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The Oxidative DNA Lesion 8,5′-(*S*)-Cyclo-2′-deoxyadenosine Is Repaired by the Nucleotide Excision Repair Pathway and Blocks Gene Expression in Mammalian Cells*

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Xeroderma pigmentosum (XP) patients with inherited defects in nucleotide excision repair (NER) are unable to excise from their DNA bulky photoproducts induced by UV radiation and therefore develop accelerated actinic damage, including cancer, on sun-exposed tissue. Some XP patients also develop a characteristic neurodegeneration believed to result from their inability to repair neuronal DNA damaged by endogenous metabolites since the harmful UV radiation in sunlight does not reach neurons. Free radicals, which are abundant in neurons, induce DNA lesions that, if unrepaired, might cause the XP neurodegeneration. Searching for such a lesion, we developed a synthesis for 8,5′-(*S*)-cyclo-2′-deoxyadenosine (cyclo-dA), a free radical-induced bulky lesion, and incorporated it into DNA to test its repair in mammalian cell extracts and living cells. Using extracts of normal and mutant Chinese hamster ovary (CHO) cells to test for NER and adult rat brain extracts to test for base excision repair, we found that cyclo-dA is repaired by NER and not by base excision repair. We measured host cell reactivation, which reflects a cell's capacity for NER, by transfecting CHO and XP cells with DNA constructs containing a single cyclo-dA or a cyclobutane thymine dimer at a specific site on the transcribed strand of a luciferase reporter gene. We found that, like the cyclobutane thymine dimer, cyclo-dA is a strong block to gene expression in CHO and human cells. Cyclo-dA was repaired extremely poorly in NER-deficient CHO cells and in cells from patients in XP complementation group A with neurodegeneration. Based on these findings, we propose that cyclo-dA is a candidate for an endogenous DNA lesion that might contribute to neurodegeneration in XP.

Xeroderma pigmentosum (XP)[1] patients in complementation groups A–G have inherited defects in nucleotide excision repair (NER) of DNA damage induced by UV radiation (1–10) or free radicals (11–14). As a result of UV radiation-induced pyrimidine dimers, XP patients develop accelerated actinic damage on sunlight-exposed tissue (2, 8, 11). Some XP patients also develop a progressive atrophic neurodegeneration, termed XP neurological disease, which is due to death of neurons resembling that seen in normal aging and in several adult-onset neurodegenerations (2, 15). The XP neurodegeneration is believed to result from the patients' inability to repair damaged DNA (2, 15, 16). However, UV radiation-induced photoproducts requiring NER are formed only by short-wavelength radiation (8) that cannot reach the central nervous system (2, 16). Therefore, it has been proposed that the DNA of neurons is damaged by reactive cellular metabolites, including oxygen free radicals abundant in neurons (2, 11, 13, 17).

Two classes of these candidate DNA lesions have been considered as possible causes of the neurodegeneration (11, 13, 17). The first class contains well studied lesions such as 8-oxodG and thymine glycols, which are typically processed by base excision repair (BER) (8, 14, 18) or, if BER capacity is overwhelmed, by NER (13, 19). The second class is composed of lesions expected to cause major helical distortion of DNA (11), *e.g.* covalent intrastrand purine dimers, which are not available for synthesis into DNA, and 8,5′-cyclopurine deoxyribonucleosides, which have recently been synthesized (20, 21). Because NER has never been studied on any lesion from the second class, we chose 8,5′-(*S*)-cyclo-2′-deoxyadenosine (cyclo-dA) as the candidate 8,5′-cyclo-2′-deoxypurine lesion for DNA repair studies in mammalian cells and their extracts. It has been shown by mass spectrometry that 8,5′-cyclo-2′-deoxyguanosine occurs in DNA of human cells (22), that its concentration is increased therein by hydroxyl radicals generated by ionizing radiation (22), and that cyclo-dA predominates over 8,5′-cyclo-2′-deoxyguanosine in irradiated dsDNA (23). The yield of cyclopurine lesions is comparable to the yield of 8-oxodG in DNA exposed to ionizing radiation (24). Furthermore, 8,5′-cyclo-2′-deoxyguanosine adducts are formed when dGMP

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[1] The abbreviations used are: XP, xeroderma pigmentosum; NER, nucleotide excision repair; BER, base excision repair; cyclo-dA, 8,5′-(*S*)-cyclo-2′-deoxyadenosine; dsDNA, double-stranded DNA; CHO, Chinese hamster ovary; HCR, host cell reactivation; TT dimer, *cis,syn*-cyclobutane thymine dimer; Luc, luciferase; DMT, dimethoxytrityl; TBDMS, *tert*-butyldimethylsilyl; THF, tetrahydrofuran; kb, kilobase(s).

is exposed to oxygen radicals generated by the Fenton reaction *in vitro* (25). Although that study (25) did not identify 8,5′-cyclo-2′-deoxyguanosine in DNA exposed to the Fenton reaction conditions, the authors suggested that failure to identify the lesion could have resulted from its inhibition of the enzymes used to cleave the DNA. Subsequent work by other groups supports this possibility (20, 21).

In this work, we first studied cyclo-dA as a substrate for NER using extracts prepared from wild-type and NER-deficient Chinese hamster ovary (CHO) cells and then for BER using extracts prepared from adult mammalian brain. In addition, we developed a novel, highly sensitive host cell reactivation (HCR) assay utilizing DNA constructs containing a single site-specific cyclo-dA or a *cis,syn*-cyclobutane thymine dimer (TT dimer) (26) on the transcribed strand of a luciferase (Luc) reporter gene downstream of the strong cytomegalovirus promoter. Transfection of these constructs into NER-deficient cells allowed us to assess the effect of single lesions on gene expression in living cells, whereas transfection into repair-proficient cells allowed us to study repair of the lesion over time.

EXPERIMENTAL PROCEDURES

Synthesis and Partial Purification of Oligonucleotides with Cyclo-dA for in Vitro Experiments and for MfeI Site HCR Experiments—All steps were conducted under subdued lighting and in the absence of UV radiation. The 30-mer lesion-free single-stranded DNA oligonucleotide had the following sequence: 3′-CCAAGGACCTTGTTAACGAAAATGTCTACG-5′ (*Mfe*I site underlined). Because the cyclo-dA phosphoramidite was a 5′-*O*-phosphoramidite, its incorporation into DNA required 5′–3′ synthesis. The cyclo-dA and control dA phosphoramidites contained identical protecting and coupling groups, except that the dA phosphoramidite had N^6-benzoyl protection. Oligonucleotides were synthesized in a 5′–3′ direction on an ABI 394 DNA synthesizer (Applied Biosystems, Inc.) using 3′-*O*-DMT-2′-deoxyribonucleoside 5′-(β-cyanoethyl)-*N,N*-diisopropylaminophosphoramidite and 3′-*O*-DMT-2′-deoxyribonucleoside 5′-succinoyl-Controlled-Pore Glass synthesis supports. All synthesis reagents were from Glen Research Corp. (Sterling, VA). DNA containing the cyclic-dA lesion was synthesized up to the position of incorporation on the DNA synthesizer, at which point we coupled manually either 3′-TBDMS-8,5′-cyclodeoxyadenosine 8,5′-(β-cyanoethyl)-*N,N*-diisopropylaminophosphoramidite (for the cyclo-dA oligonucleotide) or 3′-TBDMS-dA 5′-(β-cyanoethyl)-*N,N*-diisopropylaminophosphoramidite (for the cyclo-dA control sequence). For the coupling reaction, 10 μmol of the phosphoramidite was dissolved in 0.4 ml of 0.25 M 5-ethylthio-1*H*-tetrazole/acetonitrile and coupled using two 1-ml polypropylene syringes for 10 min. Capping and oxidation were then performed on the DNA synthesizer. Following incorporation of the 3′-TBDMS-protected base, the synthesis support was washed with anhydrous acetonitrile on the synthesizer, and the support was dried with argon. 3′-TBDMS protection was removed manually using 1 M tetrabutylammonium fluoride in tetrahydrofuran (THF) (Aldrich) over 2 h at 25 °C. A 1-ml polypropylene syringe was attached to each end of the synthesis column, and 1 ml of tetrabutylammonium fluoride/THF was passed back and forth through the column using the syringes over 2 h. The support was then washed two times each with THF and then with acetonitrile and put back on the synthesizer to complete the synthesis, leaving on the final DMT. Silyl deprotection of DNA synthesized on Controlled-Pore Glass required the use of tetrabutylammonium fluoride for reduced time to minimize cleavage of the oligonucleotide from the support. Treatment of the Controlled-Pore Glass support with triethylamine/trihydrofluoride (Aldrich) resulted in nearly quantitative cleavage of the substrate from the support. The synthetic DNAs were cleaved and deprotected using NH_4OH over 16 h at 55 °C and were partially purified on a Poly-Pak cartridge (Glen Research Corp.). The products were dried in a vacuum concentrator prior to final purification by denaturing polyacrylamide gel electrophoresis.

Synthesis, Deprotection, and Partial Purification of Oligonucleotides Containing the TT Dimer for MfeI Site HCR Experiments—All steps were conducted under subdued lighting and in the absence of UV radiation. The TT dimer phosphoramidite and oligonucleotides containing the TT dimer were synthesized as described (26). Synthesis columns containing support-bound oligonucleotides with the TT dimer were deprotected in two steps. First, *O*-methyl phosphate group protection was removed from the central phosphate of the TT dimer by treatment with thiophenol/triethylamine/THF (1:2:2) for 45 min at room temperature. The synthesis support was successively washed eight times with 1 ml of THF, five times with 1 ml of methanol, and three times with 1 ml of acetonitrile and then dried by passing argon through the column; the support was transferred to an amber vial. Second, DNA products were cleaved and deprotected by adding 1 ml of concentrated NH_4OH to the vials, sealed, and incubated at 55 °C for 16 h in the dark. Vials were then cooled on ice and decanted. The support was washed with 1 ml of H_2O; the combined solutions were filtered, diluted twice with H_2O, and purified on a Poly-Pak cartridge. Partially purified DNA was eluted with 1 ml of 20% acetonitrile/H_2O, recovered by vacuum concentration, and gel-purified.

Enzymatic characterization of DNA synthesized with our TT dimer phosphoramidite verified the lesion's stereochemistry and indicated that ~90% of the expected sites contained a TT dimer (26). Subsequent primer extension experiments demonstrated that 10% of the TT dimers reverted back to thymine (data not shown). Our dsDNA constructs containing reverted sites were cut with *Mfe*I and thereby separated from the remaining covalently closed circular constructs containing TT dimers.

Synthesis and Partial Purification of 5′-Phosphorylated Oligonucleotides for NcoI Site HCR Experiments—DNA sequences for the *Hin*dIII-*Apa*I fragment of plasmid pCMVGL3PA were as follows: 5′-AGCTTGGCATTCCGGTACTGCAGGTAAAGCCACCATGGAAGACGCCAAAAACATAAAGAAAGGGCC-3′ and 3′-ACCGTAAGGCCATGACAACCATTTCGGTGGTACCTTCTGCGGTTTTTGTATTTCTTTC-5′ (*Nco*I site underlined). The *Nco*I site contained a cyclo-dA or its control dA, and the resulting 58-mers were each annealed to the 66-mer by Oligos Etc. (Wilsonville, OR). Oligonucleotide synthesis conditions were as described above, except that (3-(4,4′-dimethoxytrityloxy)-2,2-dicarboxymethylamido)propylsuccinoylpolystyrene synthesis support (27) was used to produce 5′-phosphorylated single-stranded DNA, and 3′-TBDMS protection was removed manually using triethylamine/trihydrofluoride (28) over 5 h at 25 °C.

Synthesis of DNA for BER and NER Assays—The cholesterol-containing DNA substrate for the NER assay and the etheno-dA- and 8-oxo-dG-containing DNAs for the BER assay were synthesized in the standard 3′–5′ direction. The cyclo-dA and its dA control sequence in the NER assay were synthesized 5′–3′ as described above.

Excision Nuclease Assays—Excision nuclease assays were carried out essentially as described (29). Briefly, a 140-mer duplex DNA substrate containing a cholesterol moiety, a cyclo-dA, or dA at the central position and a ^{32}P label on the sixth phosphodiester bond 5′ to the lesion was synthesized as described (29). Substrates (5×10^4 cpm) were incubated with 25 μg of whole cell extract from AA8, UV135, or UV20 CHO cells for 3 h at 25 °C. Complementation reactions contained 12.5 μg each of UV135 and UV20 whole cell extracts. Reactions were deproteinized with proteinase K, followed by phenol/chloroform extraction; recovered by ethanol precipitation; and analyzed on denaturing 10% polyacrylamide gels, followed by autoradiography.

Glycosylase Assays—Cell nuclei from rat brain were isolated as described (30) and extracted with 50 mM Tris (pH 8.0), 50% glycerol, and 0.25 M NaCl. 10 μg of nuclear extract protein was incubated with 34-mer duplex substrates (30) that contained the indicated DNA lesions and that were labeled with ^{32}P at the 5′-end of the lesion-containing strands. Reactions contained 25 mM Tris (pH 7.5), 32 mM KCl, 5 mg/ml bovine serum albumin, 2 mM dithiothreitol, 10 mM EDTA, and 1 μl (5 μg) of nuclear extract protein in the extraction buffer described above. Following a 1-h incubation at 37 °C, 2 μl of the reaction mixture was diluted into 10 μl of 10 mM NaOH containing 95% formamide, heated to 90 °C for 3 min to cleave abasic sites, and separated on a 15% denaturing polyacrylamide gel.

HCR Constructs—We constructed the 6.0-kb pCMVGL3PA plasmid from pGL3-Control (Promega) by adding *Pst*I and *Aat*II sites at positions +263 and +286, respectively; by changing *Nar*I to *Apa*I; and by replacing its SV40 promoter (from *Bgl*II-*Hin*dIII) with the corresponding cytomegalovirus promoter from pcDNA3 (Invitrogen). For certain experiments, the *Hin*dIII-*Apa*I fragment of pCMVGL3PA was replaced (31) with either cyclo-dA or the cyclo-dA control sequence containing the *Nco*I site. pCMVGL3PAΔ3 (6.0 kb) was constructed from pCMVGL3PA by site-directed mutagenesis of all *Mfe*I sites except that at position +395. M13Luc (9.4 kb) was constructed by inserting the *Bgl*II-*Bam*HI fragment containing the complete transcription unit fragment from pCMVGL3Δ3 into M13mp18. We prepared covalently closed circular dsDNA containing site-specific lesions by annealing synthetic 30-mer DNA oligonucleotides (positions 384–413 of pGL3-Control, with or without a lesion in the *Mfe*I site) as described (32), except that T7 DNA polymerase was substituted (33). Lesion-containing M13Luc prepara-

tions were digested with *Mfe*I to linearize any non-lesion-containing constructs. M13Luc was used in most HCR experiments. For some experiments, pCMVGL3PAΔ3 phagemid DNA, with or without a lesion, was constructed as described for M13Luc using single-stranded pCMVGL3PAΔ3 prepared with M13K07 as the helper phage (34). Covalently closed circular lesion-containing and lesion-free dsDNA constructs were separated from linear and nicked constructs by electrophoresis on 1% agarose containing ethidium bromide (0.5 μg/μl), electroeluted, and recovered by ethanol precipitation.

Cell Lines—The CHO lines used were from the American Type Culture Collection (Manassas, VA) and were as follows: CRL-1859 (AA8), CRL-1862 (UV20), and CRL-1867 (UV135). The SV40-transformed human cell lines used were from the Coriell Institute for Medical Research (Camden, NJ) and were as follows: GM00637 (normal), GM04429 (XP-A) from patient XP12BE, and GM04312 (XP-A) from patient XP2OS.

Transient Transfection for Luc Assay—CHO cells were grown in Eagle's α-minimal essential medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc.). SV40-transformed cells were grown in Dulbecco's modified Eagle's medium (BioWhittaker, Inc.) containing 10% fetal bovine serum. Each well of a 96-well Microlite microtiter plate (Dynex) received 3×10^3 logarithmically growing cells. After 24 h, the medium was decanted by inversion, and the cells were washed with 200 μl of Opti-MEM I (Life Technologies, Inc.). Each of the eight wells of a vertical column received 50 ng of carrier plasmid (pUC19) and either 0.1 ng of our 6.0-kb or 0.17 ng of our 9.4-kb Luc plasmid in a mixture of Opti-MEM I and LipofectAMINE reagent (Life Technologies, Inc.). After 5 h at 37 °C, 200 μl of culture medium containing 20% fetal bovine serum was added to each well. The medium was decanted 18, 24, or 48 h after the start of transfection; each well was washed with 200 μl of phosphate-buffered saline (pH 7.4); and 50 μl of 1× Passive Lysis Buffer (Promega) was added to each well. After rocking for 45 min at room temperature, lysates were assayed in an MLX microtiter plate luminometer (Dynex), which injected luciferase reagent (100 μl/well; Promega) in accord with the manufacturers' instructions.

Calculation of Relative Luc Activity—The mean Luc activity was determined from a cell line plated in a vertical row of eight wells and transfected with either a UV-irradiated or lesion-containing construct. The mean Luc activity was also determined from the same cell line plated concomitantly in an adjoining vertical row of eight wells on the same 96-well plate, but transfected with the appropriate control construct that had received no DNA damage. The relative Luc activity of a cell line, expressed as a percent, was calculated by dividing the mean Luc activity obtained with the damaged construct by that obtained with the control construct. Assuming that the ability of a cell line to be transiently transfected in our assay is the same for the lesion-containing construct as it is for the otherwise identical undamaged construct, the relative Luc activity we calculated for one cell line can be compared with that calculated for another line in the same experiment even if the lines have different transfection efficiencies.

Statistical Analysis—Analyses of the differences between cell lines in Figs. 5–7 were based on Student's paired *t* test, comparing the logarithms of percentages obtained in the same experiment (35). Analyses of the differences between cell lines from different experiments were based on Student's unpaired *t* test, comparing the logarithms of percentages (35). Logarithms were used to lessen the impact of the skewed distributions of the percentages (35). Analyses of untransformed percentages gave similar results.

RESULTS

Synthesis of (5′S)-8,5′-Cyclo-2′-deoxyadenosine 5′-O-Phosphoramidite—To study the repair of DNA containing cyclo-dA (Fig. 1), we had to develop a synthesis (Fig. 2) for its phosphoramidite, (5′*S*)-8,5′-cyclo-2′-deoxyadenosine 5′-*O*-phosphoramidite (**9**), so we could then insert cyclo-dA into oligonucleotides not only for *in vitro* studies, but also for *in vivo* studies using the Luc gene (Fig. 3). The phosphoramidite was synthesized via photochemical conversion of a derivatized 5′-phenylthio-2′,5′-dideoxyadenosine to the desired cyclonucleoside. We prepared the 5′-*O*-phosphoramidite **9**, rather than the more conventional 5′-*O*-dimethoxytrityl-3′-*O*-phosphoramidite derivative, based upon a study of molecular models that indicated that the insertion of the bulky 4,4′-DMT group onto the (5′*S*)-hydroxyl group could prove problematic. While this study was ongoing, a

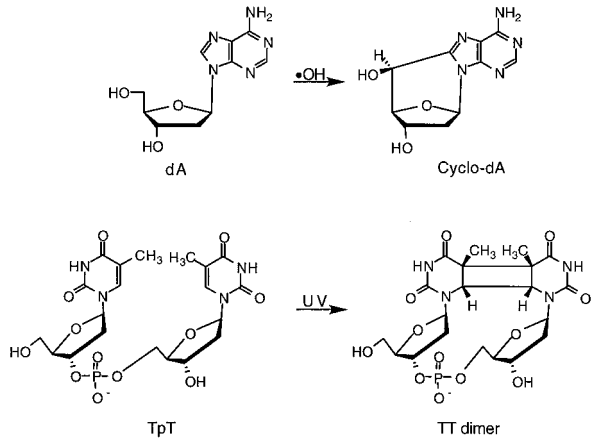

FIG. 1. **Structures of DNA lesions studied.** *Upper*, hydroxyl radicals induce formation of cyclo-dA from 2′-deoxyadenosine (*dA*). For details on our synthesis of cyclo-dA and its incorporation into DNA, see "Experimental Procedures." *Lower*, UV radiation induces formation of the TT dimer from adjacent thymines.

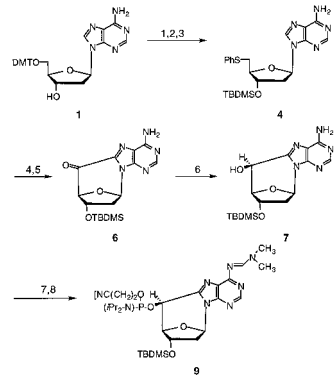

FIG. 2. **Synthesis of the cyclo-dA phosphoramidite.** For details, see "Results." **1**, TBDMS chloride (1.3 eq), 1,8-diazabicyclo[5.4.0]undec-7-ene, CH_3CN, room temperature (73%); **2**, I_2, MeOH, 30–40 °C (64%); **3**, diphenyl disulfide, $(n\text{-}Bu)_3P$, dimethylformamide (95%); **4**, λ_{254}, $P(OMe)_3$ (10 eq), CH_3CN, argon (50%); **5**, SeO_2 (2.2 eq), pyridine, reflux, 5 h (92%); **6**, KBH_4 (1.1 eq), MeOH, 2 h, room temperature (72%); **7**, dimethylformamide dimethyl acetal (10 eq), MeOH, 14 h, room temperature (93%); **8**, diisopropyl (iPr_2)-NP(Cl)[$O(CH_2)_2CN$], diisopropyl-(Et)N, THF, 0–25 °C, 2.5 h (95%). *PhS*, phenylthio.

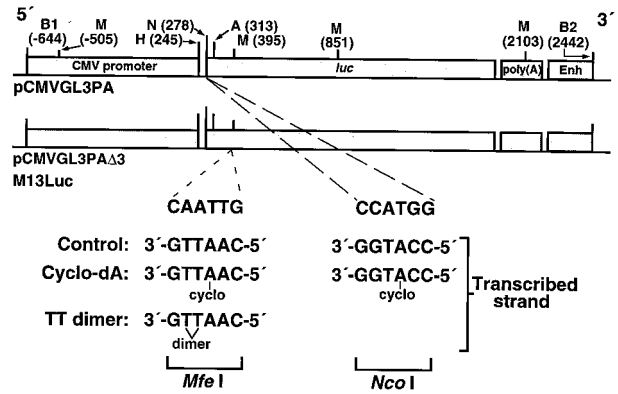

FIG. 3. **Constructs used in HCR experiments.** Relevant portions of the non-transcribed strand are diagrammed, and the original numbering was maintained. For details, see "Experimental Procedures." *M*, *Mfe*I; *H*, *Hin*dIII; *N*, *Nco*I; *A*, *Apa*I; *CMV*, cytomegalovirus; *luc*, Luc gene non-transcribed strand; *poly(A)*, SV40 late poly(A) signal; *Enh*, SV40 enhancer; *B1*, *Bgl*III; *B2*, *Bam*HI.

synthesis of a conventional 5′-*O*-DMT-3′-*O*-phosphoramidite derivative of cyclo-dA that used the same photochemical procedure was reported (20). It is interesting to note that, in this

work, the insertion of 5′-*O*-DMT was not trivial and required harsh conditions to prepare the DMT derivative in moderate yield.

In our synthesis, 5′-*O*-dimethoxytrityl-2′-deoxyadenosine (**1**) was selectively blocked at the 3′-hydroxyl by treating compound **1** with TBDMS chloride in 1,8-diazabicyclo[5.4.0]undec-7-ene to afford 3′-*O*-*tert*-butyldimethylsilyl-5′-*O*-dimethoxytrityl-2′-deoxyadenosine (73% yield). The 5′-*O*-dimethoxytrityl group was then removed to expose the 5′-hydroxyl group by treatment with iodine in warm methanol to produce 3′-*O*-TBDMS-2′-deoxyadenosine (64% yield). This derivative was subsequently converted to 5′-phenylthio-3′-*O*-TBDMS-2′,5′-dideoxyadenosine (**4**) (95% yield) (36), which was submitted to photolysis at 254 nm in acetonitrile; purged with argon; and in the presence of trimethyl phosphite, irradiated by a 400-watt medium-pressure mercury lamp with a quartz filter for 30 h to afford 8,5′-cyclo-2′,5′-dideoxyadenosine (50% yield after chromatography). Our synthesis utilized a modification of a photochemical synthesis procedure (37), yielding (5′*S*)-8,5′-cycloadenosine, to prepare the cyclo-dA system.

The 5′-hydroxyl group was generated first by oxidation of the 5′-benzylic carbon of 8,5′-cyclo-2′,5′-dideoxyadenosine by treatment with SeO_2 at reflux in pyridine to give compound **6** (95% yield). Subsequent reduction of the 5′-oxo group of compound **6** with KBH_4 in methanol furnished a stereoselective formation of the desired (5′*S*)-8,5′-cyclo-2′-deoxyadenosine (**7**) (93% yield). The assignment of 5′*S* to compound **7** was made based upon NMR analysis. The 5′-H signal appeared as a doublet at 6.40 ppm ($J_{4,5'}$ = 6.3 Hz) coupled with the 4′-H signal at 4.48 ppm. In examples of 5′*S*-derivatives of cycloadenosine, a similar coupling of the 5′-proton to the 4′-proton was observed, whereas in the 5′*R*-isomer, 5′-H is not coupled to the 4′-proton and appears as a singlet at lower field (38). Completion of the synthesis involved blocking the N^6-amine as a dimethylaminomethylidene derivative by treatment of compound **7** with dimethylformamide dimethyl acetal in methanol, and subsequent phosphitylation of the 5′-hydroxyl group using *N,N*-diisopropylaminocyanoethylphosphonamidic chloride in the presence of Hunig's base gave phosphoramidite **9** (95% yield).

Cyclo-dA Is a Substrate for NER, but Not for BER, in Vitro—We first determined whether DNA containing cyclo-dA is a substrate for the excision nuclease of the NER pathway. 140-mer duplex oligodeoxynucleotides containing a cholesterol/T base pair (39), a dA/T base pair, or a cyclo-dA/T base pair at the central position of the duplex were labeled with ^{32}P at the sixth phosphodiester bond 5′ of the central position (39). Incubation of the cholesterol-containing DNA, a known substrate for excision nuclease (39), with extract from AA8 cells resulted in the release of single-stranded DNA 24–30 nucleotides in length (Fig. 4*A*, *lane 1*, *arrowhead*) as described (39), whereas no release occurred from the lesion-free DNA (*lane 2*). The characteristic excision products of excision nuclease activity were also released from the cyclo-dA-containing duplex (*lane 3*, *arrow*).

Although release of such 24–30-mers is characteristic of NER, we used a complementation assay to seek further evidence that their release resulted from the excision nuclease. For this purpose, we assayed extracts prepared from the AA8 line and two NER-deficient lines derived from it, UV20 (lacking rodent ERCC1 protein) and UV135 (lacking XP-G protein) (8, 13). As shown in Fig. 4*B* (*lanes 1* and *2*), the two AA8 extract preparations released the 24–30-mers. Neither mutant extract demonstrated excision of cyclo-dA (*lanes 3* and *4*). However, excision occurred with a mixture of the two extracts (*lane 5*, *arrow*), albeit at a reduced rate, indicating that each extract had complemented the other (3, 8). These results confirm that

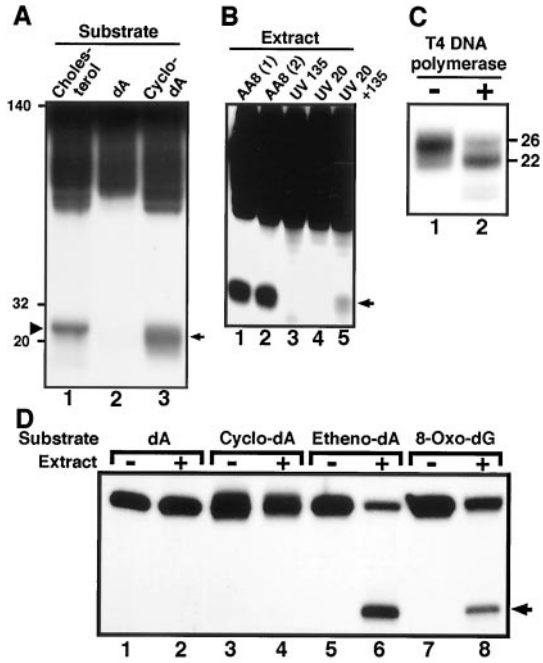

FIG. 4. ***In vitro* excision nuclease and glycosylase assays of DNA containing cyclo-dA.** *A*, NER excision nuclease assay using extracts from wild-type AA8 CHO cells. A 140-base duplex oligonucleotide containing a cholesterol moiety (39), an A/T base pair, or a cyclo-dA/T pair at the central position of the duplex and a ^{32}P label on the sixth phosphodiester bond 5′ to the lesion site was incubated with whole cell extract prepared from wild-type AA8 CHO cells, and reaction products were analyzed by polyacrylamide gel electrophoresis. Excision nuclease activity results in the release of oligonucleotides in the range of 24–30 nucleotides (*arrow*) from the cholesterol-containing substrate and cyclo-dA duplex. The cholesterol moiety is a known substrate for excision nuclease (39) and was included as a positive control. *B*, NER excision nuclease assay using extracts from the AA8 CHO line and from two NER-deficient lines (UV20 and UV135) derived from it. The 140-mer substrate containing a cyclo-dA/T pair was incubated with whole cell extract prepared from the indicated cell lines: AA8 (NER+, two separate preparations), UV20 (NER−, lacking the hamster ERCC1 homologue), UV135 (lacking the hamster XP-G homologue), or a mixture of UV20 and UV135. Excision was undetectable in either of the NER-deficient cell lines, but was detectable in a mixture of UV20 and UV135 extracts, indicating functional complementation. *C*, 3′-exonuclease activity of T4 DNA polymerase on the cyclo-dA 24–30-mer excision products recovered from a preparative scale gel. A preparative scale reaction using whole cell extract from AA8 cells similar to that shown in *B* was carried out with the cyclo-dA substrate, and the excised band was cut out of the gel. A portion was then treated with T4 DNA polymerase in the absence of dNTPs. T4 DNA polymerase-treated DNA (+) or untreated DNA (−) was separated by 20% polyacrylamide gel electrophoresis. Under these conditions, the exonuclease activity of the enzyme removes nucleotides starting from the 3′-end of the DNA and stops one nucleotide short of the position of the lesion (39), allowing determination of the location of the lesion relative to the 3′-end of the excised fragment. *D*, assay for BER activity of glycosylases in a rat brain extract. Glycosylase assays were carried out using nuclear extracts from adult rat brain. No activity was observed using the cyclo-dA/T substrate, whereas two known glycosylase substrates (etheno-dA/T (*lane 6*) and 8-oxo-dG/dC (*lane 8*)) were cleaved, demonstrating that glycosylases present in the extracts were active. *A–D* show autoradiographs of denaturing polyacrylamide gels containing the reaction products described above.

excision of cyclo-dA occurred by excision nuclease since there was no excision when either of two gene products required for NER was omitted from the reaction.

To locate the excision nuclease cleavage sites on either side of the cyclo-dA lesion, the excision products were isolated from the gel and used as substrates in the T4 DNA polymerase exonuclease assay (39). The exonuclease activity removes nucleotides starting at the 3′-end of the DNA and stops one nucleotide short of the lesion. We found that the major exci-

sion fragment was reduced in size from ~26 to 22 nucleotides (Fig. 4*C*, *lane 2*). These results indicate that the excision nuclease in the AA8 extract had cleaved the duplex approximately five nucleotides 3′ and 21 nucleotides 5′ to the cyclo-dA lesion. Similar asymmetric incision patterns have been reported for known NER substrates (8, 39).

BER is the major pathway for repairing most oxidative DNA damage (8). BER is initiated by specific DNA glycosylases that remove damaged or mismatched bases from DNA (18). To assess whether glycosylases present in brain nuclear extract could remove cyclo-dA, substrates were prepared as described (30) containing either a dA/T or cyclo-dA/T base pair at the central position of a 34-base pair dsDNA. We then incubated these duplexes with a nuclear extract from adult rat brain (30) (Fig. 4*D*). The small size of the lesion-containing substrate and the absence of magnesium in the reaction buffer preclude the action of excision nuclease in these reactions. We detected no cleavage by the extract of either the undamaged duplex (*lane 2*) or the cyclo-dA-containing duplex (*lane 4*). DNA polymerase-blocking experiments confirmed the presence of cyclo-dA at its intended position of incorporation (data not shown). Using the same extract, we readily detected glycosylase activity for the known BER substrates (18) etheno-dA/T (*lane 6*) and 8-oxo-dG/dC (*lane 8*) as well as for a T/dG mismatch and for uracil-containing DNA (dU/dA) (data not shown). We conclude that cyclo-dA is not a substrate for glycosylases present in brain nuclear extracts. However, the possibility that cyclo-dA can be removed by a glycosylase that either is unstable or requires cofactors that are unstable in our extracts cannot be ruled out.

A Novel HCR Assay Using Constructs Containing Site-specific DNA Lesions—Having shown that cyclo-dA is a substrate for NER *in vitro*, we sought to confirm this finding *in vivo* and to determine the effect of this lesion on gene expression in living cells. For this purpose, we prepared DNA constructs containing a single cyclo-dA or a TT dimer at a specific site on the transcribed strand of a Luc reporter gene contained in covalently closed circular, non-replicating dsDNA constructs. We used the Luc assay because it is more sensitive and has a larger range of linearity (>4 orders of magnitude) (40) than previously used reporter genes (1, 6, 10). Furthermore, Luc has a half-life of just 3 h in mammalian cells and therefore provides a better instantaneous monitor of transcription than more stable reporters (40). Following others (10), we chose a cytomegalovirus promoter to obtain a high level of transcription in our mammalian cells. Transfection of only 3×10^3 normal cells with 0.1 ng of undamaged plasmid resulted in as much as $1–2 \times 10^5$ relative light units, compared with 3–6 units without the plasmid (data not shown).

As shown in Fig. 3, we replaced the normal dA with cyclo-dA at either position +281 in the *Nco*I site containing the initiator methionine of the Luc gene coding sequence or at position +398 (codon 39) in the gene's *Mfe*I site. We also prepared an additional construct in which the two adjacent T residues at positions +396 and +397 of the *Mfe*I site were replaced with a TT dimer. The use of the *Mfe*I restriction site allows both lesions to be studied in a nearly identical sequence context (Fig. 3). For each lesion-containing construct, a control construct was prepared using undamaged DNA synthesized in the same manner as the lesion-containing construct. Placement of the lesions in the unique *Mfe*I restriction site enabled us to remove any lesion-free material by *Mfe*I digestion prior to final purification (4, 32) since cyclo-dA and the TT dimer prevented cleavage by the enzyme. Confirmation of the HCR constructs and of the absence of detectable non-lesion-containing molecules in the final preparations of lesion-containing constructs was obtained by restriction digestion with *Mfe*I (data not shown).

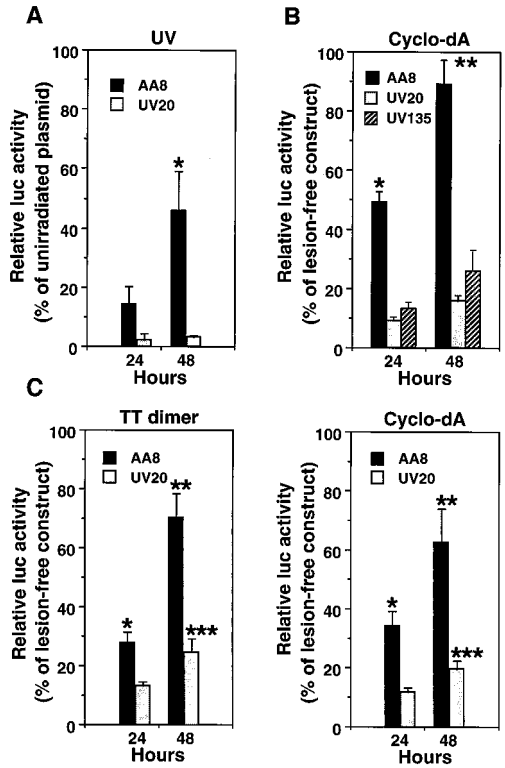

FIG. 5. **Effect of DNA lesions on gene expression in transfected CHO cells.** At each assay time of an experiment, the mean Luc activity for each construct was calculated as the mean of eight wells, and time was measured from the start of the 5-h transfection period. The relative Luc activity of a cell line, expressed as a percent, was calculated by dividing the mean Luc activity obtained with the damaged construct by that obtained with the control construct. Further details are given under "Experimental Procedures." *, **, and ***, $p < 0.02$. *A*, cells were transfected with UV-irradiated (254 nm, 304 $J \cdot m^{-2}$) or control (mock-irradiated) pCMVGL3PA (0.1 ng/well). Each *bar* represents the mean ± S.E. of two experiments. *, *versus* UV20. *B*, cells were transfected with pCMVGL3PAΔ3 (0.1 ng/well) containing cyclo-dA in its *Mfe*I site or with the plasmid's cyclo-dA control. Each *bar* represents the mean ± S.E. of 11 experiments. *, *versus* UV20 and UV135; **, *versus* UV20 and UV135. *C*, cells were transfected (0.17 ng/well) with M13Luc containing a TT dimer in its *Mfe*I site, with the M13Luc TT dimer control, or with M13Luc containing cyclo-dA in its *Mfe*I site. Each *bar* represents the mean ± S.E. of eight experiments. *, *versus* UV20; **, *versus* UV20; ***, *versus* the 24-h UV20 value.

As detailed under "Experimental Procedures," the effect of a lesion on gene expression was determined by expressing Luc activity obtained with the lesion-containing construct as a percent of that obtained with the appropriate lesion-free construct transfected into replicate wells of a cell line on the same plate in each experiment. Thus, any differences in transfection ability between cell lines will not affect the results.

A Single Cyclo-dA on the Transcribed Strand of a Gene Strongly Reduces Gene Expression and Is Repaired by NER in CHO Cells—To validate our HCR assay as an indicator of NER, we irradiated pCMVGL3PA with 254-nm UV light and transfected it into the AA8 and NER-deficient UV20 lines. As shown in the 24-h assay of Fig. 5*A*, UV radiation resulted in relative Luc activity of 15% in the AA8 line and of 5% in the NER-deficient UV20 line. However, activity increased considerably by 48 h in the AA8 line, but not in the UV20 line. We interpret these differences in relative Luc activity to reflect the lines' different NER capacities since direct evidence has been obtained showing that the TT dimer is removed from the reporter gene of UV-irradiated plasmids transfected into wild-type cells, but not into cells lacking NER, and that removal of this transcription-blocking lesion is responsible for reactivation of re-

porter gene activity (6, 10).

We then transfected the AA8, UV20, and UV135 lines with pCMVGL3PAΔ3 containing cyclo-dA in its *Mfe*I site. Both mutants showed markedly defective repair of the lesion *in vivo* (Fig. 5*B*). In contrast, the AA8 line repaired cyclo-dA efficiently, approaching complete repair at 48 h. We obtained similar results when cyclo-dA was in the *Mfe*I site of M13Luc (data not shown). These results extend the *in vitro* results (Fig. 4, *A–C*) and demonstrate that cyclo-dA is a substrate for NER *in vivo* as well as *in vitro*.

To rule out the possibility that some of the reduction of Luc activity in the AA8 and mutant lines resulted from a toxic effect induced by unrepaired cyclo-dA, we transfected cultures of each line with an undamaged plasmid, with the cyclo-dA-containing plasmid, or with both plasmids. We found that Luc activity in the cotransfected cultures of each line reached 100% of the expected sum of activities of its singly transfected cultures (data not shown). Thus, we conclude that normal and mutant CHO cells transfected with cyclo-dA retain full capacity to express Luc activity from undamaged plasmid and that the mutant lines' failure to restore Luc activity from a cyclo-dA-containing construct is due to their inability to repair the cyclo-dA by NER.

Next, we determined the effect of the TT dimer on Luc activity in the AA8 and UV20 lines. The dimer reduced Luc activity in both the AA8 and UV20 lines, markedly in the 24-h assay and less so in the 48-h assay (Fig. 5*C*, *left panel*). At each time, the AA8 line gave significantly more Luc activity than the mutant line. Similar results were obtained with cyclo-dA studied in the same experiments (*right panel*). We conclude that the two lesions have a similar effect on gene expression and that both lesions were repaired equally well in the AA8 line.

Defective Removal of Cyclo-dA from an Active Gene Is Associated with XP Neurological Disease—Patients in XP complementation group A are characterized by a severe defect in NER and by neurodegeneration (2, 15). We therefore performed HCR studies on SV40-transformed human cell lines derived from a normal individual (1) and from XP12BE, a complementation group A patient who developed XP neurological disease in childhood (2, 15). As expected (1), we found that the XP line had markedly reduced Luc activity when transfected with UV-irradiated pCMVGL3PA (data not shown). We then transfected the normal and XP-A cells with the M13Luc construct containing either a TT dimer or cyclo-dA (Fig. 6*A*). When transfected with the TT dimer construct, the relative Luc activity of the normal line was significantly greater than that of the XP-A line from patient XP12BE at 24 and 48 h, but not at 18 h, after transfection (*left panel*). In contrast, when transfected with cyclo-dA, the relative Luc activity of the normal line was significantly greater than that of the XP-A line at each assay time (*right panel*).

To provide further evidence for defective repair of cyclo-dA in XP-A patients with neurodegeneration, we studied cells from patient XP2OS, a Japanese patient who developed severe neurodegeneration in early childhood (41). As shown in Fig. 6*B*, cells from this XP-A patient also showed markedly reduced relative Luc activity with the cyclo-dA construct.

Effect of Cyclo-dA in Another Sequence Setting—To assess the generality of our results with cyclo-dA in the *Mfe*I site, we prepared an additional construct in which cyclo-dA was placed in the *Nco*I site containing the start codon of the Luc gene (Fig. 3). This construct was prepared by ligating dsDNA containing the lesion into restriction-digested pCMVGL3PA (31). The relative Luc activity with cyclo-dA in this location obtained in the XP-A cells (Fig. 7) was similar to that obtained with the lesion in the *Mfe*I site of M13Luc (Fig. 6*A*, *right panel*).

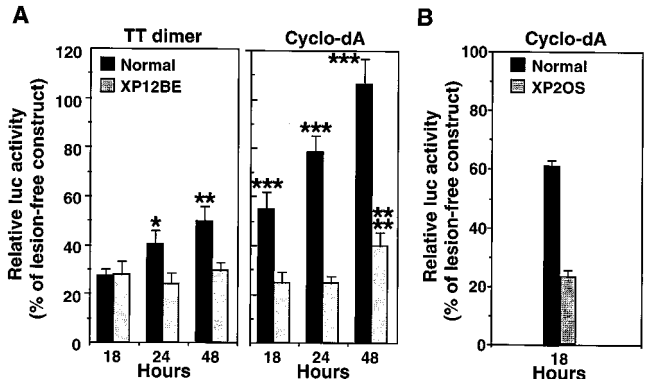

FIG. 6. **Effect of DNA lesions on gene expression in transfected SV40-transformed human cells.** At each assay time of an experiment, light for each construct was calculated as the mean of eight wells, and time was measured from the start of the 5-h transfection period. The relative Luc activity of a cell line, expressed as a percent, was calculated by dividing the mean Luc activity obtained with the damaged construct by that obtained with the control construct. Further details are given under "Experimental Procedures." *A*, normal and XP-A (XP12BE) cells were transfected (0.17 ng/well) with M13Luc containing a TT dimer in its *Mfe*I site or with its control construct (*left panel*) and with M13Luc containing cyclo-dA in its *Mfe*I site or with its control construct (*right panel*). Each *bar* represents the mean ± S.E. of seven experiments in which the TT dimer and cyclo-dA were studied concomitantly. *, **, ***, and ****, p = or < 0.05. *, *versus* the 24-h XP-A value; **, *versus* the normal 18-h value and the 48-h XP-A value; ***, *versus* the corresponding XP-A value and the other two normal values; ****, *versus* the 18- and 24-h XP-A values. *B*, normal and XP-A (XP2OS) cells were transfected (0.17 ng/well) with M13Luc containing cyclo-dA in its *Mfe*I site or with its control construct. Each *bar* represents the mean ± S.E. of three experiments. p = 0.005, normal *versus* XP2OS cells.

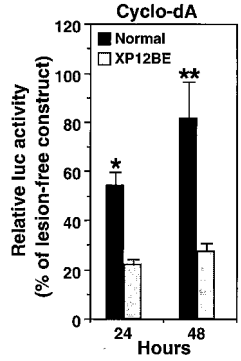

FIG. 7. **Cyclo-dA in the *Nco*I site gives results similar to those when in the *Mfe*I site.** At each assay time of an experiment, light for each construct was calculated as the mean of eight wells, and time was measured from the start of the 5-h transfection period. The relative Luc activity of a cell line, expressed as a percent, was calculated by dividing the mean Luc activity obtained with the damaged construct by that obtained with the control construct. Further details are given under "Experimental Procedures." Normal and XP-A (XP12BE) cells were transfected (0.1 ng/well) with pCMVGL3PA containing cyclo-dA in its *Nco*I site or with its control construct. Each *bar* represents the mean ± S.E. of four experiments. *, p = 0.011 *versus* the 24-h XP-A value; **, p = 0.001 *versus* the 48-h XP-A value.

DISCUSSION

In this study, we have shown that cyclo-dA is a substrate for excision nuclease in extracts from mammalian cells, indicating that it is repaired by NER. In contrast, we could not detect any evidence for glycosylase-initiated BER of cyclo-dA. In addition, using our newly developed HCR assay employing DNA constructs containing site-specific DNA lesions, we showed that a single cyclo-dA on the transcribed strand of an active gene strongly reduces gene expression and is repaired by NER in living mammalian cells. Finally, using our HCR assay in human cells from XP-A patients with neurological disease, we

found defective repair of cyclo-dA, but not of the TT dimer. Based on these findings, we conclude that cyclo-dA has the properties of a DNA lesion that could contribute to XP neurodegeneration. More definitive evidence that cyclo-dA plays such a role must await studies on other XP complementation groups as well as studies designed to detect accumulation of cyclo-dA in XP neurons.

Our results in Fig. 6*A* suggest that the normal human line repairs cyclo-dA considerably faster than the TT dimer. This situation may be analogous to that in which the (6-4) photoproduct induced by UV radiation is repaired faster than the TT dimer in human cells *in vitro* (4) and *in vivo* (42). As shown in Fig. 5*C*, the AA8 CHO line repaired the TT dimer (*left panel*) and cyclo-dA (*right panel*) at similar rates, providing another example of a difference in NER between human and CHO cells (8, 43).

With both cyclo-dA and the TT dimer, we detected significant relative Luc activity (20–30%) in the XP-A line as early as 18 h (Fig. 6*A*). This "background" activity is not due to undamaged molecules because we did not detect any undamaged molecules that could account for this activity in our lesion-containing preparations. We believe this activity is not due to NER because it did not increase from 18 to 24 h and because fibroblasts from this XP-A patient are extremely deficient in NER, having $<2\%$ of normal UV radiation-induced unscheduled DNA synthesis (44). Although we cannot conclusively rule out some type of DNA repair other than NER such as recombination, the observation of this background activity at the earliest time points studied argues against this possibility. Based on these considerations, our interpretation is that this background may reflect some bypass of the lesion by RNA polymerase II *in vivo*. Although a single TT dimer is a nearly complete block to transcription elongation *in vitro* (45), there is evidence that RNA polymerase can perform some bypass of TT dimers *in vivo* (46, 47). Even considering this background Luc activity as resulting from bypass of the dimer, our HCR results directly demonstrate that the single TT dimer we inserted within the reporter gene is a strong block to gene expression. This confirms a similar conclusion previously derived from analysis of HCR experiments using plasmid irradiated with varying doses of UV light (1, 6, 10). The amount of bypass we found with a single TT dimer would not have been apparent in the prior studies (1, 6, 10). Since unrepaired cyclo-dA also decreases gene expression in XP-A cells to the same extent as the TT dimer (Fig. 6*A*), we conclude that cyclo-dA also serves as a strong block to transcription in these cells.

We obtained similar results whether cyclo-dA was in the *Nco*I (Fig. 7) or *Mfe*I (Fig. 6*A*, *right panel*) site. Thus, in both sequence settings, Luc activity increased markedly from 24 to 48 h only in the normal line, whereas the background activity in the XP-A line at 24 and 48 h was 20–30%. Therefore, the effect of the lesion on gene expression is unrelated to the sequence in which cyclo-dA is contained or to the method used to prepare the construct in which the lesion is placed. However, the results obtained with the *Nco*I site, which contains the initiator methionine, shed additional information on the background Luc activity. In contrast to cyclo-dA in the *Mfe*I site, only error-free bypass of the lesion in the *Nco*I site by RNA polymerase, *i.e.* insertion of U opposite cyclo-dA, could result in Luc activity. We conclude this because incorporation of any other nucleotide would destroy the initiator methionine codon, and the next methionine is out of frame for Luc protein.

In conclusion, our HCR assay, in which a single DNA lesion is incorporated at a specific site on the transcribed strand of the Luc reporter gene, makes it possible to study the capacity of living cells to repair any transcription-blocking lesion that can be similarly incorporated. Although our study was confined to XP, our methods could readily be applied to cells from patients with other neurological diseases. Examples would be Cockayne's syndrome, in which defective transcription-coupled repair of oxidative DNA damage has been implicated (48, 49), and Alzheimer's disease, in which defective DNA repair has been postulated (12).

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