Single Nucleotide Patch Base Excision Repair Is the Major Pathway for Removal of Thymine Glycol from DNA in Human Cell Extracts*

(Received for publication, December 2, 1999, and in revised form, January 19, 2000)

Grigory L. Dianov‡‡, Tanja Thybo‡†, Irina I. Dianova‡, Leonora J. Lipinski‡, and Vilhelm A. Bohr‡

From the ‡‡Laboratory of Molecular Genetics, NIA, National Institutes of Health, Baltimore, Maryland 21224 and the †Department of Molecular and Structural Biology, Aarhus University, Denmark

The repair pathways involved in the removal of thymine glycol (TG) from DNA by human cell extracts have been examined. Closed circular DNA constructs containing a single TG at a defined site were used as substrates to determine the patch size generated after in vitro repair by cell extracts. Restriction analysis of the repair incorporation in the vicinity of the lesion indicated that the majority of TG was repaired through the base excision repair (BER) pathways. Repair incorporation 5’ to the lesion, characteristic for the nucleotide excision repair pathway, was not found. More than 80% of the TG repair was accomplished by the single-nucleotide repair mechanism, and the remaining TGs were removed by the long patch BER pathway. We also analyzed the role of the xeroderma pigmentosum, complementation group G (XPG) protein in the excision step of BER. Cell extracts deficient in XPG protein had an average 25% reduction in TG incision. These data show that BER is the primary pathway for repair of TG in DNA and that XPG protein may be involved in repair of TG as an accessory factor.

Exposure to ionizing radiation or cellular metabolic processes result in the formation of hydroxyl radicals that can cause oxidative damage to DNA (1). The damage to the thymine residue is of special interest, because thymine is the most easily oxidized base (2). Among the products of thymine oxidation, thymine glycol (TG) has drawn the greatest interest because of its profound biological effect. An increased level of thymine glycol has been observed in DNA after treatment of cells with UV irradiation, γ irradiation, or chemical mutagens known to generate oxygen radicals (3). TG constitutes a strong block for DNA replication, and therefore it must be efficiently removed from DNA to maintain genome integrity (4, 5). The base excision repair (BER) and nucleotide excision repair (NER) pathways are the two excision repair systems that may contribute to processing of TG in DNA. The BER pathway operates by removing either 1 or 2–6 nucleotides 3′ to a damaged base (6). In contrast, NER incises DNA on each side of the lesion, and the damaged base is released as a part of a 25–30-base-long oligonucleotide (7). Previous studies in bacterial cells have suggested that BER is the major pathway for the removal of TG from DNA (8). In bacterial cells, TG is recognized and removed by endonuclease III (Nth protein) that subsequently cleaves the phosphodiester bond 3′ to the lesion (9). Endonuclease IV, DNA polymerase, and DNA ligase complete the DNA repair process by removing a 3′ deoxyribose, filling the created one nucleotide gap, and ligating the DNA ends. Recently, human thymine glycol-DNA glycosylase (hNTH) has been cloned and purified by several groups (10–12), thus providing enzymatic basis for the expected role of BER in removal of this lesion in mammalian cells. However, there is some evidence in both bacterial and mammalian cells suggesting the involvement of other DNA repair systems in the processing of TG in DNA. First, TG induces a significant, localized structural change to the DNA double helix that may be recognized by NER (13). Second, in vitro studies have shown that both bacterial and human NER enzymes recognize TG in DNA and excise it as a part of 25–30-base-long oligonucleotide (14–16). In addition, it was recently demonstrated that general genome repair and transcription-coupled repair of TG is reduced in xeroderma pigmentosum group G/Cs primary fibroblasts GM13370 and GM13371 were obtained from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). Cells were grown in the medium recommended by Coriell.

All oligonucleotides used were purchased from Midland Certified Reagent Company (Midland, TX). Oligonucleotides were gel purified on a 20% polyacrylamide gel, 5′-end 32P-labeled, and annealed to the complementary strand as described previously (30). To generate an oligonucleotide duplex containing a single TG lesion, 2 μg of 5′-end 32P-labeled oligonucleotide containing unique thymine residue (5′-pAAAGAGAAAATCAACAGGAGGG-3′) was incubated in 100 μl of 50 mM osmium tetroxide containing 2% pyridine for 30 min at room temperature. The oligonucleotide was purified from the reaction mixture by gel filtration on a 1-ml Sephadex G-25 (Amersham Pharmacia Biotech) column and subsequently annealed with the complementary strand.

Incision Assay—Whole cell extracts were prepared by the method of Manley et al. (18). Incision reactions (15 μl) contained 0.5 nmol of...
oligonucleotide duplex, 45 mM Hepes-KOH, pH 7.8, 70 mM KCl, 1 mM dithiothreitol, 2 mM EDTA, and the indicated amount of extract protein. After incubation at 37 °C for 2 h, the reactions were terminated by the addition of 15 μl of formamide dye (0.1% xylene cyanol, 0.1% bromphenol blue dissolved in 100% formamide), incubated for 5 min at 90 °C, and separated by electrophoresis on a 20% polyacrylamide gel containing 7 M urea, 89 mM Tris-Borate, pH 8.0, and 2 mM EDTA.

Construction of Closed Circular M13 DNA Containing a Single TG Residue—The synthetic DNA duplex corresponding to the sequence 5'-TCTCTTTGTACTTTCTCT-T3' was cloned into the Smal-XbaI site of M13mp18 DNA. The recombinant single-stranded DNA, named M13TG, was purified, and the insert orientation was verified by sequencing. To prepare the single TG-containing construct, the oligonucleotide 5'-pAGAGAGAAAGTACAAAAGAGAGGG-3' was treated with osmium tetroxide as a primer. The presence of TG was verified by sensitivity of single-lesion DNA to bacterial Endo III protein. The Endo III reaction was performed in a buffer containing 70 mM Heps, pH 7.5, 70 mM KCl, 2 mM EDTA, and 0.1 mg/ml of carrier single-stranded oligonucleotide for 3 h at 37 °C. DNA Repair Synthesis Assay—Whole cell extracts were prepared from 3–5 g of cells by the method of Manley et al. (18). Standard 50-μl repair reactions contained 50–200 ng of single TG-containing DNA, 45 mM Hepes-KOH, pH 7.8, 70 mM KCl, 7.5 mM MgCl2, 40 mM phosphocreatine (di-Tris salt, Sigma), 2.5 μg of creatine phosphokinase (type I, Sigma), 3.4% glycerol (Fluka), 20 μg of bovine serum albumin, 20 μM each of three deoxyribonucleotide triphosphates, 2 μCi of α-32P-labeled dTTP (3000 Ci/mmol; American Pharmacia Biotech) adjusted to 2 μM with the corresponding cold deoxyribonucleotide triphosphate, and 100 μg of a whole cell extract protein. Reactions were carried out at 37 °C for 3 h. After the reaction, DNA was purified from the reaction mixture by phenol-chloroform extraction and ethanol precipitation. Purified DNA was treated with 20 units of the indicated restriction endonuclease for 3 h at 37 °C in the appropriate buffer supplied by the manufacturer. Reactions were stopped by addition of equal volume of formamide dye. Samples were incubated for 5 min at 90 °C and separated by electrophoresis on a 20% polyacrylamide gel containing 7 M urea, 89 mM Tris-Borate, pH 8.0, and 2 mM EDTA. The amount of radioactivity was quantified using PhosphorImager analysis provided by Molecular Dynamics.

RESULTS

Generation of TG-containing Oligonucleotide by Thymine Oxidation with Osmium Tetroxide—Osmium tetroxide reacts primarily with thymines and reacts more slowly with cytosine and guanine (20, 21). Detection of the reaction products can be accomplished by piperidine cleavage of the phosphodiester bond adjacent to the modified base (2). We analyzed alkaline-sensitive sites generated in the single thymine containing oligonucleotide (Fig. 1A) treated with osmium tetroxide and found that, under our conditions, only one damaged site (that corresponds to the hydrolysis of phosphodiester bond next to thymine) was detected after a 30-min reaction (Fig. 1B). Furthermore, when the osmium tetroxide-treated oligonucleotide was annealed to the complementary oligonucleotide and incubated with human whole cell extract, there was only one major site of cleavage, corresponding to the modified thymine residue (Fig. 1C). These data indicate that TG is the major product of osmium tetroxide modification and that this damage is recognized and excised by human cell extracts.

The TG-containing oligonucleotide was used in the construction of M13 double-stranded DNA. The constructed closed circular 7280-bp DNA contained a unique TG lesion and harbored restriction sites suitable for the analysis of repair patch size (Figs. 2A and 3A). To confirm the presence of the lesion in the substrate DNA, we demonstrated that this DNA is sensitive to Endo III, the bacterial enzyme that recognizes and removes TG in DNA (22) and cleaves of the phosphodiester bonds adjacent to the lesion (1). The substrate DNA containing TG was first digested with restriction endonucleases EcoRI and HindIII. This cleavage generates two fragments: a 62-mer containing the lesion and a 7218-bp fragment representing the rest of the DNA. The fragments were then 3'-end labeled with Klenow fragment of Escherichia coli DNA polymerase I. Filling of the protruding 3' end of the 62-mer fragment in the presence of [α-32P]dATP and dTTP generated a 66-bp duplex DNA fragment labeled in the TG-containing strand. The labeled DNA was then treated with Endo IV (Fig. 2B, lane 2) or Endo III proteins (Fig. 2B, lane 3). Cleavage of the 66-mer at the site of TG would be expected to generate a 29-mer labeled fragment (Fig. 2A). The result of the described experiment is shown in the Fig. 2B. There are no Endo IV-sensitive sites in the 66-mer fragment, and the expected 29-mer product is observed only in the TG-containing fragment after treatment with Endo III. These data indicate that the M13TG DNA contains a single TG at the defined position and is not contaminated with abasic sites in this region.

Nucleotide Excision Repair Does Not Contribute Significantly to the Removal of TG by Human Cell Extracts—There are a number of reports suggesting that the NER pathway is
involved in the repair of TG in DNA (14–16). To address the relative contribution of BER and NER in the repair of TG in DNA, we determined repair patch characteristics on our specifically designed single TG-containing DNA substrate (Figs. 2A and 3A). During base excision repair the hNTH protein removes TG from DNA, and the apurinic/apyrimidinic lyase activity of the same protein or a separate apurinic/apyrimidinic endonuclease incises the phosphodiester bond next to the apurinic/apyrimidinic site. The subsequent DNA repair events result in the replacement of one or more nucleotides in the 15-mer Csp6I-Csp6I fragment located 3’ to the damage (Fig. 3A). In contrast, during NER, the replaced region includes 20–24 nucleotides 5’ and 5–9 nucleotides 3’ to the damaged site (7, 23). Thus, in addition to the 15-mer Csp6I-Csp6I fragment, the 18-mer SalI-G1 restriction endonuclease fragment of the TG-containing DNA should be labeled during in vitro NER (Fig. 3A). Both fragments contain equal amount of dAMP residues, thus dAMP incorporation into these fragments would reflect the relative contribution of NER and BER (long patch component) to the repair of TG in DNA by cell extracts.
Fig. 4. DNA glycosylase activity in normal and XP-G/CS cell extracts. A, 25 μg of protein of whole cell extract prepared from normal (AG9387) and XP-G/CS cells were incubated with 5'-end 32P-labeled TG-containing oligonucleotide duplex for 2 h at 37 °C. XP-G1, GM13370; XP-G2, GM13371. Reactions were terminated by phenol-chloroform extraction of proteins, and after addition of an equal volume of formamide dye solution the products were analyzed on a 20% polyacrylamide gel. B, PhosphorImager quantification of the results presented in A. WT, wild type.

After incubation with whole cell extract, substrate DNA was purified from the reaction mixture, cleaved with SallG1 and Csp6I restriction endonucleases, and analyzed by gel electrophoresis. We found that because of the slow rate of TG repair in whole cell extracts, a 3-h incubation period is needed to accumulate significant incorporation into the damage-containing fragment of the TG-containing DNA (data not shown). Analysis of the gel shown in Fig. 3B indicated that after 3 h of incubation with human cell extracts, some unspecific incorporation accumulates in both damaged and undamaged DNA constructs (the band at the very top of the gel). However, when normalized to the fragment length, the incorporation into the 15-mer Csp6I-Csp6I fragment, containing TG in substrate DNA, was about 10 times more than unspecific incorporation into the rest of the DNA. The incorporation of radioactive label into the 15-mer Csp6I-Csp6I fragment was highly DNA damage-specific, i.e. very little incorporation was seen in the corresponding fragment of the control DNA that was constructed by the same procedure as the TG-containing DNA, but contained a normal T:A base pair at the same position. The quantification of the data shown in the Fig. 3B (representative gel of three independent experiments) revealed that incorporation into the 18-mer SallG1-Csp6I fragment, characteristic for NER, was similar in the TG-containing and control substrate. Most radioactive incorporation had accumulated in the 15-mer Csp6I-Csp6I fragment, indicating that the repair gap extended 3' from the TG lesion. These results indicate that BER is the major pathway for repair of TG in DNA by human cell extracts.

Analysis of BER Pathways Involved in the Repair of TG in DNA—There are two major base excision repair pathways involving distinct sets of proteins and resulting in either a 1-nucleotide or a 2–6-nucleotide repair patch (24–26). By measuring an average repair patch size, it is possible to evaluate the relative involvement of different base excision repair pathways in the removal of DNA lesions. The TG-containing DNA was incubated with cell extract in the presence of all cofactors needed for DNA repair, including radioactive dTTP or dCTP. After incubation, the DNA was purified from the reaction mixture, cleaved with Csp6I restriction endonuclease, and analyzed by gel electrophoresis (Fig. 3C). dTMP can be incorporated into the 15-mer Csp6I-Csp6I fragment during single nucleotide patch repair as well as during incorporation of the first nucleotide in long patch repair events. In contrast, dCMP incorporation solely reflects incorporation generated during long patch repair, because the first cytosine is located 3 nucleotides 3' to the lesion (Fig. 3A). The PhosphorImager quantification of the incorporation into the 15-mer Csp6I-Csp6I restriction fragment revealed that dTMP was incorporated 5–7 times more efficiently than dCMP (Fig. 3C). These results indicate that single nucleotide patch BER is the primary pathway for repair of TG in DNA by human cell extracts.

Excision of TG in XP-G/CS Cell Extracts—A slow rate of TG repair has been observed in xeroderma pigmentosum group G/Cockayne syndrome cells, and it was suggested that the XPG protein plays a role in removal of this lesion (17). Recently, it was demonstrated that excision of TG from DNA by the hNTH protein was stimulated by purified XPG protein (27, 28). However, none of these studies explored whether a deficiency in TG incision actually existed in a human cell extracts derived from patients with XP-G/CS clinical features. We thus investigated this question using cell extracts derived from XP-G/CS cells deficient in XPG protein. As a substrate we have used a 30-bp oligonucleotide duplex containing a single TG at the 16th position and 5'-end 32P-labeled at the TG-containing strand. The incision of TG in the substrate oligonucleotide duplex in cell extracts generated a 15-mer labeled product (Fig. 4). In a series of independent experiments we found a 20–25% reduction in the excision of TG-containing oligonucleotide duplex in XP-G/CS extracts when compared with wild type cell extracts. A representative gel is shown in Fig. 4A, and quantification of the results is presented in Fig. 4B.

DISCUSSION

Although it has been broadly assumed that in mammalian cells TG in DNA is processed by BER, there has been no direct evidence to support this concept. Moreover, the ability of both bacterial and human NER enzymes to recognize and remove TG in DNA has been reported (14–16), suggesting the involvement of NER in processing of TG in DNA. We have now examined the relative roles of BER and NER in the repair of this lesion in human cell extracts. Because there is a general correlation between in vivo DNA repair and in vitro repair in cell extracts (29), our data likely reflect the repair of TG in DNA in living cells. The in vitro repair system we have employed in our
experiments was optimized for NER (29) but has been demonstrated to be very effective for BER as well (30, 31). Our data show that in human cell extracts, under conditions of free competition between NER and BER repair systems, BER pathways processed the majority of TG in DNA. As has been previously demonstrated for uracil (30) and 8-oxoguanine (19, 32), we find that about 80% of TG is processed by single nucleotide BER and that the rest is removed by long patch BER. This observation clearly demonstrates that the BER pathway is the first line of cellular defense against TG in DNA. Unlike uracil, TG generates significant structural changes in the double helix DNA structure and also unlike uracil, TG represents a block to the progression of DNA polymerases (4, 33) and also can block RNA polymerases (34, 35), presumably because TG induces a local structural change in DNA (13).

Our observation is unexpected and would suggest that, at least in this incidence and contrary to the general notion, the cellular choice of BER versus NER is not reflective of the DNA distortion or polymerase blockage of a given lesion. The observed repair activity of NER enzymes on TG-containing DNA may reflect a possible back up role of this system in the repair of oxidative DNA damage.

Under our experimental conditions we observed that removal of TG is much slower than repair of uracil or abasic sites in DNA. It is possible that more TG lesions can escape DNA repair prior to transcription or replication because of the slow repair rate. Because TG may constitute a strong block for both transcription and replication, the involvement of other repair systems, such as transcription-coupled repair or repair through homologous recombination, is more likely. Little is known about the molecular mechanism of transcription-coupled repair and its role in the repair of TG. It was shown that cells derived from XP-G patients that also exhibit clinical features characteristic for CS are defective in general genome and transcription-coupled repair of TG in DNA (17). It was further reported that these repair deficiencies correlate with the inability of the cell to produce full-length XPG protein (17, 36). Based on these data it was suggested that XPG protein has a role in repair of oxidative DNA damage distinctive from its role in NER (17, 36). Recently, two independent studies have shown that XPG protein stimulates the binding of human TG-DNA glycosylase (hNTH1) protein to TG-containing DNA and accelerates the excision of TG (27, 28). In this study we also addressed the role of hNTH1 protein in repair of TG in DNA. We have used extracts prepared from human cells containing a truncated XPG protein and find a 25% decrease in the TG excision activity in these extracts. These data indicate that the XPG protein is not an essential protein in the major pathway of BER but may have a stimulatory effect as has been previously suggested (27). However, if TG located in template DNA inhibits transcription as was recently proposed by T. Bessho (28), XPG protein may function in transcription-coupled repair by recruiting the hNTH1 protein and probably other BER proteins to the site of damage in a stalled transcription complex. Our repair system is transcription uncoupled, so we would not be able to see the effect of XPG protein on transcription-coupled repair. Unfortunately, a cell free system for transcription-coupled repair is not yet available, and the role of the XPG protein in transcription-coupled removal of TG in DNA cannot be studied in vitro at this time.

Acknowledgments—Robert M. Brosh and Simon G. Nyaga are thanked for critical reading of the manuscript.

REFERENCES

1. Demple, B., and Harrison, L. (1994) Annu. Rev. Biochem. 63, 915–948
2. Palecek, E. (1992) Methods Enzymol. 212, 139–155
3. Dizdaroglu, M. (1992) Mutat. Res. 275, 331–342
4. Klysik, J., Rippe, K., and Jovin, T. M. (1990) Biochemistry 29, 9831–9839
5. Dianov, G., and Lindahl, T. (1994) J. Biol. Chem. 269, 1069–1076
6. Klysik, J., and Lindahl, T. (1997) EMBO J. 16, 3341–3348
7. Dianov, G., and Lindahl, T. (1994) J. Biol. Chem. 269, 1069–1076
Single Nucleotide Patch Base Excision Repair Is the Major Pathway for Removal of Thymine Glycol from DNA in Human Cell Extracts
Grigory L. Dianov, Tanja Thybo, Irina I. Dianova, Leonora J. Lipinski and Vilhelm A. Bohr

*J. Biol. Chem. 2000, 275:11809-11813.*

*doi: 10.1074/jbc.275.16.11809*

Access the most updated version of this article at [http://www.jbc.org/content/275/16/11809](http://www.jbc.org/content/275/16/11809)

**Alerts:**
- When this article is cited
- When a correction for this article is posted

**Click here** to choose from all of JBC's e-mail alerts

This article cites 36 references, 13 of which can be accessed free at [http://www.jbc.org/content/275/16/11809.full.html#ref-list-1](http://www.jbc.org/content/275/16/11809.full.html#ref-list-1)