Invited Review

Detection of the Excised, Damage-containing Oligonucleotide Products of Nucleotide Excision Repair in Human Cells†

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ABSTRACT

The human nucleotide excision repair system targets a wide variety of DNA adducts for removal from DNA, including photoproducts induced by UV wavelengths of sunlight. A key feature of nucleotide excision repair is its dual incision mechanism, which results in generation of a small, damage-containing oligonucleotide approximately 24 to 32 nt in length. Detection of these excised oligonucleotides using cell-free extracts and purified proteins with defined DNA substrates has provided a robust biochemical assay for excision repair activity in vitro. However, the relevance of a number of in vitro findings to excision repair in living cells in vivo has remained unresolved. Over the past few years, novel methods for detecting and isolating the excised oligonucleotide products of repair in vivo have therefore been developed. Here we provide a basic outline of a sensitive and versatile in vivo excision assay and discuss how the assay both confirms previous in vitro findings and offers a number of advantages over existing cell-based DNA repair assays. Thus, the in vivo excision assay offers a powerful tool for readily monitoring the repair of DNA lesions induced by a large number of environmental carcinogens and anticancer compounds.

INTRODUCTION

DNA is constantly under assault by endogenous and exogenous agents, and therefore the maintenance of genomic integrity is essential for the survival and function of living cells. A major environmental source of DNA damage is ultraviolet (UV) light from the sun, which induces the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts induced by UV wavelengths of sunlight. A key feature of nucleotide excision repair is its dual incision mechanism, which results in generation of a small, damage-containing oligonucleotide approximately 24 to 32 nt in length. Detection of these excised oligonucleotides using cell-free extracts and purified proteins with defined DNA substrates has provided a robust biochemical assay for excision repair activity in vitro. However, the relevance of a number of in vitro findings to excision repair in living cells in vivo has remained unresolved. Over the past few years, novel methods for detecting and isolating the excised oligonucleotide products of repair in vivo have therefore been developed. Here we provide a basic outline of a sensitive and versatile in vivo excision assay and discuss how the assay both confirms previous in vitro findings and offers a number of advantages over existing cell-based DNA repair assays. Thus, the in vivo excision assay offers a powerful tool for readily monitoring the repair of DNA lesions induced by a large number of environmental carcinogens and anticancer compounds.

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MECHANISM OF HUMAN NUCLEOTIDE EXCISION REPAIR

The basic mechanism of excision repair involves three basic steps: (1) damage recognition and the assembly of repair factors at the damage site; (2) a dual incision event at sites bracketing the lesion to release the damage in the form of a small DNA oligonucleotide; and (3) repair synthesis and ligation within the resulting gap. Unlike in the general or global genome repair pathway that utilizes RNA polymerase stalling at the lesion and the subsequent recruitment of the CSA and CSB (Cockayne syndrome A and B) proteins. Because of the lack of in vitro systems for reconstituting transcription-coupled repair, less is known about its molecular mechanism in comparison with the general repair system. Nonetheless, following damage recognition, the remaining steps of excision repair are thought to be identical in both repair pathways. The XBP and XPD helicase subunits of TFIH unwind the DNA to open the helix locally at the damage site and form a stable intermediate termed preincision complex 1 (PIC1). XPG is then recruited into the complex and XPC leaves...
the damaged DNA, resulting in the formation of preincision complex 2 (PIC2). Finally, XPF-ERCC1 is recruited to the complex, which leads to the formation of preincision complex 3 (PIC3). XPG makes an incision 3′ to the lesion and XPF-ERCC1 mediates the 5′ incision, which results in release of the lesion from DNA in the form of a small oligonucleotide. Nearly 25 years ago, in vitro biochemical studies using human cell-free extract and a UV-damaged DNA substrate first demonstrated that the excision process involves incisions at the 20th (±5) phosphodiester bond 5′ and the sixth (±3) phosphodiester bond 3′ to the DNA lesion, which release an oligonucleotide 24–32 nucleotides in length that contains the DNA lesion (10–12). The resulting gap is filled during repair synthesis by polymerase δε and replication proteins (RPA, RFC and PCNA), and the remaining nicks are sealed by DNA ligase (13,14). Comprehensive, detailed overviews of the molecular mechanism of nucleotide excision repair have been presented elsewhere (1–4). In this review, we therefore focus on assays for detecting the excised oligonucleotide products of repair, which provide a convenient way to monitor the biochemical activity of the nucleotide excision repair machinery.

IN VITRO SYSTEMS FOR DETECTING EXCISED OLIGONUCLEOTIDE REPAIR PRODUCTS

Numerous studies both in vitro and in vivo have provided us with our current understanding of the mechanism of nucleotide excision repair. Indeed, the entire concept of excision repair was based on seminal studies by Howard-Flanders and Setlow in which radiolabeled thymidine incorporated into genomic DNA of *E. coli* cells was found to be released (excised) from DNA into the acid-soluble fraction of cells following UV irradiation (15,16). However, it took nearly 20 years to identify the core genes and purify the proteins (UvrA, UvrB and UvrC) responsible for this phenomenon and to ultimately reconstitute the bacterial nucleotide excision repair system in vitro (17). This accomplishment demonstrated that UV photoproducts and other related bulky adducts are removed from DNA by a dual incision mechanism in the form of a 12 to 13 nucleotide-long oligonucleotide.

It took an even longer period of time for the human nucleotide excision repair system to be characterized, and this process
Table 1. The six core factors of human nucleotide excision repair and their functions.

| Factor | Subunit | Function |
|--------|---------|----------|
| XPA    | XPA/p31 | Damage recognition, binding to a damaged strand, facilitates repair complex assembly |
| RPA    | p70     | Damage recognition, binding to single-stranded DNA |
|        | p32     | Binding to single-stranded DNA |
|        | p11     | Binding to single-stranded DNA |
| XPC    | XPC/p106| Damage recognition, DNA binding |
|        | HR23B/p58| Molecular matchmaker binds to XPC and stimulates activity |
| TFIH   | XPB/ERCC3/p89| 3′-5′ DNA helicase, unwinding the duplex |
|        | XPD/ERCC2/p80| 5′-3′ DNA helicase, kinetic proofreading |
|        | p62     | TFIH subunit stimulates XPB |
|        | p52     | TFIH subunit |
|        | p44     | TFIH subunit stimulates XPD |
|        | p34     | DNA binding |
| XPG    | XPG/ERCC5/p135| DNA endonuclease for 5′-incision |
| XPF- | XPF/ERCC4/p112| DNA endonuclease for 5′-incision |
| ERCC1  | ERCC1/p33 | Structure-specific endonuclease |

DETECTION OF EXCISED OLIGONUCLEOTIDE REPAIR PRODUCTS IN VIVO

To address these issues, methods for detecting and isolating the UV photoproduct-containing oligonucleotide products of excision repair in human cells were recently developed (28,29). The approach is relatively straightforward and overwhelmingly confirms two decades of findings with in vitro approaches, including important observations regarding the length of the excised oligonucleotide (24–32 nt), the locations of the dual incision events relative to the damaged bases (17–22 phosphodiester bonds 5′ and 5–8 phosphodiester bonds 3′ to the lesion) and the release of excised oligonucleotides in a tight complex with TFIH. To detect the generation of excision repair products containing UV photoproducts in vivo, low molecular weight DNA is extracted from UV-irradiated cells and then purified. The DNA is then subjected to immunoprecipitation with antibodies specific for (6-4)PPs or CPDs. The oligonucleotides containing UV photoproducts are subsequently radiolabeled at the 3′-terminus with terminal transferase and a radionucleotide, electrophoresed on a urea-polyacrylamide sequencing gel and detected via phosphorimaging. Using this approach, small DNA oligomers in the range of ~18–32 nucleotides in length are observed following UV irradiation, and these DNAs comprise both the primary excision products approximately 30 nt in length and the secondary products that have undergone partial nucleolytic degradation.

The in vivo excision assay has also been applied to better understand the postexcision processing of the excised oligonucleotide products of repair, including both their localization in the nucleus and the role that gap filling DNA repair synthesis and ligation play in excision repair (30). Moreover, using oligonucleotide standards, the in vivo excision assay can be utilized in a quantitative manner to study excision repair activity.

involved the development of a variety of methods over the past few decades. In particular, the identification of excision repair genes and the establishment of in vitro excision repair systems using cell-free extracts and later purified repair factors led to characterization of the individual repair proteins and ultimately to a detailed elucidation of the molecular mechanism of nucleotide excision repair (7,8,18). The notion that UV photoproducts are removed from DNA in humans in the form of a small DNA oligonucleotide as in bacteria was first demonstrated using in vitro assays with human cell-free extracts and a plasmid substrate carrying thymine dimers near a radioisotope label. The results revealed that a series of DNA fragments consisting of 24 to 32 nucleotide oligomers were released from the DNA substrate (10). Human excision repair was then reconstituted in a highly defined system with the six purified repair factors (RPA, XPA, TFIH, XPC, XPF-ERCC1 and XPG) that were shown to be sufficient for the excision reaction in vitro (7,8). These in vitro excision repair systems with defined DNA substrates and cell-free extracts or purified proteins have subsequently been applied for measuring DNA repair activity toward a large variety of DNA adducts, including CPDs, (6-4)PPs, benzo[a]pyrene-guanine adducts, acetylaminofluorene-guanine lesions and cisplatin (GpG) diadducts (19–21). Moreover, the establishment of such a reliable in vitro repair assay has provided considerable insight into the biochemical steps of human excision repair. However, the relevance of these findings with in vitro systems to the mechanism of excision repair in vivo remained unclear. Similarly, the ultimate fate of the excised oligonucleotide products of excision repair had not been widely considered (22).

Recently, this latter issue was examined in greater detail through the use of the classical in vitro excision repair assay that employs an internally radiolabeled substrate containing a UV photoproduct and mammalian cell-free extracts and purified human excision repair factors (23). Interestingly, this work demonstrated that the excised oligonucleotide products of repair were found to be released from DNA in a tight complex with the repair factor TFIH. The excised oligonucleotides were found to ultimately be slowly released from TFIH and to associate with RPA, which is the major single-stranded DNA binding protein in eukaryotic cells, or become degraded in cell-free extract. However, the extent to which these in vitro findings regarding the excised oligonucleotide recapitulate what takes place in vivo was not clear owing to a lack of assays for detecting the excised oligonucleotide products of repair in living cells.

Furthermore, in vitro systems using purified repair proteins or cell-free extracts have primarily utilized naked DNA substrates and may therefore not accurately represent the repair of DNA damage that is embedded in nucleosomes and higher-order chromatin in cells in vivo. Previous studies had shown that nucleosomes can have inhibitory effects on various DNA transactions (24–26), and the Sancar laboratory indeed found that a DNA lesion in the nucleosome core is refractory to human excision repair by both the purified human excision proteins and mammalian cell extracts (27). Considering the lack of methodologies for monitoring excision repair and specifically the excised oligonucleotide products of the repair reaction in vivo, many questions remained regarding the mechanism of excision repair within chromatin and the status and fate of excised oligonucleotides in living cells.
and kinetics (29). The assay has also provided a mechanism for characterizing the process of nucleotide excision repair in previously unstudied organisms (31) and on novel potential substrates in genomic DNA in vivo (32). Furthermore, taking advantage of the association of excised oligonucleotides with TFIIH and next-generation sequencing, the development of the XR-Seq method has provided detailed maps of nucleotide excision repair events across the genome in UV-irradiated cells (33,34).

DEVELOPMENT OF A NONRADIOISOTOPIC IN VIVO EXCISION ASSAY

Although DNA lesion and TFIIH immunoprecipitation along with radioisotopic DNA labeling allow for the first detection of the excised oligonucleotide repair products of excision repair in UV-irradiated cells in vivo, the method involves several time-consuming steps and additional safety matters associated with the use of radioisotopes. We have therefore established a novel, nonradioisotopic approach that improves the applicability of the in vivo excision assay for studying UV photoproduct repair in living cells (29). The experimental procedure, which is outlined in Fig. 2, is similar to the radioisotopic approach in many regards, but instead utilizes biotin labeling of the excised oligonucleotides and detection with horseradish peroxidase-conjugated streptavidin and chemiluminescence. Furthermore, this novel methodology allows for the detection of the excised oligonucleotide products of repair in human cells without the need for immunoprecipitation with antibodies specific for repair factors or photoproducts that were used in previous studies in vitro and in vivo described above.

Following UV irradiation, cells can be lysed using the Hirt method (35) that is widely used for extraction of low molecular weight DNAs from mammalian cells. However, we found that other cell lysis buffers containing nonionic or ionic detergents can also be used for this approach, indicating that the excised oligomer products of excision repair are highly soluble and readily extractable from cells. For the nonradioisotopic DNA labeling, we use terminal transferase and the nucleotide analog biotin-11-dUTP to label the 3' end of the excised oligonucleotides (36). The labeled DNAs are then subjected to denaturing urea–polyacrylamide gel electrophoresis and transfer to a nylon membrane. Finally, the DNAs are visualized by HRP-conjugated streptavidin. This methodology readily visualizes DNA oligomers in the range of ~20–30 nt in length. Furthermore, the signals are...
DNA excision repair events that are induced by nonlethal UV irradiation are extremely high sensitivity of the methodology for detecting nucleotide excision repair of DNA oligomers. Excised oligomers are readily detectable within minutes after UV irradiation. The repair assay is capable of detecting nucleotide excision repair of a broad range of DNA lesions that are substrates for repair, including benzo[a]pyrene-7,8-dihydriodiol-9,10-epoxide (BPDE), N-acetoxy-2-acetylaminofluorene (AAF), formaldehyde, mitomycin C (MMC) and cisplatin. These results demonstrate that our in vivo excision assay has the potential to become a routine approach in studies examining cellular responses to DNA damage that include DNA repair, checkpoint and apoptosis using a single preparation of cells.

CONCLUSIONS AND PERSPECTIVES

The study of DNA damage and associated repair requires highly sensitive methods for detecting DNA repair events. The high sensitivity and versatility of the in vivo excision assay therefore offer researchers interested in a wide variety of DNA-damaged compounds a novel way to quantitatively measure repair with low, nontoxic doses of compounds and within short time frames of damage formation. Thus, we believe that the in vivo excision assay has the potential to become a routine approach in studies examining cellular responses to DNA damage that is targeted for repair by the nucleotide excision repair system in human cells.

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