Materials and Methods

Preparation of Constructs

Oligonucleotides containing either a single cyclo-dA lesion or a single CPD in an unique MfeI site were prepared as described previously (Brooks et al., 2000). We note that the two lesion-containing oligonucleotides we used were prepared in different laboratories, from phosphoramidites synthesized by entirely different methods, and using different oligonucleotide synthesis chemistries, as described in detail previously (Brooks et al., 2000). After obtaining the initial results demonstrating the 5′A mutation, the location of the cyclo-dA lesion in the oligonucleotide was verified by Dr. Jacob Theruvathu using mass spectrometry as described (Jaruga et al., 2004).

The plasmid used, pCMV-I-GL3 contained a transcription unit consisting of a CMV promoter followed by a chimeric intron, the luciferase coding sequence, the SV40 late polyadenylation signal, and an SV40 enhancer. The construction of this plasmid was described previously (Marietta et al., 2002). Preparation of single-stranded (ss) DNA, annealing of the oligo to ssDNA, DNA synthesis using T7 DNA polymerase, and ligation were done as described (Marietta et al., 2002). Purification of the lesion-containing constructs was done using an adaptation of the enzymatic method described previously (Marietta et al., 2002). After DNA synthesis and ligation, constructs containing either a cyclo-dA or TT-dimer lesion were treated with 10 U MfeI (NEB) for 1 hour at 37°C to remove any lesion-free plasmid. At the same time, shrimp alkaline phosphatase (Sigma) was also added to a final concentration of 20 U/mL. Lesion-free
constructs were incubated in parallel but without adding MfeI. The completeness of the digestion was confirmed by adding 100 ng of a tester plasmid to a 20 uL aliquot of the MfeI digest, followed by agarose gel electrophoresis. After the digestion was complete, enzymes were heat inactivated at 65°C for 15 minutes. Then, T7 DNA polymerase (NEB) and Plasmid-Safe ATP-Dependent DNAse (Epicentre) were each added to a final concentration of 0.1 U/uL, and incubated at 37°C for 1 hour. An aliquot of the reaction was analyzed by agarose gel electrophoresis to confirm the absence of any material other than covalently-closed circular DNA. The enzymes were then heat-inactivated at 65°C for 20 minutes, and plasmids stored at 4°C until transfection.

**Cell Culture, Transfection and RNA Isolation**

The SV40 transformed fibroblast cell lines GM04429 (XPA, XP12BE) and GM08207 (XPD, XP6BE), were obtained from Coriell Institute for Medical Research (Camden, NJ, USA). SV40 transformed cells from a CSB patient, CS1BE, were a gift from Dr. Kiyoji Tanaka, Osaka University. The mutations in these patients are described at [www.xpmutations.org](http://www.xpmutations.org).

Cells were plated on 12 well plates and transfection was carried out using Lipofectamine (Invitrogen) as described (Marietta et al., 2002). Harvesting of cells took place 48 hours (Table 1) or 24 hours (enrichment experiments and Table 2) after transfection by the addition of 500 μL of RNABee (Tel-Test, Inc; Friendswood, TX, USA) or lysis with 500 μL of 1X passive lysis buffer (Promega) followed by ethanol precipitation and isolation of RNA from the precipitates using RNA Bee. Forty μg of glycogen was added to each RNA sample as a carrier. Luciferase assays carried out on aliquots removed from samples of XPA cells prepared in passive lysis buffer prior to
RNA precipitation verified that the effect of the cyclo-dA lesion on luciferase activity (60-90% reduction), was consistent with previous observations (Brooks et al., 2000 and unpublished observations).

RNA pellets were dissolved in 50 µL of 5 mM DTT in DEPC - treated water, frozen on dry ice, and kept at -80 °C until further processing. Isolated RNA was subjected to two rounds of DNAse treatment, first using an Ambion RNA free kit, followed by DNA-FREE RNA Kit (ZYMO Research). The RNA was frozen on dry ice and stored at -80 °C until use.

RT-PCR and Gel Purification

RT-PCR was performed using the One-Step RT-PCR Kit (Qiagen) with luciferase primers 871 (5’GTCGTGAGGCACTGGGCAGGT/GT) and 1190 (5’ACGATTCTGTGATTTGTATT). The / in the sequence of primer 871 indicates the location of the splice site. RT-PCR was performed using either a Perkin Elmer GeneAmp 9600 PCR or 9700 PCR machine (Applied Biosystems, Foster City, CA USA). The RT was done for 30 minutes at 50 °C followed by incubation at 95 °C for 15 minutes to inactivate reverse transcriptase and activate Taq. This was followed by 35 cycles of 94 °C for 30 sec, annealing at 57°C for 30 seconds, and an elongation at 72°C for 1 min. Following a final elongation of 10 min at 72°C and cooling to 4°C, the RT-PCR products were run on a 2 % agarose gel. The 357 bp band (Fig. 1C) resulting from spliced RNA transcripts was cut from the agarose and gel purified using the Promega Wizard SV Gel and PCR Clean-up System. The gel purified PCR product was cloned into pCR4 using
the pCR4-TOPO cloning kit from Invitrogen (Invitrogen). The gel purified PCR product was mixed with the pCR4-TOPO vector and incubated overnight at 4°C, then transformed into competent *E. coli* (One Shot DH5α or Top10, Invitrogen), and plated on LB agar plates containing 100 μg/ml ampicillin. Individual colonies from the transformed plates were selected and grown in LB-ampicillin, and plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (Qiagen). Plasmids were screened for mutations by *Mfe*I digestion followed by agarose gel electrophoresis. *Mfe*I resistant clones were sequenced using automated sequencing methods.

To increase the efficiency of detecting cDNAs corresponding to mutant transcripts, an *Mfe*I enrichment step was included. RT-PCR products were inserted into pCR4-TOPO, and the mixture used to transform competent *E. coli*. The transformation mixture was grown overnight in LB with ampicillin, and plasmid DNA isolated. The isolated plasmid DNA was digested with *Mfe*I and transformed into the Top 10 or DH5α cells, followed by plating on LB-ampicillin plates. Individual colonies of *Mfe*I-resistant clones were picked and plasmid DNA isolated and sequenced as described above.

**Sequencing**

Automated DNA sequencing was carried out on plasmids using 96-well plate capillary sequencing apparatus, either by a commercial facility (Macrogen, Inc) or by Gary Jenkins and Dr. Zhifeng Zheng, Laboratory of Neurogenetics, NIAAA.

The Chi-square test was used to determine whether the frequencies of the 5’A and MND transcripts obtained from different cell lines transfected with the cyclo-dA plasmid (Table 2) were significantly different. For this analysis, all types of MND transcripts
were pooled. The analysis was carried out using a Web-based chi-square calculator http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html.

