Fidelity of replication of the leading and the lagging DNA strands opposite N-methyl-N-nitrosourea-induced DNA damage in human cells

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ABSTRACT

Semi-conservative replication of double-stranded DNA in eukaryotic cells is an asymmetric process involving leading and lagging strand synthesis and different DNA polymerases. We report a study to analyze the effect of these asymmetries when the replication machinery encounters alkylation-induced DNA adducts. The model system is an EBV-derived shuttle vector which replicates in synchrony with the host human cells and carries as marker gene the bacterial gpt gene. A preferential distribution of N-methyl-N-nitrosourea (MNU)-induced mutations in the non transcribed DNA strand of the shuttle vector pF1-EBV was previously reported. The hypermutated strand was the leading strand. To test whether the different fidelity of DNA polymerases synthesizing the leading and the lagging strands might contribute to MNU-induced mutation distribution the mutagenesis study was repeated on the shuttle vector pTF-EBV which contains the gpt gene in the inverted orientation. We show that the base substitution error rates on an alkylated substrate are similar for the replication of the leading and lagging strands. Moreover, we present evidence that the fidelity of replication opposite O-6-methylguanine adducts of both the leading and lagging strands is not affected by the 3' flanking base. The preferential targeting of mutations after replication of alkylated DNA is mainly driven by the base at the 5' side of the G residues.

INTRODUCTION

The accuracy of DNA replication of the human genome is guaranteed by the concerted action of multiple proteins (for a review see 1). However, DNA replication is an asymmetric process with a leading and a lagging DNA strand (2) as well as different DNA polymerases that replicate the two strands (3, 4, 5). This provides a considerable opportunity for non-random error rates. An interesting issue to be resolved concerns the biological consequences of these asymmetries. Asymmetric error rates for the two strands have been invoked to explain differences in substitution rates in sequences of the β-globin complexes of six species of primates (6) and asymmetries in nucleotide composition of the two strands of several mammalian DNA sequences (7). On the other hand, there is now evidence that during SV40 origin-dependent DNA replication in HeLa cell extracts the base-substitution error rates of the leading and lagging strands are similar, at least at one template position (8). These results suggest that proofreading operates at comparable rates during replication of both DNA strands. An additional question to be answered concerns the effects of these asymmetries when the replication machinery encounters DNA adducts. Strand-specific formation of mutations has been reported following exposure of mammalian cells to UV light (9, 10, 11). To explain this phenomenon a model has been proposed which hypothesizes that the synthesis of the leading strand opposite UV-damaged template is more error-prone than the synthesis of the lagging DNA strand (9, 12).

In a previous study we have reported that N-methyl-N-nitrosourea (MNU)-induced mutations are preferentially located in the non transcribed DNA strand of the gpt gene carried by the EBV-derived shuttle vector pF1 EBV, stably replicating in human cells (13). The EBV-derived shuttle vectors are supercoiled plasmids, nucleosome associated and the cellular DNA polymerases replicate these genomes in synchrony with host cell DNA (14, 15). The direction of replication from the viral origin of replication, oriP, has been established (16) allowing the identification of the leading and lagging DNA strands. Therefore they may be a good model to study the processing of DNA damage by the replication machinery of eukaryotic cells.

In this paper we tested whether the different fidelity of DNA polymerases synthesizing the leading and the lagging DNA strand might contribute to MNU-induced mutation distribution. We constructed a shuttle vector, pTF-EBV, in which the direction of replication of the marker gene was inverted as compared to pF1-EBV plasmid. The analysis of the mutational spectra revealed that both the frequency of mutation and their sequence-context are unaffected by the direction of replication.

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MATERIALS AND METHODS

Construction of the EBV-derived shuttle vectors

The shuttle vector used in this study, pTF-EBV, is a derivative of plasmid pFl-EBV (17). To obtain pTF-EBV, the pFl-EBV plasmid was digested with BamHI, filled in with Klenow fragment enzyme and subsequently digested with HindIII. The HindIII- blunt end fragment containing the bacterial gpt gene under the control of the mouse metallothionein I (MT-I) promoter was inserted into the polylinker of pBlueScript KS+ (Fig. 2). This construct was digested with BamHI and XhoI to release the gpt transcriptional cassette which was then ligated with the pFl-EBV BamHI-Sall fragment containing the oriP and EBNA sequences of the Epstein-Barr virus (EBV), the ampicillin resistance gene and the origin of replication of pBR322, and the hygromycin (hyg) resistance gene. As a result of these subcloning steps the EBV-derived shuttle vector pTF-EBV (12.6 kb) contained the bacterial gpt gene downstream of the inducible MT-I promoter oriented in the same direction as the EBNA and oriP sequences (Fig. 2).

In pFl-EBV shuttle vector the non transcribed strand of the gpt gene is the leading strand; in the new construct, pTF-EBV, the non transcribed strand of the gpt gene is the lagging strand.

Cell culture and DNA transfection

Human embryonic kidney 293 cells (18) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% foetal calf serum (Flow Laboratories), penicillin and streptomycin in a 10% CO2 incubator. The plasmid pTF-EBV was introduced into the cells by the calcium phosphate coprecipitation technique (19) and the precipitate was left on the cells overnight. Three days after transfection, hygromycin B (200 µg/ml, Sigma) was added to the growth medium. Resistant cell colonies were detected one week after the addition of the selective drug. The cells were collected by cloning rings and propagated into mass culture. Cell lines containing the EBV-derived shuttle vector were maintained in medium containing 200 µg/ml of hygromycin B. One clonal cell line, clone 7, harbouring pTF-EBV was selected for mutagenesis studies.

Cell treatment and analysis of mutant plasmid DNA

N-methyl-N-nitrosourea (MNU) (Sigma) was dissolved in DMEM medium without serum at pH 6 shortly before use and quickly diluted in growth medium at the required concentration. Clone 7 cells, at approximately 30% confluence, were exposed to the mutagen that, being a short-lived chemical, was left in the growth medium. The cells were cultured until they reached confluence. The plasmid DNA was isolated from clone 7 cells by an alkaline extraction method (20). The plasmid containing extract was incubated with RNaseA (Sigma) followed by sequential phenol-chloroform extractions and by precipitation with ethanol. Plasmid DNA was further purified by drop dialysis and then transformed into the E. coli DT2 strain (gpt+, pur+) by electroporation (21) (Bio-Rad electroporation system, Bio-Rad Lab., Richmond, CA). Transformants were plated on minimal salt plates supplemented with ampicillin (50 µg/ml) and 6-thioguanine (6-TG, 54 µM final concentration, Sigma) (MATG) to select for colonies containing mutated (gpt−) plasmid DNA as previously described (17). A small portion of mutant plasmid DNA was amplified by Taq polymerase (Cerus) with a 5' biotinylated primer. Sequencing reactions applying Sanger's dideoxy chain termination procedure (22) were performed on single-stranded DNA bound to magnetic beads (Dyanabeads M280 Streptavidin).

Assay for gpt transcription

Total cytoplasmic RNA was isolated from 5×10^6 clone 7 cells by using the guanidinium/cesium chloride method (23). To achieve high level of gpt transcription, clone 7 cells were also incubated for 5 hr with zinc acetate (100 µM final concentration) prior RNA extraction. The concentration of RNA was measured and its integrity was checked by agarose gel electrophoresis. Serial two-fold dilutions of each RNA were spotted on nylon filter and hybridized with the appropriate 32P-nick translated probe. After hybridization, filters were washed twice for 15 min at room temperature in 2×SSC/0.1% SDS and twice for 15 min at 60°C in 0.1% x SSC.

RESULTS

Characterization of a human cell line stably transformed with pTF-EBV. Direction of replication of the reporter gene

1) Expression of the gpt gene in human clone 7 cells. In a previous study we have shown that human cells containing the EBV-derived shuttle vector pFl-EBV synthesize gpt mRNA and the expression of the gene is inducible by heavy metal ions (17). To verify that the new construct pTF-EBV was able to confer the same characteristics to human cells, the level of specific gpt transcripts in total cytoplasmic RNA of cells harbouring pTF-EBV plasmids was determined by dot-blot analysis. RNA samples from a clonal cell line stably transformed with pTF-EBV, clone 7, were analysed. As shown in Fig. 1, panel A, clone 7 cells transcribed the gpt gene (lane 1) and the level of transcription was significantly increased after cell treatment for 5 hr with 100 µM zinc acetate (lane 2). RNA from the host cells without the plasmid exposed to 100 µM zinc acetate for 5 hr did not hybridize with the gpt probe (data not shown). The same RNAs were probed with a fragment from the actin gene as a control for the relative amounts of RNA bound to the nylon filter (Fig. 1, panel B).

![Figure 1. Dot-blot analysis of gpt-specific RNA. Total cytoplasmic RNA (8 µg) was serially diluted in two-fold steps, spotted on nylon filters and hybridized with the appropriate 32P-labeled nick-translated probe. (A) The filter was probed with a nick-translated 1.8 kb HindIII-BamHI gpt fragment spanning the coding region of the bacterial gpt gene. (B) A parallel filter with the same RNAs was probed with a 2.3 kb PstI actin fragment as a control for the relative amounts of bound RNA. Lane 1: pTF-EBV transformed 293 cells (clone 7); lane 2: after 5 hr exposure to 100 µM zinc acetate.](image-url)
These data indicate that the gpt gene carried by an EBV-derived shuttle vector is actively transcribed in human cells regardless of the direction of transcription relative to the viral origin of replication, oriP.

2) Identification of the leading and lagging DNA strands of the gpt gene in pTF-EBV. To identify the leading and lagging DNA strands of the gpt gene in pTF-EBV shuttle vector the restriction endonuclease map of the vector was analysed and compared to pFl-EBV map. The EBV oriP contains two components, a dyad symmetry element which is the site of initiation of DNA replication, oriP. The location of this site was used as a reference to establish the direction of replication of the gpt gene in the two recombinant plasmids. As shown in Fig. 2, when pTF-EBV was digested with EcoRV and BamHI (lane 1) three fragments were obtained (from A to C) as expected from the cloning of the gpt transcriptional cassette immediately adjacent to the dyad symmetry element of oriP (fragment D is undetectable on etidium bromide-stained agarose gels). In this configuration the transcription of the EBV sequences and the gpt gene are coordinated and the transcribed strand of the target gene is the leading strand. The double digestion with EcoRV and BamHI of pFl-EBV plasmid (lane 4) yielded five fragments (from A to E) as expected from the cloning of the 5’ sequences of the MT-I promoter downstream of the hyg resistance gene. In this construct the transcription of the EBV sequences and the gpt gene are head-to-head and the transcribed strand of the gpt gene is the lagging strand. Fig. 2 also shows the pattern of digestion of the two plasmids with EcoRV (lane 2 and 5) and BamHI (lane 3 and 6) alone.

Spontaneous and MNU-induced mutagenesis in human cells carrying pTF-EBV. Molecular analysis of MNU-induced mutations

Plasmid pTF-EBV was transfected into 293 cells. Several clones harbouring the plasmid were isolated and expanded. Plasmid DNA was extracted from individual cell clones and transformed into E.coli gpt− DT2 cells which were plated on medium containing 6-TG. A clonal cell line, clone 7, with a low gpt− mutation frequency (1.5×10−5) was selected for this study. Clone 7 cells were exposed to increasing MNU doses (from 50 to 150 μg/ml) and the mutation frequency at the gpt gene was determined. As shown in Fig. 3, a dose-response relationship was observed with a 100-fold increase over background at the dose of 150 μg/ml of MNU. Fig. 3 displays also the mutation frequency data previously obtained at the same doses with the shuttle vector pFl-EBV replicating in clone 6NT (13).

The similarity between the MNU-induced mutation frequencies observed with the two recombinant plasmids indicates that the direction of replication of the gpt gene does not affect the rate of mutation.

DNA sequence analysis of MNU-induced pTF-EBV mutants was performed. As shown in Tab. 1, MNU induced exclusively

Table 1. DNA sequence analysis of MNU-induced mutations

<table>
<thead>
<tr>
<th>Siteb</th>
<th>Sequencec</th>
<th>Change</th>
<th>Amino acid</th>
<th>Occurrences</th>
</tr>
</thead>
<tbody>
<tr>
<td>−93</td>
<td>ACTG G TTTT</td>
<td>GC-AT</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>ACAT G AGCG</td>
<td>GC-AT</td>
<td>Met-Ile</td>
<td>1</td>
</tr>
<tr>
<td>27</td>
<td>CTCG G GACA</td>
<td>GC-AT</td>
<td>Trp-Stop</td>
<td>1</td>
</tr>
<tr>
<td>87</td>
<td>AATTG G AAAG</td>
<td>GC-AT</td>
<td>Trp-Stop</td>
<td>3</td>
</tr>
<tr>
<td>107</td>
<td>GTAA G CCCT</td>
<td>GC-AT</td>
<td>Ser-Asn</td>
<td>1</td>
</tr>
<tr>
<td>113</td>
<td>GTGT C GGCT</td>
<td>GC-AT</td>
<td>Gly-Asp</td>
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</tr>
<tr>
<td>116</td>
<td>GGCG G TCTG</td>
<td>GC-AT</td>
<td>Gly-Asp</td>
<td>1</td>
</tr>
<tr>
<td>128</td>
<td>CCGG G TGCG</td>
<td>GC-AT</td>
<td>Gly-Asp</td>
<td>3</td>
</tr>
<tr>
<td>185</td>
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<td>GC-AT</td>
<td>Ser-Asn</td>
<td>2</td>
</tr>
<tr>
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<td>GC-AT</td>
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<td>2</td>
</tr>
<tr>
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<td>GC-AT</td>
<td>Asp-Asn</td>
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</tr>
<tr>
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<tr>
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<td>1</td>
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<td>418</td>
<td>GTGG G ATAT</td>
<td>GC-AT</td>
<td>Asp-Asn</td>
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</table>

a Mutants obtained after treatment of human cells with 100 μg/ml of MNU.
b The first base of gpt coding sequence is considered at position number 1.
c Sequence of the non transcribed strand.

Figure 2. Identification of the direction of replication of the gpt gene in pTF-EBV and pFl-EBV shuttle vectors. Plasmid DNA was digested with the appropriate restriction endonuclease and analysed by 2% agarose gel electrophoresis. Lane 1: pTF-EBV DNA after digestion with EcoRV and BamHI; lane 2: after digestion with EcoRV; lane 3: after digestion with BamHI; lane 4: pFl-EBV DNA after digestion with EcoRV and BamHI; lane 5: after digestion with EcoRV; lane 6: after digestion with BamHI; lane 7: phi X174 DNA Haelll digest; lane 8: lambda DNA HindIII digest.
GC to AT transitions. Assuming that the premutagenic lesion responsible for this base change is \( \text{O}^6\)-methylguanine (\( \text{O}^6\text{-meGua} \)), the mutation strand distribution can be established. A significantly higher number of mutations was detected in the non transcribed DNA strand (26/30) as compared to the transcribed one (4/30) and almost 50% of all mutations were detected at non-sense sites and Glycine codons. The non transcribed strand was also highly mutated in pFl-EBV mutants (13) indicating that MNU-induced mutation distribution is independent of the directionality of the replication complex. These data confirm that the functional constraints of the GPT protein play a major role in DNA strand-specificity of MNU-induced mutations (24, 25, 26).

Comparison of the mutational spectra induced by MNU as a function of the direction of replication of the gpt gene

To identify the potential contribution of the fidelity of replication of the leading and the lagging DNA strands to mutational spectra, the site and sequence location of MNU-induced mutations in pTF-EBV and pFl-EBV mutant progeny was compared. Because the majority of mutations in both shuttle vectors were targeted in the non transcribed DNA strand the analysis was restricted to this DNA strand which is the lagging strand in pTF-EBV and the leading strand in pFl-EBV. Moreover, the recovery of mutations localized in this strand is not significantly affected by the structure of the GPT protein (26). Fig. 4 shows the striking similarity between the distribution of mutations in the non transcribed DNA strands of the two vectors with 50% of the gpt target sites in common. It is interesting to note that only 20 out of 162 mutable (via a GC to AT transition) guanine residues were involved in the induction of this type of mutations. As shown in Fig. 5 the mutated G's displayed a common feature: the majority were preceded in 5' by another purine (often a G). Our data indicate that the DNA polymerase insertion fidelity is not involved in the 5'-nearest neighbor effect and support the hypothesis that this phenomenon is mainly due to site-specific alkylolation of DNA (27, 13, 28). It is known that DNA polymerase insertion fidelity may be affected by 3' neighboring bases (29).

However, when the 3' flanking bases of the mutated G's in the leading and the lagging gpt strands were analyzed (Fig. 5) a random localization of mutations at 3'-purine-G and 3'-pyrimidine-G sequences was observed.

These results suggest a similar mechanism of misinsertion of T versus C opposite template \( \text{O}^6\text{-meGua} \) residues during DNA replication of the leading as well as the lagging DNA strand.

**DISCUSSION**

When cells are exposed to monofunctional alkylating mutagens a variety of repair mechanisms act to remove DNA lesions ahead of the replication fork. The suicide protein \( \text{O}^6\text{-meGua-DNA-methyltransferase (AGT)} \) very rapidly transfers to its own cysteine residue the methyl groups from the \( \text{O}^6 \) position of guanine (for a review see 30). \( \text{O}^6\text{-meGua} \) has been shown to delay in vitro DNA replication by human cell extracts (31) and has the potential to block the initiation (32) and extension (33) steps of DNA replication when placed in specific DNA sequences. However, when G is alkylated at the \( \text{O}^6 \) position its base-pairing specificity is altered and the G-T mispair is strongly stimulated allowing replication to proceed although with diminished fidelity. A fast removal of 3-methyladenine (3-meAde) adducts also occur by a specific DNA glycosylase (34). 3-meAde is able to inhibit DNA replication (35) presumably by distorting DNA and affecting the geometric recognition DNA polymerase-template. In E.coli cells the introduction of blocking lesions into DNA induces the SOS response which allows translesion replication. The molecular analysis of mutations induced by methylating agents would suggest that in physiologically normal bacterial and mammalian cells the bypass of 3-meAde lesions is error-free.

The 'safety mechanism' which guarantees the integrity of the genome relies upon the base insertion selectivity and the editing functions of DNA polymerases together with the possible additional editing by the mismatch repair complex. As inferred from SV40 model systems, DNA polymerases delta and alfa are believed to carry out leading and lagging strand replication, respectively (36, 37). A third DNA polymerase, DNA polymerase epsilon, has been recently discovered in yeast (38, 3) and in eukaryotic cells (38) but its functional role in replication is still undefined (40). Cellular polymerase alfa is required for lagging strand synthesis based on its association with RNA primase activity. Whether or not polymerase alfa has an associated 3' exonuclease activity remains an open question, while polymerase delta has been shown to contain an associated 3'-5' exo nuclease activity (41). Polymerase delta is more accurate than polymerase alfa-primase complex from both HeLa cells and calf thymus (42) suggesting that DNA polymerase exonuclease editing has a key role in mutagenesis. In fact, in bacterial cells, mutations in DNA polymerase proofreading exo nucleasees have been correlated with mutator and antimutator phenotypes (43).

In this paper we analysed MNU-induced mutations as a function of the direction of replication of the reporter gene to have some clues on the effects of the eukaryotic asymmetric replication machinery on error rate and type during replication of alkylated DNA.

We have demonstrated that the base substitution error rates on an alkylated substrate are similar for the replication of the leading and lagging strands. The same mutation frequency at the gpt gene was observed independently of gpt strand orientation. If one makes the assumption that the GC to AT transitions induced by MNU are due to the miscoding properties of \( \text{O}^6\text{-meGua} \) residues, our results suggest also that the frequency of misinsertion of an incorrect nucleotide (T versus C) opposite \( \text{O}^6\text{-meGua} \) adducts is similar for the DNA polymerases
synthesizing the leading and the lagging strands. However, we cannot exclude that additional editing by the mismatch repair system contributes to obliterate differences in the synthesis accuracy of the two strands.

Several studies indicate that DNA polymerase insertion fidelity is affected by flanking neighboring bases. In in vitro experiments it has been shown that, in the absence of repair, \( \theta^6 \)-meGua can induce widely different mutation frequencies depending on the base at the 3' side (44). We present evidence that the fidelity of replication opposite \( \theta^6 \)-meGua residues of both the leading and lagging strands is not affected by the 3' flanking neighbors, but the base at the 5' side is indeed the main determinant for the preferential targeting of mutations after replication of alkylated DNA. The majority of GC to AT mutations recovered in the leading and the lagging strands present a purine 5' to the mutated G residue. G's which are flanked 5' by a purine are more frequently alkylated by MNU (45) and less susceptible to repair by AGT than guanines preceded by a pyrimidine (46, 47). Sequence-dependent factors such as electrostatic potential of a site or base stacking might be envisaged as responsible for the availability of specific sites for alkylation and/or repair of alkylated bases.

Another interesting basic difference in the organization of the two shuttle vectors used for this study is the direction of transcription of the reporter gene relative to replication fork movement. The transcription of the gpt gene proceeds in the same direction as replication initiating from the dyad symmetry element in pTF-EBV plasmid and in the opposite direction in pF1-EBV plasmid. Brewer (48) proposed that the direction of transcription and replication fork movement must be coordinated in E.coli to avoid 'polymerase collision'. In fact, of the chromosomal E.coli genes that have been analysed, 74% are oriented with their 5' ends proximal to the E.coli origin of replication. Recently it has been reported that in E.coli strand-specific mutation induction by 1-aminopyrene is coupled to the direction of transcription relative to replication (49). Our data show that in human cells the frequency and the type of alkylation-induced mutations is unaffected by the relative direction of DNA and RNA polymerases. This might well be due to the asynchronous timing of transcription and replication of the majority of the genes in eukaryotic cells.

In conclusion, our data show a similar fidelity of synthesis of alkylated DNA template by both leading and lagging DNA strand polymerases. Similarly, the nature of the DNA polymerase involved in the replication of specific strands is not a primary determinant of the hotspots of mutation at photoproduct sites.
observed in a shuttle vector plasmid replicating in UV-irradiated Xeroderma Pigmentosum (50). All these findings might have implications for the composition of the eukaryotic replication apparatus. Recently, a replication model has been proposed where the leading strand is replicated by a processive DNA polymerase (polymerase delta or epsilon) and the Okazaki fragments of the lagging strands are synthesized to completion by the same DNA polymerases (3, 5). Such a replication apparatus, which is reminiscent of the asymmetric DNA polymerase III holoenzyme of E.coli, would guarantee the conservation of the eukaryotic genome.

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