Role of Nucleotide Excision Repair in Processing of O^4-Alkylthymines in Human Cells*

(Received for publication, May 4, 1994)

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O^4-Alkylthymines have been implicated as potential carcinogenic DNA lesions. We have studied the effects of O^4-methylthymine, O^4-ethylthymine, and O^4-n-propylthymine in a model system in which a single lesion was located at a defined position on a SV40-based shuttle vector and have found large differences in the effects of these lesions in repair-proficient and nucleotide excision repair-deficient cells. In repair-proficient human HeLa cells, all 3 residues were highly mutagenic; a mutation frequency of \( \sim 20\% \) was found for both O^4-methylthymine and O^4-ethylthymine, whereas that of O^4-n-propylthymine was \( \sim 12\% \). These frequencies were independent of the activity of the O^6-alkylguanine DNA alkyltransferase. All three O^4-alkylthymines induced T \( \rightarrow \) T transitions exclusively. In nucleotide excision repair-deficient XP-A cells, however, these lesions were not mutagenic but strongly inhibited plasmid replication (>90%). These results indicate that O^4-alkylthymine residues are efficiently recognized by the nucleotide excision repair system and cause a complete cessation of plasmid replication if this system is deficient. Nevertheless, proficiency in the nucleotide excision repair pathway correlates with a high frequency of mutation induction by these lesions.

Carcinogenic alkylating agents induce a variety of aberrant structures upon reaction with DNA. These DNA lesions (N- or O-adducted bases or O-alkylphosphate lesions) may have different mutagenic and carcinogenic properties, depending on the position and size of the alkyl group, which can be modulated by the presence of specific repair enzymes in a certain cell type and the DNA sequence context of the lesion (1-4).

Alkyl adducts at O-4 of thymine (O^4-AlkT) have been implicated as highly mutagenic lesions that cause transitions by mispairing during replication (5). From experiments using alkylated DNA and DNA polymerases in vitro, indirect evidence was obtained that the miscoding potential of these residues might be very high (up to 80-90%) (4-6). During DNA replication in HeLa cells, however, the frequency of mutations induced by O^4-ethylthymine (O^4-EtT) appears to be much lower (<20% overall or >40% per adducted strand) (7). Other studies showed that in mammalian cells the persistence of O^4-AlkT lesions varies with the size of the alkyl group (8-10); the half-life of O^4-methylthymine (O^4-MeT) in DNA of cultured cells or rat liver varies from 2 to 20 h, whereas O^4-EtT has a half-life of 2-20 days. The processing of these lesions thus appears to depend on the size of the alkyl group (11). These observations indicate that O^4-AlkT residues are actively repaired in mammalian cells.

This repair could be mediated by O^6-alkylguanine DNA alkyltransferases, nucleotide excision repair, or an as yet unknown mechanism (4, 9, 12, 13). In Escherichia coli O^4-AlkT lesions are substrates for the ada- and ogt-encoded alkyltransferases that normally act on O^6-alkyldeoxyguanosine residues in DNA (9, 14, 15). O^4-EtT can also be removed by nucleotide excision repair if alkyltransferases are not induced and there is virtually no repair of these lesions in bacterial cells that lack both the alkyltransferases and the nucleotide excision repair system (16).

The mammalian alkyltransferase efficiently removes alkyl groups from the O-6 of dG, and, while it was thought that this protein could not or could very poorly repair O^4-AlkT (9, 17, 18, 19), one study reported the presence of low levels of a O^4-MeT-specific transferase-like activity in human liver (12), whereas another suggested that the removal of O^4-EtT residues occurs by a non-transferase mode of repair (15). Recently it was shown that the yeast and human alkyltransferase can bind to double-stranded alkydeoxygenucleotides that contain O^4-MeT, although with a low affinity (20). Evidence for the actual removal of O^4-MeT by both of these alkyltransferases is now emerging (21, 22). In mammalian cells, it might also be that alkylated nucleotides are repaired by nucleotide excision repair. Evidence for the removal of O^4-alkyldeoxyguanosine by this repair mechanism in human cells has been published (23-25).

In the present study, we investigated whether O^4-AlkTs in human cells are substrate for nucleotide excision repair by comparing the mutagenic effects of individual O^4-MeT, O^4-EtT, and O^4-nPrT moieties during replication in HeLa cells, normal (SV40-transformed) human fibroblasts and excision repair-deficient xeroderma pigmentosum cells of complementation group A (XP-A). All residues were located at a single position of a SV40-based plasmid that was transfected into the various cell lines and allowed to replicate transiently. We also determined the mutagenicity of O^4-MedG in relation to the activity of the alkyltransferase in the same set of cell lines. Our experiments...
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provide evidence that the mutagenicity of O'-AlkTns in human cells is primarily caused by incomplete excision repair.

MATERIALS AND METHODS

Enzymes and Cells—All enzymes used were purchased from Boehringer Mannheim. HeLa cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum. The SV40-transformed excision proficient human fibroblasts (SV40 wild type fibroblasts, denoted as PL-A fibroblasts) and SV40-transformed XP-A cell lines (XP2OS-SV-2) were a gift from Dr. R. J. Hoeijmakers (Erasmus University, Rotterdam). The XP2OS-SV revertant cells (clone A24) were a gift from Dr. P. B. G. M. Blei (Vrije University, Amsterdam). The XP-A cells were grown in a modified Eagle's medium with 10% fetal calf serum.

Hanahan-resistant E. coli DH5α cells (transformation efficiency 10⁻⁶/μg) were obtained from Life Technologies, Inc. and used as recommended.

Plasmids—The plasmids used were described earlier (27, 28); both contained the complete SV40 early region and origin of replication. pSVsupF carried the alkylated nucleotide at the unique ClaI site and contained the β-lactamase (Amp') gene for selection in bacteria. Plasmid pSVetr served as internal unmodified control and carried the bacterial chloramphenicol resistance (Cm') gene as selection marker.

Synthesis and Analysis of O'-AlkylTn Oligodeoxynucleotides—Unmodified oligonucleotides (5'-dAATTCACTGATATCTA-3', O'-AkkT, and O'-MedG containing oligomers (5'-dAATTCA(04-EtT)CGATATCTA-3' and 5'-dAATTCTAC(04-MeG)ATATCTA-3') were chemically synthesized using the solid phase phosphoramidite method described by Roelen et al. (27, 28). The oligomer with both an O'-EtT and a N-de-ethoxyaminosine-8-vсложened acetylated (dG-CS-AAF) residue was obtained by reacting the purified thiolated oligodeoxynucleotide with N-acetoxy-AAF as described previously (26). All oligodeoxynucleotides were extensively purified and analyzed on a Pharmacia PEPRC reversed phase fast protein liquid chromatography column using a buffer of 0.1 M sodium bicarbonate, 0.1 M sodium acetate, 5 mM EDTA, 0.3 M sodium acetate, 3 mM dithiothreitol containing 5 μg/ml of DNase I and 0.05 units/ml of phosphatase (27, 28). The oligomer with both an O'-EtT and a N-de-ethoxyaminosine-8-vсложened acetylated residue at the unique ClaI site was obtained after ligation of chemically synthesized oligodeoxynucleotides carrying just one O'-EtT residue at the unique ClaI site (see forward and reverse oligomers).

Site-specific Modified Plasmids— Constructs with O'-AkkT, O'-MedG, or both O'-EtT plus dG-CS-AAF residue at the unique ClaI site were obtained after ligation of chemically synthesized oligodeoxynucleotides carrying just one of the modified nucleotides into plasmid molecules that contain a gap at the desired region (see "Materials and Methods"). A schematic representation of the region of modification of the constructs is given in Fig. 1.

Before ligation, all oligomers were extensively purified using reversed phase fast protein liquid chromatography. Levels of contaminations were below detection limits (<1%) with analytical fast protein liquid chromatography (Fig. 2a). The modification of the oligomers is also demonstrated by the altered migration on plasmid pSVsupF. These modified nucleotides were all located in a single-stranded oligodeoxynucleotide (5'-dAATTCACTGATATCTA-3', broken underline) which was inserted into gapped duplex forms of the plasmid. Within the oligomer sequence, the modified residues were placed at the unique ClaI site (solid underline). The ClaI cleavage sites are indicated by vertical arrows. The leading and lagging strand during replication are indicated with ←→, respectively.

Enzymatic hydrolysis of the alkylated oligodeoxynucleotides was performed essentially as described elsewhere (32, 33). Cells were trypsinized, washed with phosphate-buffered saline, and pelleted by centrifugation for 5 min at 1500 rpm. Cells were resuspended (2 x 10⁶ cells/ml) in a buffer of 50 mM Tris-HCl (pH 7.3), 1 mM EDTA, 0.3 M dithiothreitol containing 5 μg/ml leupeptin and disrupted by sonication (10 s at 10 μF followed by 10 s at 16 μF) after which point phenylmethylylsulfonyl fluoride (57 μg/ml) was added. Cellular debris was removed by centrifugation for 10 min at 13,000 rpm at 4 °C. Aliquots of the supernatants were incubated for 1 h at 37 °C with calf thymus DNA (4.15 mg/ml) that had been methylated before treatment with [3H]MNU. Specific activities were calculated from the final pool of methyl-3H transferred to normalized amounts of protein. Values represent the mean of five independent determinations.

RESULTS

Analysis of Site-specific Modified Plasmids— Constructs with an O'-AkkT, an O'-MedG, or both an O'-EtT plus dG-CS-AAF residue at the unique ClaI site were obtained after ligation of chemically synthesized oligodeoxynucleotides carrying just one of the modified nucleotides into plasmid molecules that contain a gap at the desired region (see "Materials and Methods"). A schematic representation of the region of modification of the constructs is given in Fig. 1.

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FIG. 2. a, reversed phase fast protein liquid chromatography analysis of purified unmodified and modified oligodeoxynucleotides; b, HPLC profiles of enzyme hydrolyzed oligodeoxynucleotides. These carried the wild type undamaged sequence (wt) or contained a single O⁴-AlkT or an O⁶-MedG. Samples contained the correct relative amounts of nucleosides as was determined in previous experiments (27, 28). See "Materials and Methods" for experimental details. c, UV absorption spectra of the modified nucleosides.
fied T or dG (27, 28). Deviations from the correct values were maximally 3.7% and, on average, 2.3% (27, 28). These are due to experimental variations in the hydrolysis and chromatography steps. The modified nucleosides had the appropriate UV absorption characteristics (Fig. 2c) and comigrated with connected reference nucleosides. The O4-EtT containing oligomer sample was also analyzed by 32P postlabeling in previous studies (7) and appeared to contain less than 1% impurities.

Purified adducted plasmids were subjected to restriction fragment analysis. Plasmids carrying a lesion at the ClaI site could not be cleaved by ClaI, whereas unmodified controls could. Enzymes recognizing adjacent sequences cleaved both unmodified and adducted plasmids.

**Mutagenicity of Individual O4-AlkT Lesions in Human Cells**—Unmanipulated (wild type) plasmids, controls obtained after insertion of an unmodified oligomer or site-specific modified plasmids were transfected into the different repair-proficient and repair-deficient human cell lines. Plasmids were allowed to replicate transiently and were reisolated; unreplicated material was excluded from further analysis. Replicated plasmids were analyzed for the presence of mutations at the original region of modification (ClaI restriction site) and mutation frequencies were established from the relative numbers of mutated plasmids among the total amount of progeny (see "Materials and Methods").

Table I shows the mutation frequencies found after replication of the different constructs in the chosen set of cell lines. Except for the clones derived from wild type plasmids, we verified the presence of a mutation on a large number of clones by means of restriction fragment analysis. The reliability of the ClaI selection was also verified by the fact that cotransfected control plasmids (also carrying a ClaI site) were completely linearized. Progeny of untreated plasmids isolated from all cell lines carried (less than) 0.2–1% mutations. These clones were not analyzed further.

Insertion of an unmodified oligomer slightly increased the mutation frequency to ~2%. This frequency was found with all cell lines tested. Table II shows the spectrum and distribution of the mutations found after sequencing of the mutant clones. The mutations, single base pair substitutions or small deletions, appeared to be randomly distributed over the region of modification. In addition, multiple mutations were found with a characteristic pattern consisting of base pair substitutions with small deletions. These mutations have also been found in previous studies (7).

The presence of an O4-MeT or an O4-EtT increased the mutation frequency to ~20% among progeny plasmids derived from repair proficient HeLa cells and WtA fibroblasts. Almost all mutations were found at the position of the alkylated T and consisted of a substitution into dC. The remainder of the mutations were multiple mutations of the type described above (see Table II).

The mutation frequency induced by O4-nPrT in the two repair proficient cell lines was approximately half of that induced

| Construct | Cell type | Mean mutation frequency ± S.E. | No. of experiments | Total no. of mutants | Mean no. of progeny plasmid clones/μg DNA ± S.E. |
|-----------|-----------|-------------------------------|--------------------|----------------------|-----------------------------------------------|
| Wild type plasmid | HeLa | 0.2 ± 0.06 | 5 | 6 | 5500 ± 1700 |
| Fibroblasts | 1 ± 0.3 | 4 | 31 | 5800 ± 2300 |
| XP-A (20S) | 1 ± 0.3 | 5 | 32 | 3100 ± 1100 |
| +Unmod. ol. | XP-A (20S) | 2 ± 0.8 | 5 | 13 | 3900 ± 1600 |
| +O4-MeT XP-A | 2 ± 0.8 | 5 | 7 | 2500 ± 600 |
| +O4-EtT | HeLa | 17 ± 0 | 2 | 460 | 7700 ± 2800 |
| Fibroblasts | 19 ± 2 | 2 | 150 | 3700 ± 870 |
| XP-A (20S) | ~0 | 2 | 0 | 240 ± 140 |
| +O4-nPrT | HeLa | 21 ± 2 | 9 | 890 | 7300 ± 1800 |
| Fibroblasts | 18 ± 3 | 6 | 550 | 3600 ± 1300 |
| XP-A (20S) | 1 ± 1 | 6 | 2 | 160 ± 60 |
| +O4-nPrT | Fibroblasts | 11 ± 3 | 7 | 420 | 6400 ± 1000 |
| XP-A (20S) | 12 ± 2 | 7 | 520 | 2400 ± 710 |

* Unmanipulated plasmid.
† Normal WtA fibroblasts.
‡ + unmod. ol., constructs containing an unmodified chemically synthesized oligonucleotide.

Table II
Specificity and frequency of mutations present in mutant clones derived from pSVsupF plasmids with site-specific O4-AlkT residues after replication in different human cell lines.

| Cell type + construct | Targeted T → C | Other point mutations | Multiple mutations |
|-----------------------|---------------|-----------------------|--------------------|
| HeLa + unmodified oligonucleotide | 0.2 | 1 | 0.8 |
| HeLa + O4-MeT | 17 | 0 | 0 |
| HeLa + O4-EtT | 20 | 0 | 1 |
| HeLa + O4-nPrT | 10 | 0 | 1 |
| Fibroblast† + unmodified oligonucleotide | 0 | 2 | 0 |
| Fibroblast† + O4-MeT | 19 | 0 | 0 |
| Fibroblast† + O4-EtT | 18 | 0 | 0 |
| Fibroblast† + O4-nPrT | 12 | 0 | 0 |
| XP-A (20S) + O4-EtT | 1 | 0 | 0 |

* Values for the mutational specificity of O4-EtT in HeLa cells were in previous studies: 21% T to C transitions, 1% other point mutations, and 2% multiple mutations (7). The multiple mutations consisted of a number of single base pair substitutions combined with small deletions as described previously (7, 26).
† Normal WtA fibroblasts.
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| Construct   | Cell type  | Mean mutation frequency ± S.E. | No. of experiments | Total no. of mutants | Mean no. of progeny plasmid ± S.E. | clones/µg DNA |
|-------------|------------|--------------------------------|--------------------|----------------------|-----------------------------------|--------------|
| Wt plasmid  | XP-20S     | 1 ± 0.3                        | 5                  | 32                   | 3100 ± 1100                       | 1300 ± 900   |
|             | XP-20S rev | 0.5 ± 0.3                      | 3                  | 2                    | 3000 ± 1200                       | 160 ± 60     |
| +O\(^{\bullet}\)-EtT | XP-12RO | 0.2 ± 0.1                      | 4                  | 1                    | 400 ± 150                         | 690 ± 220    |
|              | XP-20S     | 1 ± 1                          | 6                  | 2                    |                                   |              |
|              | XP-20S rev | 24 ± 12                        | 2                  | 11                   |                                   |              |
|              | XP-12RO    | 26 ± 5                         | 4                  | 34                   |                                   |              |

These frequencies were also determined for damaged (Wt) plasmids. Values were calculated from the relative numbers of progeny plasmids harboring an sequence alteration at the region of modification by O\(^{\bullet}\)-MeT and O\(^{\bullet}\)-EtT (on average 12 versus 20%) (Table I). In all sets of parallel transfections, we reproducibly found lower mutation frequencies for O\(^{\bullet}\)-nPrT than for O\(^{\bullet}\)-MeT or O\(^{\bullet}\)-EtT, and for the mutation frequencies of O\(^{\bullet}\)-EtT and O\(^{\bullet}\)-nPrT in HeLa cells, the difference was statistically significant (p < 0.02). O\(^{\bullet}\)-EtT also exclusively induced T → C transitions. The few other mutations found consisted of the multiple mutations that were also observed with the analysis of controls containing unmodified oligomers (Table II).

We could not determine the mutagenicity of O\(^{\bullet}\)-AlkT in XP-A cells, since all 3 residues extensively inhibited plasmid replication (see below). The few replicated clones that were isolated carried almost exclusively the wild type sequence. From O\(^{\bullet}\)-EtT-adducted plasmids, only two clones were obtained that carried the specific T → C transition. However, since plasmid replication was so severely impaired, it is not clear whether these two mutants were specifically induced by O\(^{\bullet}\)-EtT.

Interference with Plasmid Replication in XP20S (XP-A) Cells—In order to measure selective loss of the plasmids due to replication inhibition by the modifications, we calculated the ratios for the (normalized) amounts of progeny derived from adducted pSVsupF plasmids and the corresponding unmodified pSVsupF controls in parallel transfections. These amounts were determined using E. coli transformations (as is described under "Materials and Methods"). Mean ratios from at least two independent sets of experiments are depicted in Fig. 3. In addition, adducted plasmids were always cotransfected with equal amounts of an unmodified control plasmid (pSVctrr). We also determined the ratios relative to the amount of progeny derived from pSVctrr control plasmids (Fig. 4).

In both of the excision repair-proficient cell lines, adducted constructs and controls carrying an unmodified oligonucleotide yielded approximately equal amounts of replicated plasmids (ratios were ~0.9 for HeLa and ~0.7 for WtA fibroblasts, Fig. 3). When ratios were established relative to the replication of cotransfected pSVctrr plasmids in these cells, we also found similar values (~0.4 for HeLa and ~0.5 for WtA fibroblasts). However, in XP20S cells, plasmids carrying either one of the O\(^{\bullet}\)-AlkT lesions were less well replicated relative to both of the controls (undamaged pSVsupF and cotransfected pSVctrr), the amount of progeny obtained from adducted constructs was only 5–8% (ratios of 0.05–0.08) of that obtained from wild type plasmids transfected in parallel experiments, while controls carrying an unmodified oligodeoxynucleotide replicated almost as well as the untreated plasmids (Fig. 3).

Effects of O\(^{\bullet}\)-EtT in Other XP-A cells and a XP-A Revertant Cell Line—To ascertain that the replication inhibition in XP20S cells was indeed caused by the nonfunctional XP-A factor, we introduced constructs with an O\(^{\bullet}\)-EtT into another XP-A cell line (XP12RO-SV) and revertant cells (clone A24) from the XP20S-SV line.

XP20S cells are homozygous for a splicing mutation in intron 3, which results in two abnormally spliced XPAC mRNAs; the smallest lacks exon 3 entirely and carries a new stop codon in exon 4, the largest only contains the extra stop codon in exon 4 (34, 35). Both mRNAs yield largely truncated XP-A factors. The XP20S revertant cells have not been characterized at a molecular level, but their UV survival capacity is nearly equal to wild type levels (36). XP12RO cells contain severely reduced amounts of normal sized XPAC mRNA. However, this tRNA contains a nonsense mutation in the fifth exon, giving rise to a truncated (207-amino acid) XP-A protein that lacks the 87 C-terminal amino acids (34, 35).

The replication of wild type and O\(^{\bullet}\)-EtT-adducted pSVsupF plasmids relative to the replication of cotransfected pSVctrr controls in all cell lines is shown in Fig. 4. The relative amounts of the two unmodified plasmids undergoing replication appear to be cell line specific. Similar ratios were found for adducted plasmids replicating in excision repair-proficient HeLa cells, fibroblasts, and XP20S revertant cells. Adducted plasmids failed to replicate only in the XP20S-SV cell line (ratio 0.01) and were very inefficiently replicated in the XP12RO-SV cells (ratio 0.06) in comparison to undamaged plasmids, which produced ratios of 0.25 and 0.98, respectively. This implies that in XP12RO cells a relatively larger fraction of adducted plasmids has replicated.

The mutation frequency of O\(^{\bullet}\)-EtT in XP12RO was 25% (Table III). Mutations were again T → C transitions. In XP20S revertant cells, the mutation frequency of O\(^{\bullet}\)-EtT was 24%, which is comparable with the frequency found for the other repair proficient cell types. The majority (~21%) of the mutations were T → C transitions.

Comparison with the Mutagenicity of O\(^{\bullet}\)-MedG—To exclude a possible influence of the alkyltransferase on the mutagenicity of O\(^{\bullet}\)-AlkT, we transfected plasmids carrying a single O\(^{\bullet}\)-MedG at the ClaI site into the same cell lines. As is shown in Table IV, the mutation frequency of O\(^{\bullet}\)-MedG varied considerably among the different cell types. The mutagenicity in HeLa cells was comparable with that of O\(^{\bullet}\)-MeT and O\(^{\bullet}\)-EtT (mutation frequencies of 14 and 17–21%, respectively), whereas in WtA fibroblasts and XP20S cells, the mutation frequency (2–5%) hardly exceeded background levels (2%). The majority of the mutations consisted of a G → A transition at the former position of the adducted dG (Table V), which is in good agreement with the mutational specificity of O\(^{\bullet}\)-MedG reported in literature (17).

The specific activity of the alkyltransferase varied from 18 fmol/mg of protein for the XP20S cells to 768 fmol/mg of protein for the WtA fibroblasts (see Table IV). The mutation frequencies were in agreement with the alkyltransferase activity in the two excision repair proficient cell lines; the high mutation frequency of O\(^{\bullet}\)-MedG in HeLa cells corresponds with the relatively lower alkyltransferase activity in these cells and the much lower mutation frequency in WtA fibroblasts with the higher alkyltransferase activity.

The low mutation frequency of O\(^{\bullet}\)-MedG in XP20S cells does
**FIG. 4.** Relative replication of unmanipulated (—→) or O^4^-EtT added pSVsupF modified plasmids as compared with cotransfected unmodified pSVctr controls in different human cell lines. This is expressed as normalized amounts of Amp' versus Cm' clones. Solid bars represent the average ratios from at least two independent experiments; error bars represent standard error. The different cell lines are indicated with H (HeLa), F (normal fibroblasts, SV40 Wt Amsterdam), Xlr (XP2OS-SV revertant clone A24), and X1 (XP-A cell line XP2OS-SV). See “Materials and Methods” for experimental details.

**FIG. 3.** Replication of pSVsupF plasmids containing a single O^4^-AlkT or an unmodified oligonucleotide (unm) as compared with the replication of unmanipulated pSVsupF in different human cell lines. Adducted plasmids and controls were transfected in parallel experiments. Normalized amounts of progeny plasmids were determined for each transfection from the number of Amp' clones. Solid bars represent mean ratios from three independent experiments; error bars represent the S.E. The different cell lines are indicated with H (HeLa), F (normal fibroblasts, SV40 Wt Amsterdam), Xlr (XP2OS-SV revertant clone A24), and X1 (XP-A cell line XP2OS-SV). See text for experimental details.

**DISCUSSION**

We have determined the mutagenicity of O^4^-MeT, O^4^-EtT, and O^4^-nPrT residues in human cells with different repair capacities using site-specific modified plasmids. All three lesions were highly mutagenic in excision repair-proficient cells (~20% for methyl and ethyl adducts and ~12% for n-propyl adducts) and specifically induced T → C transitions.

The mutational specificity of the O^4^-AlkT residues is as expected and in accordance with our previous studies on the mutagenicity of O^4^-EtT in HeLa cells (7). The same transitions were also found by other groups investigating the effects of O^4^-MeT and O^4^-EtT during replication in _E. coli_ and in _in vitro_ assays (5, 37–39). The mutation frequencies induced by these lesions in our system, however, were lower than those calculated from _in vitro_ experiments (4, 6). This suggests that there is repair of O^4^-AlkTs in human cells, although this may be inefficient.

In order to investigate what type(s) of repair could be involved, we used different cell lines that varied largely with respect to the repair activities of the O^4^-alkylguanine DNA alkyltransferase or nucleotide excision repair system (AT^-/-NER+, AT^-/-NER-, AT^-/-NER-). In _E. coli_, O^4^-AlkTs are primarily removed by the O^4^-alkylguanine DNA alkyltransferase (9, 15, 33), but it appears unlikely that this is the case in human cells. In our studies, alkyltransferase activities varied widely in the different cell lines and correlated well with the mutation frequency of O^4^-MedG, but not at all with that of O^4^-AlkT. The alkyltransferase activity in HeLa cells appeared relatively low as compared with that in WtA fibroblasts, and this is reflected in the much higher mutation frequency of O^4^-MedG in HeLa cells. However, mutation frequencies of each of the O^4^-AlkT lesions were similar in both HeLa cells and WtA fibroblasts and approximately equal to those reported for O^4^-MedG (O^4^-Me-, O^4^-EtT) and O^4^-EtdG (O^4^-nPrT) in alkyltransferase-deficient CHO cells (17).

It has been reported recently that the human alkyltransferase is able to bind with very low affinity to O^4^-MeT in _in vitro_ but not to O^4^-EtT (20). Hence, it was suggested that the human alkyltransferase may also repair O^4^-MeT to some extent _in vivo_ (20), and this may explain the differences in biological halflives of O^4^-MeT and O^4^-EtT in chromosomal DNA as observed with other experimental systems (t_{1/2} of 2–20 h and 2–20 days, respectively) (8–10). Such repair might have influenced the mutation frequency of O^4^-MeT in our system as well, but this frequency appeared to be similar to the mutation frequency of O^4^-EtT (~20%). Therefore, alkyltransferases do not seem to play a prominent role in the removal of any of the O^4^-AlkTs in human cells.
Our data indicate that O'-AlkT moieties are recognized by nucleotide excision repair. Remarkably, we found these lesions to be inhibitory to plasmid replication and, consequently, not to be mutagenic in nucleotide excision repair-deficient XP-A cells. The replication inhibition of O'-EtT adducted plasmids was observed with two different XP-A cell lines (XP2OS-SV and XP12RO-SV), both of which are highly deficient in nucleotide excision repair (41). Our data suggest that the excision of the dG-C8-AAF adducts is a prerequisite for replication inhibition. The presence of the adducts in the damaged strand is incised, and subsequent repair replication uses the undamaged strand as the template and introduces the mutation frequency to increase with adduct size, as both the E. coli and mammalian alkyltransferases are known to repair larger O'-alkyl groups less efficiently (43, 44, 45). We observed the opposite. The decrease in mutation frequency we found can only be consistent with removal by nucleotide excision repair since this type of repair has been shown to recognize distortions of the DNA double helix caused by the adducts rather than the adducts themselves (46). With increasing adduct size, the distortion of the helix might become more extensive, allowing a more efficient repair. The effect is most pronounced with dG-C8-AAF, which has been shown to cause large alterations in the DNA conformation and which is indeed efficiently removed by nucleotide excision repair (26, 47).

Finally, it is known from literature that different cell lines of the XP-A complementation group fail to replicate plasmids carrying other carcinogen-DNA adducts, which have been shown to be substrates for nucleotide excision repair (41, 48, 49). Previous experiments in our laboratory demonstrated that lesions that are substrates for glycosylase repair (i.e. 8-oxo-dG) do not inhibit DNA replication in XP-A cells (26). From the present studies, it appears that even the smallest O'-AlkT adducts completely prevent bypass of replication, indicating that nucleotide excision repair is the predominant repair mode operating on these adducts.

The fact that the observed inhibition of plasmid replication in XP-A cells is (almost) 100% indicates that the alkylated thymines are very efficiently recognized by the nucleotide excision repair system. This is unexpected because from the high mutation frequencies in repair-proficient cells, one would have anticipated a rather poor recognition of O'-AlkTs. Apparently, recognition and incision/excision are somehow uncoupled processes, since all lesions (small and large) are recognized efficiently but not removed with equal efficiency. It may be that the smaller O'-AlkT lesions do not permit the specific identification and incision of the damaged strand but that, instead, the undamaged strand is incised, and subsequent repair replication using the damaged strand as the template introduces the mutation opposite O'-AlkT. Removal of the lesion by the proper operation of the excision repair system might then allow normal plasmid replication to proceed. Alternatively, the nucleotide excision repair factors in proficient cells might interact with the damage in a way that does not completely suppress replication, whereas in repair-deficient cells this interaction is more extensive and completely blocks replication.

The lower mutation frequencies of the larger (O'-nPrT and dG-C8-AAF) adducts suggest that the incision/excision process is more reliable with larger adducts. When the strand carrying an O'-EtT was marked with an additional C8-AAF adduct on a neighboring dG, it might have been predicted that this would reduce the mutagenicity of the adjacent O'-EtT and indeed, with the excision of dG-C8-AAF the O'-EtT was simultaneously
removed and the mutation frequency concomitantly decreased to background levels.

The recognition of O\textsuperscript{4}-AlkTs by nucleotide excision repair has also been shown to occur in E. coli (16). However, Bronstein et al. (25) reported no differences between excision repair deficient and proficient human cells with respect to the $t_{1/2}$ values for the removal of O\textsuperscript{4}-AlkTs residues from DNA, and they concluded that these lesions were not substrates for nucleotide excision repair. We have not measured the actual removal ($t_{1/2}$) of these lesions in our system. Our experiments also differ with respect to the position of the lesions (plasmid versus chromosomal DNA), the cell lines used, and their rates of division. Therefore, the apparent discrepancy between their conclusions and ours probably relates to gross differences in the experimental systems that were utilized.

Altogether, our experiments demonstrate that O\textsuperscript{4}-AlkT residues can be highly mutagenic in human cells, in spite of the fact that they appear to be efficiently recognized by the nucleotide excision repair system. Surprisingly, the nucleotide excision repair system seems instrumental in the induction of mutations at the position of O\textsuperscript{4}-AlkT lesions. This clearly designates these lesions as potential inducers of neoplastic transformation.

Acknowledgments—We thank Pim van Dijk for technical assistance with the HPLC analysis and Joes Domen, Hein te Riele, Chris Saris, and Gerard Westra for helpful and stimulating discussions. We are grateful to Dr. J. Hoeijmakers and Dr. P. Belt for providing us with the XP cell lines and fibroblasts.

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