of these, XPG, incises the NER bubble on the 3' side of the lesion and is also required non-enzymatically for the second incision on the 5' side of the lesion by ERCC1/XPF. The lesion-containing oligonucleotide is then released and a PCNA-dependent replicative polymerase, either Pol d or e, synthesizes a repair patch to fill the gap. The patch is then ligated to restore an intact strand.

Base Excision Repair (BER). As a distinct pathway, BER repairs base damage that, in contrast to lesions removed by NER, generally does not cause severe distortion of the helix. A large variety of damaged or inappropriate bases, including alterations resulting from spontaneous deamination, alkylation or oxidation from endogenous sources, replication errors, or radiation damage, are recognized by a battery of lesion-specific glycosylases (reviewed in Krokan et al. 1997). There are two classes of

these glycosylases, differentiated by their enzymatic activities. The first, monofunctional glycosylases, cleaves the glycosidic bond to release the damaged base, leaving an apurinic/apyrimidinic (AP) site. The second class, bifunctional glycosylases, has in addition a lyase activity that cleaves the phosphodiester backbone on the 3' side of the newly generated AP site to give a 5'-phosphate but an α,β -unsaturated aldehyde group on the 3'-end. Curiously, all glycosylases that remove oxidative DNA damage, both in bacteria and eukaryotic cells, are of the bifunctional type. AP endonuclease (APE) recognizes either of these glycosylase products and either cleaves on the 5' side of the AP site or excises the 3' α,β -unsaturated aldehyde, respectively. However, while the latter activity is robust in the *E. coli* enzyme, it is weak in human APE1 (Mitra et al. 1999; Ramana et al. 1998). For the subsequent step of BER, two pathways for repair synthesis that are differentiated by their patch size have been detected *in vitro*, although their roles *in vivo* remain to be clearly elucidated (Fortini et al. 1998; Frosina et al. 1996; Kim et al. 1998). Short patch repair requires Pol β and a DNA ligase, either DNA ligase I or XRCC1/DNA ligase III, to make a single nucleotide patch. Long patch repair (LP-BER), in contrast, replaces 2-10 nucleotides with DNA Pol δ or ϵ , PCNA, RFC, FEN1, and DNA ligase I. Available evidence suggests that the long patch pathway primarily operates when BER is initiated by a simple glycosylase such as those involved in repair of uracil or alkylation damage, followed by strand scission by APE. This incision event leaves a 3'-OH but a 5' terminus blocked by the resulting deoxyribosephosphate group that, if not removed by the lyase activity of pol β , results in strand displacement synthesis since ligation cannot occur (Klungland and Lindahl 1997; Matsumoto and Kim 1995). The resulting flap is cleaved by FEN-1, a structure-specific endonuclease the activity of which is stimulated by PCNA (Gary et al. 1999). Because this sequence of events would not be expected in repair of oxidative lesions for which strand scission is by the AP lyase mechanism, the short patch pathway is thought to be the primary one in this case, consistent with results from *in vitro* studies with cell extracts (Fortini et al. 1999). However, early passage cells from pol β knockout mice are hypersensitive to alkylating agents but not to IR or hydrogen peroxide (Miura et al. 2000; Sobol et al. 1996), and hence the mechanism of the resynthesis step in repairing oxidative damage in the cell thus remains an open question, as does the biological role of LP-BER.

Consistent with an essential role for BER in repair of endogenous damage in mammals, embryonic lethality in mice is caused by disruptions of the genes encoding several of the proteins required for steps in BER, including AP endonuclease, DNA pol β , and the XRCC1 protein that, as a complex with Ligase III, is important for rejoining of strand breaks (Fig. 1; reviewed by Wilson and Thompson 1997). There is a significantly milder phenotype for knockouts of particular glycosylases, *e.g.* AAG (Engelward et al. 1997), OGG1 (Klungland et al. 1999b; Minowa et al. 2000), and UNG (Nilsen et al. 2000). These observations could imply either a relative lack of toxicity of their respective substrates or, perhaps more likely, the presence of backup repair enzymes or processes not readily detected by assaying cell extracts.

Transcription-Coupled DNA Repair: Multiple Excision Pathways

Strand-specific repair of transcribed strands of active genes was initially discovered for repair of UV damage in mammalian cells. Such transcription-coupled NER (TC-NER) has been shown to occur in *E. coli* (Mellon and Hanawalt 1989), *S. cerevisiae* (Leadon and Lawrence 1992; Smerdon and Thoma 1990; Sweder and Hanawalt 1992), and rodent and human cells (Leadon and Lawrence 1991; Mellon et al. 1987). XPC is the only protein required for global NER that is not also required for TC-NER (Evans et al. 1993b; Nospikel et al. 1997; Venema et al. 1991; Venema et al. 1990). This finding has two implications. First, lesion removal in TCR of UV and other bulky lesions is apparently performed by NER enzymes. This conclusion agrees with the finding that the patch size of 30 nt for TC-NER is similar to the patch size of global NER (Bowman et al. 1997). Second, since XPC is required for recognition of the lesion in global NER (Sugasawa et al. 1998), identification of the lesion must occur by another means in TC-NER. Active on-going transcription by RNA pol II is required for TCR (Leadon and Lawrence 1991; Leadon and Lawrence 1992; Sweder and Hanawalt 1992), and it is postulated that it is stalling of RNAP II at a lesion that serves as the signal for initiating preferential repair by TC-NER. Transcription by RNA pol I and pol III apparently does not lead to TC-NER (Christians and Hanawalt 1993; Dammann and Pfeifer 1997; Vos and Wauthier 1991).

Transcription-Coupled Repair is a Process Distinct from NER. One of the first indications that TCR might also direct BER in addition to NER was the finding of more rapid repair of lesions produced by ionizing radiation (IR) in the TS compared to the NTS of an active gene in normal human cells, as

shown by a method that follows the insertion of repair patches (Fig. 2A; Leadon and Cooper 1993). Much of the DNA damage induced by IR results indirectly from attack by reactive oxygen species (ROS) produced by ionization of water molecules and hence resembles endogenous damage to DNA from ROS produced by the cellular metabolism. While a large variety of base damages and strand breaks with non-ligatable blocked 3'-ends result from ROS attack on DNA, most of these are repaired by BER. Direct examination of removal of a particular prominent, stable oxidatively damaged base, thymine glycol (Tg), by use of a monoclonal antibody against the lesion revealed that these lesions were repaired much faster in the TS than the NTS in both the yeast *S. cerevisiae* (Leadon and Lawrence 1992) and normal human cells (Fig. 2B; Cooper et al. 1997) after treatment with hydrogen peroxide. Tg is a lethal lesion that has been shown to block both prokaryotic DNA and RNA polymerases *in vitro* (Evans et al. 1993a; Hatahet et al. 1994), and its removal in human cells is initiated by an evolutionarily conserved glycosylase/AP lyase activity closely related to *E. coli* endonuclease III, or NTH (Aspinwall et al. 1997). Indeed, the observed TCR of IR damage in general (Leadon and Cooper 1993) and of Tg in particular (Cooper et al. 1997; Le Page et al. 2000b; Leadon et al. 1995) was found not to depend on NER. Thus, TCR of oxidative damage occurs at the same rapid rate in TS of normal human cells or those having either defective XPA or XPF, defects in the incision function of XPG, or defects in the NER function of XPD (Fig. 2A,B).

An oxidatively damaged form of guanine, 7,8-dihydro-8-oxoguanine (8-oxoG, or GO), is an important pre-mutagenic lesion due to its potential to mispair with A, thus generating GC to TA transversions, and its biological significance is underscored by the existence of an evolutionarily conserved three-tiered "GO system" to prevent its presence in the genome (reviewed in Boiteux and Radicella 1999). Its removal in human cells is initiated primarily by OGG1, a glycosylase/AP lyase that is functionally equivalent to the *E. coli* Fpg (MutM) protein (Radicella et al. 1997; Roldán-Arjona et al. 1997; Rosenquist et al. 1997). That this particular damaged base is also repaired by TCR was demonstrated using an SV40-based shuttle vector containing a single 8-oxoG in the 3'-UTR of the T antigen (TAg) gene. Transcription of the sequence containing the lesion was determined by presence or absence of the SV40 early promoter in the vector, which was transfected into various human cell strains and recovered after incubation for analysis of persistence of the lesion (Le Page et al. 2000b). The site-

specifically placed 8-oxoG was removed faster when in a transcribed sequence than when not transcribed in both normal human cells and cells defective in NER (Fig. 2C), providing further support for the concept of transcription-coupled BER (TC-BER). While this NER-independent transcription-coupled pathway has thus now been demonstrated for both Tg and 8-oxoG, it is evident from the extent of repair synthesis in transcribed strands in human cells after IR (Fig. 2A) that other oxidative lesions must also be subject to TC-BER, but their identities are not yet known.

The Link Between Transcription-Coupled Repair and Human Genetic Disease

Cockayne Syndrome. XP patients have inherited mutations affecting genes required for NER and are extremely sensitive to sunlight, have pronounced skin changes in sun-exposed areas, and are highly prone to skin cancer due to inability to carry out either global or transcription-coupled repair of UV-induced lesions in DNA. A distinctly different sun-sensitive hereditary disease, Cockayne syndrome (CS), is characterized by small size at birth followed by postnatal developmental failure involving a complex clinical picture that includes wasting, profound retardation, progressive and severe neurological deterioration during infancy and early childhood, and early death (Nance and Berry 1992). Unlike XP, the sensitivity of the skin to sunlight does not lead to a predisposition to skin cancer. CS most often arises from mutations in the *CSA* or *CSB* genes. Involvement of these genes in TCR (and in fact the first hint of the existence of a repair pathway preferential for transcribed DNA) was implied by the observation that, while cells from normal individuals recover from the inhibition of RNA synthesis that occurs after UV irradiation, CS-A or CS-B cells do not (Mayne and Lehmann 1982) although their global NER is normal. Both types of mutant cells were later shown to be defective in TC-NER (Leadon and Cooper 1993; van Hoffen et al. 1993), and it is now clear that both *CSA* and *CSB* proteins are essential for this process. However, early observations with CS cells showed them to be unusual in displaying cross-sensitivity to both UV and IR (Chan and Little 1981; Deschavanne et al. 1984; Deschavanne et al. 1981), and indeed TCR of IR-induced damage and of both Tg and 8-oxoG removal was found also to depend on *CSB* (Fig. 2; Cooper et al. 1997; Le Page et al. 2000b; Leadon and Cooper 1993). In contrast, although *CSA* is stringently required for TC-NER, CS-A cells are able to preferentially repair oxidative damage in TS, albeit at a reduced rate relative to cells from normal

individuals (Fig. 2A; Leadon and Cooper 1993). Apparently both CSA and CSB play indispensable roles in TC-NER but only the latter is strictly required for TC-BER (Table 1).

Cockayne Syndrome combined with Xeroderma Pigmentosum. In rare cases, CS is due to mutations in either the *XPB*, *XPD*, or *XPG* genes that encode proteins essential for NER and that usually result in XP when defective (Fig. 1; Table 1). Because XP-A patients completely lacking NER do not exhibit clinical features of CS, the involvement of these three genes in CS is *a priori* unlikely to be due to their roles in NER. For XP-G patients, there is a striking correlation between the nature of the mutation and the clinical symptoms. Mutations that confer severe infantile CS (class 3) are those that lead to extreme truncation of the protein, whereas conservative single amino acid substitutions or other changes that severely reduce or eliminate XPG enzymatic activity but allow the production of full-length protein result in XP only (class 1) or XP with limited or late onset CS-like symptoms (class 2) ((Nospikel et al. 1997) and unpublished data). Since class I *XPG* mutations eliminate NER by severely reducing the enzymatic activity of XPG but do not lead to CS symptoms, the endonucleolytic function of XPG must not be related to CS (Cooper et al. 1997). Similarly, most mutations in *XPD* that inactivate its helicase function, which is essential for unwinding around the lesion in NER, result in XP but not CS. Since both *XPB* and *XPD* are essential for transcription initiation as part of TFIIH, only conservative mutations in these genes are consistent with viability, and probably for this reason analysis of the *XPD* mutations in the very few XP-D/CS patients has not been particularly informative thus far (Thompson 1998). Nonetheless, for the set of distinct mutations in either *XPD* or *XPG*, the striking difference in severity of the clinical phenotype of XP vs. XP/CS is consistent with alterations in different functions of the encoded protein. An obvious potential candidate for the relevant non-NER function of both *XPB* and *XPD* is their requirement in transcription initiation as components of the basal transcription factor TFIIH, especially since *XPD* helicase activity is known not to be required for its role in initiation (Winkler et al. 2000). However, there is no known similar requirement for *XPG* in transcription, and in fact XP-G/CS individuals are among the most severely affected of all CS patients.

Defective Transcription-Coupled Repair of Oxidative Damage in CS. An alternative explanation for the CS-related, non-NER functions of *XPB*, *XPD*, and *XPG* has been provided by studies of TCR of oxidative lesions in cells from CS patients with mutations in these genes. Like CS-B cell lines, XP/CS

cells all lack preferential TS repair of damage induced by ionizing irradiation (detected as repair patches), of Tg induced in the genome by H₂O₂, or of a defined 8-oxoG in a transfected shuttle vector, whether the causative mutation is in *XPB*, *XPD*, or *XPG* (Table 1; Fig. 2; Cooper et al. 1997; Le Page et al. 2000b). This is in contrast to normal levels of TC-BER observed for the XP-only mutations of the same genes and for other XP mutations (XP-A and XP-F cells) and has highlighted a correlation between deficient TC-BER and disease phenotype. This correlation is strengthened by the observation that XP-G class 2 cells with an intermediate clinical phenotype also have intermediate capacity for TC-BER, with significantly reduced but not absent strand-specific repair of 8-oxoG, Tg, and ionizing radiation-induced DNA damage ((Le Page et al. 2000b) and unpublished observations). A notable difference in the repair properties of XP-G/CS (class 3) cells as compared to CS-B, XP-B/CS, or XP-D/CS cells is that the former not only cannot preferentially repair oxidative lesions in transcribed strands but actually repair lesions in both TS and NTS more slowly than lesions in NTS or in the genome overall in normal cells (compare lines b and c, Fig. 2A,B). It thus seemed likely that XPG facilitates global BER in addition to its evident requirement in TC-BER (Cooper et al. 1997; Le Page et al. 2000b), and in fact purified XPG protein has been shown to stimulate hNTH glycosylase activity on a Tg-containing substrate up to seven-fold in a reconstituted BER reaction (Bessho 1999; Klungland et al. 1999a). Consistent with our cellular repair studies, this function of XPG is also distinct from its incision activity in NER, since proteins with inactivating mutations of the catalytic site (Constantinou et al. 1999) retain this property. The decreased rate of removal of 8-oxoG in NTS in XP-G/CS cells suggests that XPG also similarly stimulates the OGG1 glycosylase (compare lines b and c1, Fig. 2C).

In view of the correlation between presence of clinical features of CS and the inability to carry out transcription-coupled repair of oxidative damage in every case examined to date, it seems likely that the relationship is a causal one, as is discussed further in the last section. In addition, the reduction in global BER of oxidative DNA damage in the absence of XPG in XP-G/CS cells, all of which have truncating mutations, may contribute to the additional clinical severity of XP-G/CS.

Proteins Required for TCR in Human Cells.

In addition to the previously known requirement for CSA and CSB to carry out TCR, studies of the repair of oxidative DNA damage in human cells have thus allowed the description of required

discrete functions in TCR of both XPG and TFIIH (through its component helicases XPB and XPD) that are separable by particular mutations from their roles in NER. At the risk of stating the obvious, there is also a requirement for active RNA polymerase II, which may not necessarily be only a passive participant in TCR once it has stalled. The mismatch repair (MMR) proteins hMSH2 and hMLH1 (Leadon and Avrutskaya 1997; Mellon and Champe 1996) and the two breast-cancer associated suppressor gene products, BRCA1 and BRCA2 (Gowen et al. 1998; Le Page et al. 2000c) have been directly implicated in TCR by virtue of loss of strand-specific preferential repair in mutants defective in them. Of these, only hMSH2 has been implicated in both TC-NER and TC-BER, while hMLH1 is involved only in the former, and BRCA1 and BRCA2 are required only for transcription-coupled repair of oxidative lesions. The role of BRCA2 in TC-BER is more fully discussed in the article in this volume by S.A. Leadon. While neither the precise mechanism of TCR nor the roles of any of these required proteins in it (summarized in Fig. 1) have been elucidated, it is useful to consider what is currently known about the participants in order to devise testable models for TCR.

CSA and CSB Proteins. CSA belongs to the WD-repeat family of proteins (Henning et al. 1995) whose members have regulatory rather than catalytic roles in many cellular functions including cell division, signal transduction, mRNA modification, and transcription. These repeats structurally form a beta-propeller, first observed in the G-protein transducin b subunit (Sondek et al. 1996). CSB contains a region of multiple ATPase/helicase motifs characteristic of the expanding and diverse SWI2/SNF2 protein family (Troelstra et al. 1992), various members of which are implicated in chromatin remodeling during transcription. It is a DNA-dependent ATPase (Citterio et al. 1998) but like other members of this family does not have DNA helicase activity. However, CSB has recently been shown to modulate the conformation of double-stranded DNA and to have ATP-dependent chromatin remodeling activity, and it binds directly to core histones (Citterio et al. 2000a). CSA and CSB antibodies microinjected into cells partially inhibit TCR after UV irradiation, with CSB antibodies having a significantly greater effect than those against CSA. The microinjected antibodies have no effect on transcription detectable by autoradiography (van Gool et al. 1997a), although a modest effect of CSB on transcript elongation *in vitro* has been observed (Selby and Sancar 1997a). From *in vitro* pulldown experiments, CSA was reported to associate with CSB and RNA polymerase II (Henning et al. 1995), but in cell free extracts

CSB did not detectably interact with CSA (van Gool et al. 1997a). In any case, it is clear from both *in vitro* and *in vivo* observations that CSB exists in a complex with RNA polymerase II (Selby and Sancar 1997b; Tantin et al. 1997; van Gool et al. 1997a).

TFIIH, an Essential Basal Transcription Initiation Factor and Repair Protein. The repair/transcription factor TFIIH is required both for promoter opening during transcription initiation by RNA polymerase II and for helix opening around lesions in NER, in addition to its separable role in TCR uncovered by studying repair of oxidative lesions. The nine subunit TFIIH complex (consisting of a six subunit core of XPB, XPD, p62, p52, p44, and p34 plus the CAK kinase subunit containing cdk7, cyclinH, and MAT1) contains two helicases, the 89 kDa XPB which has 3' to 5' activity and the 80 kDa XPD which has 5' to 3' activity. Both helicases are required in NER for formation of an open complex of approximately 25 nt asymmetrically surrounding the lesion (Evans et al. 1997a; Evans et al. 1997b). Only the XPB helicase is essential for promoter opening to initiate transcription, with the role of XPD in initiation evidently involving stabilization of the CAK kinase subunit of the TFIIH complex, which is required for phosphorylation of the carboxy terminal domain (CTD) of RNAP II, and possibly stabilization of the whole complex (Araujo et al. 2000; Tirode et al. 1999; Vermeulen et al. 2000; Winkler et al. 2000). Since NER-defective XP-D cells in which the helicase activity of XPD is inactivated are still competent for TC-BER, this function must not be required for TCR *per se* (Le Page et al. 2000b).

XPG, a Multi-Functional Repair Protein. XPG is a 134 kDa protein having endonuclease activity specific for single-strand/double-strand junctions in DNA in which the ssDNA is 5' to the junction (O'Donovan et al. 1994). This activity is essential for the required role of XPG in making the first incision in NER. However, XPG is also required in a non-enzymatic capacity for occurrence of the second, 5' incision by the ERCC1/XPF heterodimer, another structure-specific endonuclease of opposite polarity, and for full opening of the helix around the lesion by TFIIH (Constantinou et al. 1999; Evans et al. 1997b; Wakasugi et al. 1997). There are thus now four known functions of XPG: (1) its activity as a structure-specific endonuclease in NER; (2) a non-catalytic activity in NER; (3) its activity as a cofactor for global BER of oxidative lesions, attributable at least in part to its stimulation of the hNTH glycosylase-AP lyase; and (4) its requirement in TCR. Consistent with these multiple roles, XPG has

been reported to interact with a number of other proteins including TFIIH components (XPB, XPD, p62, p44), PCNA, RPA, RNA Pol II, CSB, and hNTH (Araujo et al. 2000; Bessho 1999; Gary et al. 1997; He et al. 1995; Iyer et al. 1996; Klungland et al. 1999a; Maldonado et al. 1996). Because XPG has roles in both NER and BER throughout the genome, it may serve a role in TCR that would be analogous to that of a bacterial transcription-repair coupling factor (Selby and Sancar 1991; Selby and Sancar 1993) in recruiting the appropriate repair machinery to lesions that have blocked the progress of transcription. In any case, its stimulatory role in global BER and its required role in TC-BER place it in a central position for understanding the mechanism of TC-BER.

Sequence comparison of XPG with its smaller paralog FEN1, a flap endonuclease involved in removing RNA primers from Okazaki fragments (Li et al. 1995; Lieber 1997), highlights significant regions of homology and equally significant differences (Fig. 3). Although the two proteins have similar structure-specific endonuclease activities, both the roles that XPG plays in oxidative damage repair and its functions in NER are unique and likely to involve interactions mediated by regions of the protein not found in FEN1. This notion is supported by the nature and location of TCR-inactivating mutations in XP-G/CS and class 2 XP-G patients ((Nospikel et al. 1997) and unpublished results) and by a preliminary identification of TFIIH-interacting regions of XPG (Iyer et al. 1996). A likely candidate for such interactions is the distinctive R-(for “recognition”) domain of XPG that separates the active site N and I domains (Fig. 3). It is about 600 residues long and larger than the entire FEN1, in which the corresponding region is only 18 residues. The other potential region of interest is the C-terminus of XPG, which is approximately 180 residues longer than that of FEN1. These domains are unique to XPG and are predicted to be involved in its protein-protein and protein-DNA interactions that are critical for NER, BER, and TCR.

Other Proteins Involved in TCR. A requirement for the human homolog of the bacterial MutS mismatch binding protein in TCR of both UV damage and oxidative lesions has been demonstrated by absence of TCR in cells defective in MSH2, a subunit of both the Mut Sa and MutSb heterodimers. These complexes initiate mismatch repair by binding to single base mismatches and loops, respectively, but have also been shown to bind to certain oxidative lesions (Duckett et al. 1996; Mu et al. 1997). Two-hybrid screens in yeast have detected association of MSH2 with a number of NER/TCR-related

proteins: RAD2 (XPG), RAD1 (XPF), RAD10 (ERCC1), RAD14 (XPA), RAD25 (XPB), and RAD3 (XPD) (Bertrand et al. 1998). Whether the role of the mismatch binding proteins in TCR involves their recognition and binding of the lesion, perhaps thus contributing to stalling of the RNA polymerase, is presently not known. Similarly, the function of the breast-cancer associated proteins BRCA1 and BRCA2 in TCR is not understood, but it is significant that they are required only for TCR of oxidative damage, since BRCA1 has been directly implicated in double-strand break repair (Moynahan et al. 1999) and both co-localize in the cell with Rad51 protein required for homologous recombination (Bhattacharyya et al. 2000; Chen and al. 1998; Scully et al. 1997). BRCA2 interacts directly with Rad51 (Chen et al. 1998; Katagiri et al. 1998), and BRCA1 probably co-localizes by virtue of its interaction with BRCA2 (Chen et al. 1999). In addition, BRCA1 associates with the hRad50-hMre11-p95 complex (Zhong et al. 1999) also involved in homologous recombination and responses to ionizing radiation and with the mismatch repair proteins MSH2, MSH6, and MLH (Wang et al. 2000). Whether these interactions imply a direct relationship between TCR and repair of double-strand breaks or alternatively reflect a common signaling pathway in response to oxidative damage remains to be determined.

The potential existence of a class of novel proteins not yet associated with any human disease but essential for both transcription and TCR is suggested by the recent identification of one such protein, XAB2 (Nakatsu et al. 2000), whose function is not yet understood. In addition, it is highly likely that TCR also involves other as-yet unidentified disease-related proteins, as indicated by the existence of a number of CS-like and UV-sensitive human syndromes in which the genetic defect remains unknown, for example UVs syndrome (Itoh et al. 1996; Ohta et al. 1999).

Does TCR of Oxidative Lesions Proceed by BER?

Because TCR of UV damage requires all the proteins needed for NER with the exception of XPC, it is clear that NER preferentially directed to sites of blocked transcription must be the means by which the photoproducts are removed. In contrast, our studies of oxidative lesions have revealed no defect in TCR in cells in which NER is completely non-functional, thus strongly suggesting that repair of these lesions in transcribed strands proceeds by BER just as it does in NTS. However, cells from OGG1 knockout mice that are completely defective in removal of 8-oxoG from non-transcribed sequences have only a modest reduction in the rate of repair in TS (Le Page et al. 2000a). This retention

of functional TCR probably explains the weak phenotype of the knockout mice themselves (Klungland et al. 1999b). The fact that there is reduced repair of TS in the absence of OGG1 establishes that indeed BER can be targeted preferentially to transcribed strands but begs the question of the mechanism for the remaining TCR of 8-oxoG. Since there is evidently no functional complement to OGG1 for global BER of 8-oxoG, one interpretation is that strand-specific repair of 8-oxoG does not proceed primarily by the BER pathway. There are two formal possibilities: (1) existence of alternate glycosylases for 8-oxoG that function primarily in TCR, are optimized for interaction with the TCR machinery, and hence are not detectable *in vitro*; or (2) repair pathways other than BER function in TCR of oxidative lesions.

With respect to the first possibility, there is at least one potential candidate for an alternate 8-oxoG glycosylase that may have specialized applications (Hazra et al. 1998), and the existence of two glycosylases for removal of 8-oxoG in *E. coli* (Hazra et al. 2000) lends support to the idea that mammalian cells could be expected to have more than one. The second possibility is not ruled out but seems unlikely for a number of reasons. If the putative alternative repair mechanism is NER, it must be completely redundant to BER, since no effect of its absence is detectable in cells that are competent for BER, whether the NER defect is due to mutations in *XPA*, *XPD* (without CS complications), *XPF*, or *XPG* (class 1). Given the requirement for MSH2 for TCR, mismatch repair could conceivably be directly involved in lesion removal. However, since TCR of oxidative lesions is not affected by defects in MLH1 that eliminate mismatch repair (Leadon and Avrutskaya 1997), this is unlikely to be the mechanism. Similarly, given the requirement for BRCA1 and BRCA2, a direct role for homologous recombination in TCR of oxidative damage could be proposed. This is even less plausible, however, for two reasons: (1) the assay for repair of Tg in the genome directly detects loss of the damaged base itself, and if the lesion were exchanged by recombination to allow transcription to proceed, it would still be present in the genome rather than removed; and (2) the assay for 8-oxoG involves use of a shuttle vector containing a single damaged base at a defined location, so that there is no undamaged homolog available for recombination.

Definitive assignment of BER as the operative repair mechanism in TCR of oxidative damage will require either an assay that detects release of damaged bases during TCR in the cell or construction of a cell line with known BER defects that result in loss of TCR. Meanwhile, it is a good working

assumption that preferential repair of oxidative lesions in transcribed strands does occur by TC-BER, especially since XPG, which is absolutely required for TCR of oxidative lesions, also has a clear role in global BER, and application of "Occam's razor" suggests that its involvement in both processes should involve related mechanisms. It is likely that TCR of oxidative lesions in the cell involves preferential recruitment of the global BER pathway to transcription-blocking lesions by the TCR machinery.

Mechanism of TCR: Many Remaining Questions and Some Answers

Although the process of transcription-coupled repair was identified some fifteen years ago, its mechanism is still largely unknown, and it is even likely that some of the players still remain to be identified. Progress in elucidating the mechanistic details of TCR has been hampered by the lack of an efficient *in vitro* system to study it, but current efforts in a number of laboratories to identify and characterize the relevant protein-protein interactions and the multi-protein complexes likely to be involved, coupled with new methods for visualizing events in living cells, hold considerable promise. These efforts will be aided by consideration of the conceptual framework resulting from the cellular studies of TCR that help to define the outlines of the mechanism.

Requirement for release of the stalled RNA polymerase. A striking feature of the loss of TCR for oxidative lesions in XP/CS or CS cells in the case of a single 8-oxoG in a shuttle vector is that not only is the lesion not preferentially repaired when it is in a transcribed sequence, but no removal at all is observed over the course of the experiment, even when the plasmid is resident in the host human cell for three days (shown in Fig. 2C, line c2 for early times; Le Page et al. 2000b). This is so despite the fact that the cells are obviously proficient for the relevant repair process *per se*, as evidenced by complete removal of the lesion by 12 hours when it is in a non-transcribed sequence in both normal and mutant cells. The complete loss of 8-oxoG repair of the transcribed strand in XP-G/CS and CS-B cells suggests not only the absence of TCR but more remarkably, the prevention of global BER access to the 8-oxoG lesion. This blockage of 8-oxoG repair was correlated with prevention of transcription through the lesion when RT-PCR of a sequence containing the 8-oxoG lesion was compared to that of an earlier region of the same gene in normal cells or in XP-G/CS cells. While both strains transcribed the upstream sequence efficiently, only normal cells transcribed the 8-oxoG-containing sequence. No transcript that spanned the lesion was detectable in XP-G/CS cells despite active transcription upstream

of the lesion (Le Page et al. 2000b). The simplest interpretation of these observations is that RNA polymerase is stalled at the lesion and that it remains there in cells defective in TCR, preventing access to the lesion even by global repair processes. Why the impediment to repair imposed by the stalled RNAP has not been detectable in most experiments in which damage is introduced into the genome itself (*e.g.*, compare line b in Fig. 2A,B to line c2 in 2C), is not entirely clear but presumably has to do with lack of sufficient resolution of affected lesions due to the relatively high levels of damage and the stochastic nature of its introduction. Importantly, however, the same conclusion can be drawn from measurements of repair of UV-induced lesions at nucleotide resolution in active chromosomal genes in yeast, in which an impediment to NER caused by blocked transcription was detected in the absence of Rad26, the yeast homolog of CSB (Tijsterman and Brouwer 1999).

These results have thus provided strong experimental support for the model that TCR has two functions: (1) recruitment of appropriate repair proteins to lesions that are blocking transcription, thus allowing them to be repaired more rapidly than lesions elsewhere in the genome, and (2) perhaps more importantly, removal of the stalled RNAP to allow repair to occur. A number of different scenarios can be envisioned for how the RNAP might be removed, including release and degradation of the stalled RNAP (Bregman et al. 1996; Ratner et al. 1998), release of the stalled polymerase accompanied by its dephosphorylation to regenerate the hypo-phosphorylated form required for transcription initiation, back-up of the RNAP by transcript shortening (Tornaletti et al. 1999), or even leap-frogging forward, presumably at the cost of an error in the nascent transcript. Recently reported experiments have demonstrated that initiation of transcription on an undamaged template by extracts from UV-irradiated cells is reduced by 1 hr post-irradiation, concurrent with loss of the hypo-phosphorylated form of RNAP II (IIa) and accumulation of the hyper-phosphorylated form (IIo) associated with elongation (Rockx et al. 2000). The ability to initiate transcription recovers along with reappearance of IIa in extracts prepared from normal cells at 6 hr post-UV but not in CS cells, in which the accumulation of IIo is even more pronounced. A scenario that is entirely consistent with these observations is transient blockage of IIo at lesions in irradiated normal cells, with release occurring by any of the listed mechanisms, but a permanent block in CS cells such that eventually all available RNA polymerase II is tied up as the IIo form in a “traffic jam” behind the unrepaired lesions.

A current model to explain the loss of TCR in CS cells invokes reduced transcription initiation after damage and hence less opportunity for transcription arrest at downstream lesions (Rockx et al. 2000; van Oosterwijk et al. 1996). The reduced initiation is attributed to a postulated direct, non-repair-associated role of the CS proteins in regeneration of the hypo-phosphorylated, initiation-competent form of RNA polymerase from the elongation-associated form. Aside from the fact that this hypothesis might predict significantly decreased transcription initiation in undamaged CS cells, which is not observed, the 8-oxoG results in the shuttle vector system indicate to the contrary that the defect in TCR is directly related to release of the blocked RNAP, since despite the presence of active upstream transcription no repair of the lesion on the transcribed strand can occur in CS cells. Thus the observed accumulation of hyper-phosphorylated RNAP II is most likely reflective of a requirement for the CS proteins for the release *per se*.

Sequence of events in TCR. As depicted in Fig. 4 (right), TCR is likely to proceed in a stepwise fashion. (1) RNA polymerase must be blocked at a lesion, either by the lesion itself, with or without some processing, or by a protein bound to the lesion. (2) The stalled RNA polymerase must be recognized by the TCR machinery. (3) The polymerase must either be shifted away from the lesion on the DNA or completely displaced. (4) The presence of a lesion and the type of lesion must be determined. (5) The appropriate repair pathway must then be recruited and the lesion must be repaired. (6) Transcription must resume, either by re-elongation of a displaced polymerase or by re-initiation from the promoter. These steps will be considered in order.

A clear requirement of the model is that lesions repaired by TCR must be lesions that block elongation by RNA polymerase II. While this requirement has been shown to be satisfied *in vitro* for cyclobutane pyrimidine dimers induced by UV for both prokaryotic and mammalian RNA polymerases, the situation is much less clear for oxidative lesions, and indeed many of them including 8-oxoG would not be expected to block transcription since they are readily copied by DNA polymerases (reviewed in Tornaletti and Hanawalt 1999). One possibility is that such lesions are converted into transcription blocks by binding of a protein, with mismatch binding proteins being a rather obvious possibility. Such a role could explain the requirement for MSH2 in TCR, particularly TC-BER. Interestingly, mouse ES cells deficient in MSH2 had increased survival after exposure to ionizing radiation due to a failure to

induce apoptosis (DeWeese et al. 1998). Since blockage of RNAP II is a potent signal for apoptosis, (Ljungman and Zhang 1996), this result would be consistent with failure to block transcription in the MSH2-deficient cells.

A number of lines of evidence suggest that CSB is involved in recognition of the stalled polymerase. An attractive model is that the known interaction of CSB with RNAP II targets it to sites of blocked transcription. Such a model is strongly supported by *in vitro* studies that further suggest that CSB can recruit TFIIH to the ternary complex with DNA and RNA (Tantin 1998; Tantin et al. 1997). Whether XPG is recruited along with TFIIH by virtue of their direct interaction or whether it too may participate early in the recognition step is not known. What is clear, however, is that at a minimum CSB, XPG and TFIIH are all required for release of the stalled RNA polymerase, since the lesion does not become accessible for repair in the absence of any of these (Le Page et al. 2000b). Examination of TCR at nucleotide resolution in both yeast and human cells has revealed that CSB is not required for repair of transcribed DNA near the transcription initiation site, prior to the point at which TFIIH dissociates from the elongating RNAP II. This result suggests that CSB (and CSA in the human cells) is dispensable in TCR if TFIIH is already present at the polymerase when it stalls (Tijsterman et al. 1997; Tu et al. 1997). It further implies that the stalled RNA polymerase is released by TFIIH, perhaps together with XPG, although the involvement of other as yet unknown proteins is not ruled out.

Release of the RNA polymerase exposes the lesion for repair, but since either a bulky lesion handled by NER or an oxidative lesion handled by BER may have been the initiating event, there must be some mechanism for lesion verification and determination of which repair machinery to recruit. Since the hallmark of TCR is that repair in TS is faster than repair elsewhere in the genome, this recruitment must be more efficient than in the global process, and/or the repair activities must be stimulated in the context of TCR. For lesions repaired by the NER pathway, recruitment may be relatively straightforward since TFIIH and XPG, which along with XPA and RPA are thought to perform lesion verification following damage recognition by XPC-HR23B in global NER (Sugasawa et al. 1998), are already present. Thus only XPA and RPA would need to be recruited in addition. For lesions repaired by the BER pathway (Fig. 4, right), it is likely that XPG is involved in recruitment of the glycosylase, since NTH catalytic activity and DNA binding are strongly stimulated by XPG *in vitro*

(Bessho 1999; Klungland et al. 1999a), and the decreased global BER of 8-oxoG in XP-G/CS cells suggests that XPG also stimulates OGG1 (Le Page et al. 2000a). Notably, APE1 is also a favorable candidate for recruitment to the lesion and stimulation by the TCR machinery, in particular XPG, since the 3'-phosphodiesterase activity of APE1 that is required for removal of the 3'-phosphomoiety left by AP lyase action or ionizing radiation-induced 3'-blocked ends is relatively weak and may be the rate-limiting step in BER of oxidative damage (Izumi et al. 2000; Mitra et al. 1997; Ramana et al. 1998). A potential role for XPG in stimulation of the glycosylase/AP lyase and the 3'-phosphodiesterase steps in global BER as well as in TC-BER of oxidative damage is depicted schematically in Fig. 5.

The Molecular Basis for Cockayne Syndrome

A considerable challenge to defining relationships between molecular defect and patient phenotype is that different mutations affecting either the XPG structure-specific endonuclease or the XPD helicase component of the basal transcription initiation factor TFIIH can result in very different clinical outcomes (XP or CS). Exactly what causes the severe clinical symptoms in CS patients is still controversial, with one prevalent theory centering on a subtle reduction in the capacity for basal transcription and another relating the symptoms to loss of ability to perform transcription-coupled repair of endogenous DNA damage, particularly oxidative lesions. Either theory should be able to account for the involvement of all five of the genes known to give rise to CS when defective, i.e. *CSA*, *CSB*, *XPB*, *XPD*, and *XPG*, and either should also account for the developmental progression of the disease.

The transcription hypothesis is based on the requirement for TFIIH in transcription initiation (Tirode et al. 1999) and is supported by the finding that purified TFIIH containing mutant XPB or XPD proteins similar to those found in XP/CS patients is less competent to initiate transcription *in vitro* (Coin et al. 1999; Hwang et al. 1996). According to this hypothesis, the repair defect in CS cells is secondary to a transcription deficiency, and the lack of TCR is attributed to relatively low levels of transcription. However, any effect of CS-B defects on transcription in the cell would have to be very subtle, since there is no discernible effect of injecting neutralizing antibodies into normal cells and since knockout mice are viable (van der Horst et al. 1997; van Gool et al. 1997a). Furthermore, there is no known role for XPG in transcription, and indeed upstream transcription of the TAg gene on a shuttle vector was quite active in XP-G/CS cells, indicating that there is no overall deficit in transcription in these cells (Le

Page et al. 2000b). Perhaps the most serious problem with the transcription hypothesis, however, is that CS is primarily a postnatal syndrome. Thus, although CS babies are relatively small at birth, the major developmental failure appears in infancy, childhood, or occasionally even later in less severe cases. This time course of the clinical symptoms is not consistent with the primary defect being in basal transcription.

An alternative theory that better fits with these facts is based on the demonstrated correlation of clinical CS with inability to carry out TCR of oxidative damage. The TCR defect is manifested by failure to release RNA polymerase stalled at endogenous lesions with a secondary inability to repair the lesions even by global BER, thus resulting over time in cumulative loss of RNA polymerase available for transcription initiation. One consequence would be eventual transcription insufficiency, but in addition the blocked transcription would provide a powerful signal for apoptosis, resulting in destruction of those cell types in which apoptosis occurs. Since the oxygen tension is presumably much higher after birth than *in utero*, the relatively normal prenatal development with profound and progressive postnatal developmental failure (as unrepairable oxidative lesions and stalled polymerases accumulate, and unscheduled apoptosis is initiated) is consistent with this explanation. The most metabolically active tissues and those most susceptible to apoptosis would thus be expected to be the most affected in CS patients, and this prediction does fit the clinical picture (Nance and Berry 1992). TCR machinery may also be involved in maintaining efficient transcription elongation on non-damaged DNA by the same mechanism employed for remedying stalling of RNA polymerase at lesions, whether derived from endogenous or environmental sources. Examples of situations in which this might occur are strong natural pause sites or regions of strong secondary structure in the nascent transcript (Yu et al. 2000). In this repair-based model developed here and elsewhere (van Gool et al. 1997b), the primary molecular defect in Cockayne syndrome is an inability to repair endogenous oxidative lesions that stall RNA polymerase, but this repair defect is ultimately manifest as a defect in transcription.

Conclusion

The process of transcription-coupled repair is being revealed as an intricately choreographed and dynamic network involving an unexpectedly large number of ensembles and being more intimately connected to the life of the cell than could have been envisioned when the process was discovered in the

laboratory of Dr. Philip Hanawalt at Stanford some 15 years ago. It now seems that the TCR machinery can direct either NER or BER to lesions that are particularly threatening because they are blocking progress of RNA polymerase, and in doing so it also calls mismatch repair and possibly homologous recombination proteins into play. Further insights into the mechanism by which this fascinating and essential process allows us to survive the perils of an oxygen world will almost certainly depend on approaches that allow understanding of the organization and dynamics of large multi-protein complexes.

Acknowledgments

This work was supported by grant CA63503 from the National Cancer Institute of the USPHS and by the U.S. Department of Energy, Office of Biological and Environmental Research through contract DE-AC03-76SF00098.

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Figure Legends

Figure 1. Proteins required for BER, TC-BER, TC-NER, and NER. Asterisks highlight proteins whose mutation or deletion leads to embryonic lethality in mice or to XP, CS, or XP/CS diseases in humans. In the case of TC-BER and TC-NER, it is assumed that the transcription-coupled repair event itself also requires the proteins listed for the BER and NER pathways, respectively. Involvement of the XPG, XPB, and XPD proteins in TC-NER in a role that is separate from their functions in NER is inferred from their demonstrated requirement in TC-BER, although it cannot be shown directly for repair of UV damage due to the absolute requirement for their NER functions.

Figure 2. TCR of oxidative lesions in XP, XP/CS, and CS cells. The rates of repair of oxidative lesions in transcribed strands (TS) and non-transcribed strands (NTS) were determined by measuring (A) increases in DNA repair patches in an active gene (metallothionein IA, *MTIA*) after X- or γ -irradiation; (B) decreases in thymine glycol (Tg) content in *MTIA* after treatment with H_2O_2 ; or (C) removal of 8-oxoG uniquely placed in the 3'-UTR of the gene encoding large T antigen (Tag) on a shuttle vector that was transfected into human cells. The curves are representative of data published in (Cooper et al. 1997; Le Page et al. 2000; and Leadon and Cooper 1993). In all cases the repair rate of oxidative lesions in the TS is indistinguishable for normal and for XP cells (line a), being much faster than that of the NTS in the same strains. Repair in the NTS is identical for all cell strains except for XP-G/CS, which is significantly reduced (panels A and B, line c; panel C, line c1). There is no preferential repair of lesions in the TS in CS-B, XP-B/CS, XP-D/CS, and XP-G/CS, in all of which the TS of *MTIA* is repaired at the same rate as the NTS (panels A and B, lines b and c). In contrast, preferential TS repair still occurs in CS-A cells, although at a reduced rate relative to normal (panel A only, line d). For the case of single 8-oxoG lesions on a shuttle vector (panel C), there was no detectable removal of the lesion from the TS in XP-G/CS and CS-B cells (line c2) despite the fact that global BER is still functional (lines b and c1).

Figure 3. Schematic of XPG and FEN1 showing conserved regions and the location of mutations and of identified protein-protein interaction regions in XPG. Mutations are divided into those that affect the incision activity and hence NER only and those that give rise to clinical CS features by disrupting other functions of XPG. Mutations in the conserved active site domains N and I either

eliminate (D77A and E791A) or greatly reduce (A792V) the endonuclease activity of XPG (Constantinou et al. 1999). The latter is a naturally occurring mutation in the class 1 XP-G patient XP125LO. Also shown are mutations found in three XP-G/CS patients, all of which would result in a truncated protein (Nospikel et al. 1997).

Figure 4. Pathways for Base Excision Repair and Transcription-coupled Base Excision Repair.

Global BER is the major pathway for repair of base lesions, and has been shown *in vitro* to result in insertion of either short (1 nt) or long (2-10 nt) patches, as described in the text. TCR of lesions is initiated with the stalling of RNA pol II at a lesion and requires at a minimum TFIIH, CSB, XPG, MutS, and BRCA1 and BRCA2 (not shown). The stalled RNA polymerase must be released from the lesion in order to make the lesion accessible to repair. BER enzymes are likely recruited to repair the lesion, allowing the resumption of transcription. Whether the original transcript can be re-elongated or whether transcription must be re-initiated from the promoter has not been established.

Figure 5. A schematic model for participation of XPG in global BER and TC-BER. In global BER, XPG assists in loading NTH and potentially APE1 onto the lesion and may also stimulate their enzymatic activities but does not form a stable ternary complex. In TC-BER, XPG is first itself recruited to the stalled RNA polymerase and may then from its position on the transcription bubble recruit the BER enzymes to the lesion, as well as stimulating their enzymatic activities.

Table I. Correlation between clinical phenotype and repair properties of cells from patients with repair deficiency diseases. Clinical features and the ability to perform global NER, TCR of IR damage measured by amount of repair patches, TCR of Tg, and global BER of Tg show defined grouping of mutations that show linkage between the disease phenotype and the type of DNA repair deficiency.

Category	Clinical Features	NER	TCR of IR Damage (BU-patches)	TCR of Thymine Glycol	GGR of Thymine Glycol
Normal	Normal	++++	++++	++++	++++
XP-G Class 1	Mild cutaneous	None	++++	++++	++++
XP-G Class 2	Severe cutaneous; Neurologic	None	++	++	++
XP-G/CS Class 3	Severe CS	None	None	None	+ / ++
XP-D	Severe XP	None (lesion removal)	++++	++++	++++
XP-D/CS	Severe CS	None (lesion removal)	None	None	++++
XP-B/CS	XP; Moderate CS	None	None	None	++++
CS-B	Severe CS	GGR +++++ TCR - none	None	None	++++
CS-A	Severe CS	GGR +++++ TCR-none	+++	N/D	N/D
XP-A	Severe XP (Late neurologic)	None	++++	++++	++++

Figure 1

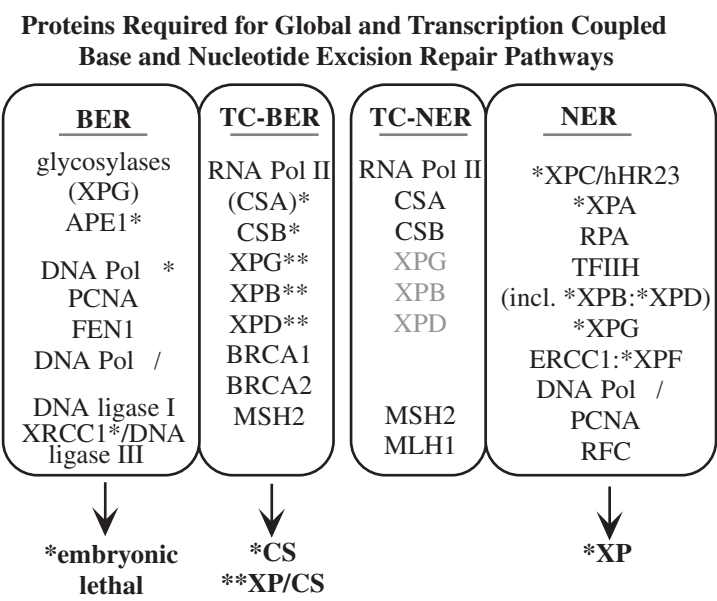


Figure 2

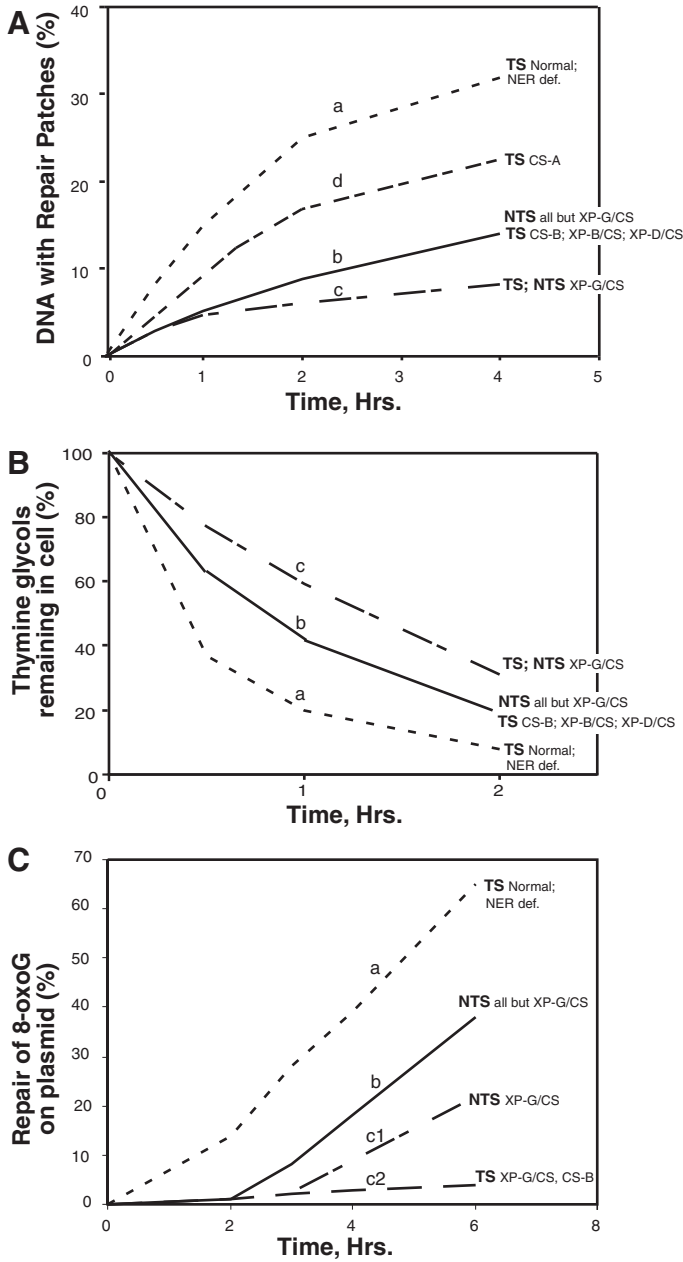


Figure 3

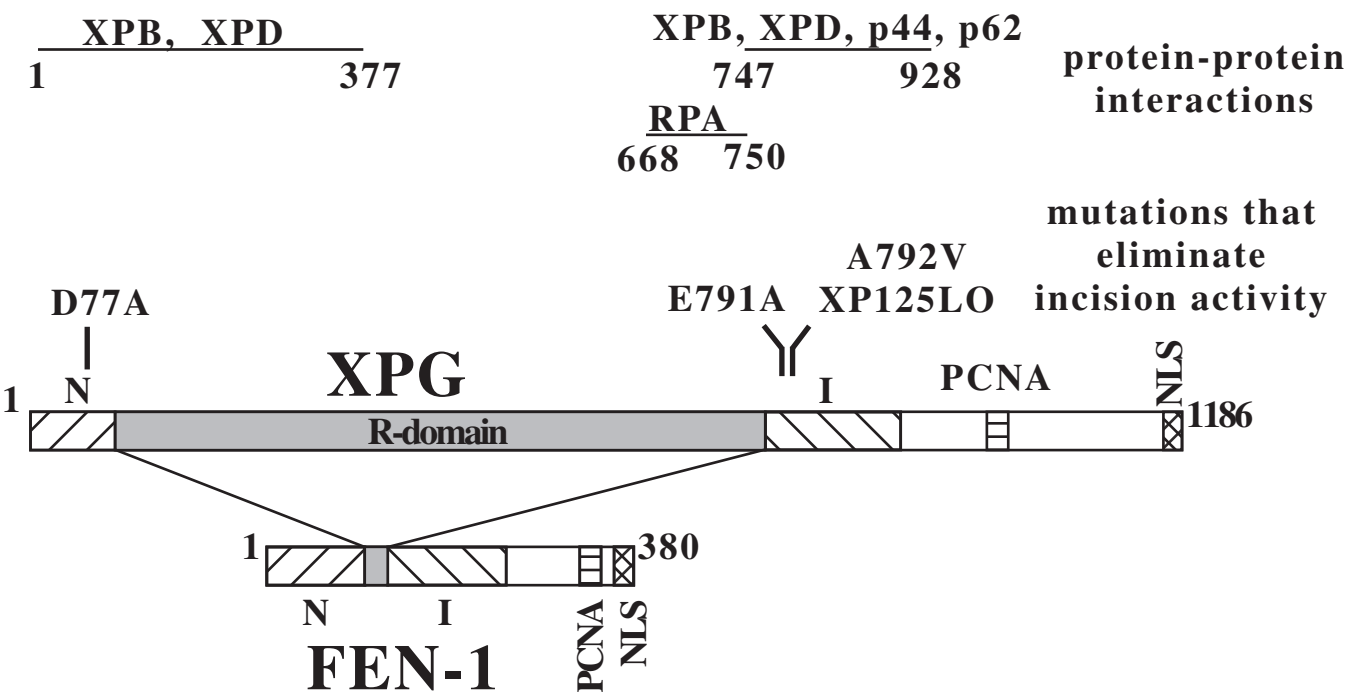


Figure 4

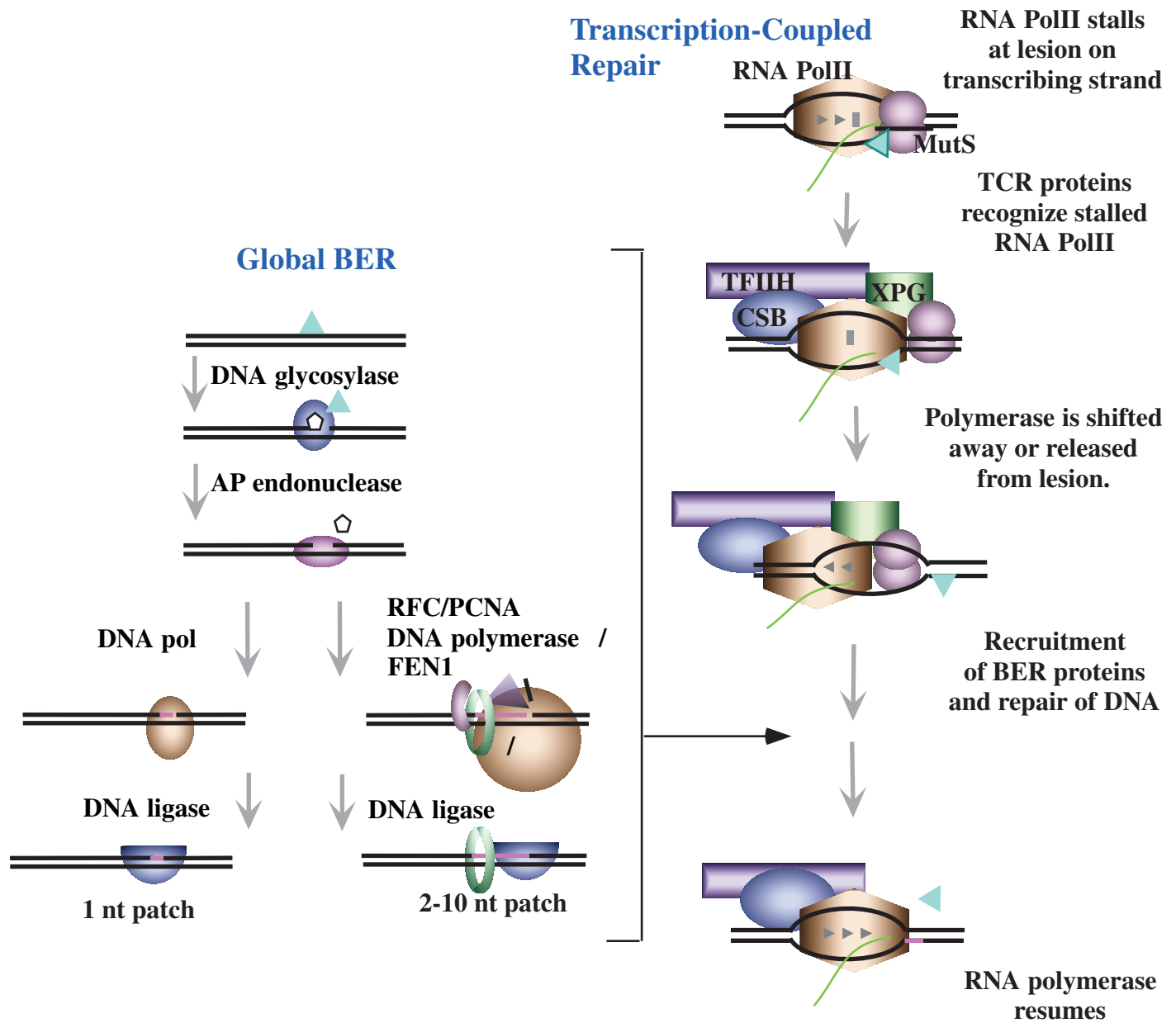


Figure 5

