Transcriptional bypass of bulky DNA lesions causes new mutant RNA transcripts in human cells

Cheryl Marietta & Philip J. Brooks⁺

Section on Molecular Neurobiology, Laboratory of Neurogenetics, National Institute on Alcohol Abuse and Alcoholism, Bethesda, Maryland, USA

Here, we characterize the mutant transcripts resulting from bypass of an 8,5'-cyclo-2'-deoxyadenosine (cyclo-dA) or cyclobutane pyrimidine dimer (CPD) by human RNA polymerase II (Pol II) *in vivo*. With the cyclo-dA lesion, we observed two new types of mutant transcripts. In the first type, the polymerase inserted uridine opposite the lesion and then misincorporated adenosine opposite the template deoxyadenosine downstream (5') of the lesion. The second type contained deletions of 7, 13 or 21 nucleotides (nt) after uridine incorporation opposite the lesion. The frequency of the different types of transcript from the cyclo-dA lesion in mutant human cell lines suggests that the Cockayne syndrome B protein affects the probability of deletion transcript formation. With the CPD-containing construct, we also detected rare transcripts containing 12 nt deletions. These results indicate that RNA pol II in living human cells can bypass helix-distorting DNA lesions that are substrates for nucleotide excision repair, resulting in transcriptional mutagenesis.

Keywords: RNA polymerase; Cockayne syndrome; *xeroderma pigmentosum*; cyclopurine

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INTRODUCTION

DNA lesions that cause significant helical distortion are repaired by the nucleotide excision repair (NER) pathway (Andressoo & Hoeijmakers, 2005). Such lesions are strong blocks to DNA replication and transcription. During DNA replication, blocking lesions are avoided by switching to specialized polymerases that can bypass DNA lesions (Friedberg *et al*, 2005a). However, this mechanism is not possible for RNA polymerases owing to the requirement for initiation at promoter sequences.

Prokaryotic RNA polymerases have been shown to bypass non-bulky DNA lesions including 8-oxoguanine and uracil in a process known as transcriptional mutagenesis (TM; Doetsch, 2002). We

have previously shown that two helix-distorting DNA lesions—the cyclobutane pyrimidine dimer (CPD) and 8,5'-cyclo-2'-deoxyadenosine (cyclo-dA)—are strong but incomplete blocks to transcription in NER-deficient human cells (Brooks *et al*, 2000), indicating that these lesions might also cause transcriptional mutagenesis. Here, we describe a new method for studying transcriptional mutagenesis in living human cells. Our results indicate that both lesions stimulate the production of new types of mutant RNA transcript.

RESULTS

Studying lesion bypass by RNA polymerase II *in vivo*

We investigated the bypass of cyclo-dA and CPD in human cells as shown in Fig 1. First, constructs containing a single lesion or control (lesion-free) constructs were prepared and transfected into simian virus 40 (SV40)-transformed cells from NER-deficient XPA (xeroderma pigmentosum, complementation group A) patient XP12BE (Kraemer *et al*, 1975). The lesions were placed in a unique *MfeI* restriction enzyme site (5'-CAATTG-3'). This allowed a restriction digestion stage to remove any detectable lesion-free molecules before purification and transfection, and also provided a rapid method to screen for complementary DNAs derived from mutant transcripts. The constructs used did not contain any mammalian DNA replication sequences.

To detect transcriptional mutagenesis, we carried out reverse transcription-PCR (RT-PCR) of RNA isolated from cells transfected with lesion-containing or control constructs by using a primer pair that spanned the location of the lesion. The upstream primer also spanned the splice site, thus precluding the possibility of amplifying lesion-containing plasmid DNA, which could complicate the analysis. The cDNA amplification products corresponding to the spliced RNA (Fig 1C) were purified, cloned into plasmids and transformed into bacteria. Plasmid DNA was isolated from individual colonies, screened for mutagenesis by *MfeI* digestion and the *MfeI*-resistant plasmids were sequenced.

A new type of misincorporation mutation from cyclo-dA

We analysed a total of 81 colonies from two RNA preparations from replicate wells transfected with the cyclo-dA construct. The results are summarized in Table 1. The most frequent *MfeI* site mutation had the sequence 5'-CAATAG-3'. As illustrated in Fig 2,

Section on Molecular Neurobiology, Laboratory of Neurogenetics, National Institute on Alcohol Abuse and Alcoholism, 5625 Fishers Lane, Room 3S-32, MSC 9412, Bethesda, Maryland 20892, USA

⁺Corresponding author. Tel: +1 301 496 7920; Fax: +1 301 480 2839;

E-mail: pjb Brooks@mail.nih.gov

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Fig 1 | An experimental strategy for detecting lesion bypass by RNA. (A) Structures of the DNA lesions cyclo-dA and CPD investigated in this study. (B) Strategy for analysis of transcriptional mutagenesis by cyclo-dA or a CPD *in vivo*. Double-stranded DNA constructs containing site-specific DNA lesions are prepared and transfected into NER-deficient cells. The **V** on the construct represents the location of the intron. **A** indicates the location of the cyclo-dA and **T-T** indicates the location of the CPD on the transcribed strand of the luciferase gene. The spliced RNAs (**Δ** in the messenger RNA represents the splice site) containing mutations resulting from bypass of the lesion (**X**) in the cell are amplified by RT-PCR, cloned into plasmids, screened for mutations by *MfeI* digestion and the mutants are sequenced. (C) RT-PCR analysis of RNA from cells transfected with cyclo-dA (lane 1), CPD (lane 2) or lesion-free constructs (lane 3). No band was detected in the absence of input RNA (lane 4). The band is the expected size of the PCR product derived from spliced RNA (357 bp), whereas no band is detectable at the position where amplified plasmid DNA (489 bp) would migrate. The PCR conditions used are not suitable for quantitative comparisons. CPD, cyclobutane pyrimidine dimer; cyclo-dA, 8,5'-cyclo-2'-deoxyadenosine; NER, nucleotide excision repair; RT-PCR, reverse transcription-PCR.

this sequence was consistent with RNA polymerase II (Pol II) having incorporated the 'correct' ribonucleotide, i.e. uridine (U), opposite the cyclo-deoxyadenosine (cyclo-dA) lesion, followed by misincorporation of an adenosine (rA) opposite the next nucleotide downstream (5') to the lesion, which was a dA. We refer to these as 5'A mutations. PCR products containing this sequence could not have arisen from PCR contamination because no molecule containing this sequence had been produced previously in our laboratory. In addition, in the sequence context used here, the 5'A mutations do not change the predicted amino-acid sequence of the luciferase protein (Fig 2C).

Table 1 | Mutant RNA transcripts resulting from bypass of a 8,5'-cyclo-2'-deoxyadenosine lesion

Construct	Number of clones
<i>Cyclo-dA</i>	81
<i>MfeI</i> digested	36*
<i>MfeI</i> resistant	45 [‡]
WT	4
5'A	24
–7	7
–13	2
–21	1
5'-CAAATG-3'	1
5'-CAATCG-3'	1 [§]
Control (no lesion)	30
<i>MfeI</i> digested	26
<i>MfeI</i> resistant	4
WT	4

*The WT sequence is 5'-CAATG-3'. [‡]Five of the *MfeI*-resistant clones did not give interpretable sequence data. [§]This sequence could be explained by a T to C transversion during PCR, which is the most common type of mutation produced by *Taq* polymerase (Tindall & Kunkel, 1988). ^{||}All four of the *MfeI*-resistant clones from cells transfected with lesion-free constructs were found to be WT on sequencing. 5'A, misincorporation of adenosine opposite the template dA 5' to the lesion; cyclo-dA, 8,5'-cyclo-2'-deoxyadenosine; WT, wild type.

Cyclo-dA generates MND transcripts

In addition to 5'A mutations, we also observed a series of RNA transcripts that had deletions of 7, 13 or 21 nt (Fig 3). The sequence of the –7 deletion transcripts could, in theory, have resulted from Pol II incorporating a U opposite the lesion, misincorporating rA opposite the next downstream base and then skipping 8 nt downstream to the template dG. However, the sequence of the –13 and –21 transcripts would not be consistent with this explanation. Therefore, as indicated in Fig 3, we interpreted these deletion transcripts as resulting from incorporation of a U opposite the cyclo-dA lesion and then reinitiating transcription at a template dA nucleotide 7, 13 or 21 nt downstream. We refer to these as multiple nucleotide deletion (MND) transcripts.

Transcripts generated from lesion-free control constructs

We also analysed 30 colonies prepared from wells transfected with lesion-free constructs, and all were found to be wild type (see Table 1).

MND transcripts from a CPD

The same approach was used to search for mutant transcripts resulting from bypass of the CPD lesion; however, we were unable to detect any mutants in the screening of 40 individual colonies by *MfeI* digestion. Therefore, we modified our protocol to include an *MfeI* digestion enrichment step before transforming bacteria and picking colonies for sequencing.

To validate the *MfeI* digestion enrichment method, we carried out an experiment with RNA prepared from cells transfected with

Fig 2 | An 8,5'-cyclo-2'-deoxyadenosine lesion stimulates a novel type of misincorporation event when transcribed by polymerase II. (A) The sequences of the 5'A and WT transcripts are indicated above the double-stranded DNA construct, showing the location of the cyclo-dA lesion (A). (B) Sequence electropherogram of the cDNAs corresponding to the WT or 5'A transcripts. (C) The predicted open amino-acid sequences of the WT and 5'A mutant transcripts are the same. 5'A, misincorporation of adenosine opposite the template dA 5' to the lesion; cyclo-dA, 8,5'-cyclo-2'-deoxyadenosine; WT, wild type.

a new preparation of cyclo-dA-containing plasmid. Sequence analysis of the 24 clones showed 63% to be 5'A mutation clones and 29% to be -7 deletion clones. The relative ratio of these two mutations is similar to that observed previously (Table 1), thereby replicating the original finding and validating the *MfeI*-enrichment method. In addition, this analysis showed two novel mutant transcripts: one containing a deletion opposite the cyclo-dA lesion and the other a 9 nt deletion beginning after incorporation of U opposite the lesion.

By using the *MfeI* pre-enrichment method with RNA prepared from cells transfected with the CPD-containing construct, 40% (4 out of 10) of the *MfeI*-enriched products were found to contain a deletion of 12 nt (Fig 3B), with the remainder being wild type. The sequence of these mutant transcripts indicated that the polymerase incorporated rA opposite the 3' thymidine (T) of the CPD, followed by skipping 12 nt downstream before reinitiating transcription. Consequently, this mutation is qualitatively similar to the deletion mutations observed with the cyclo-dA-containing constructs. As the actual sequences are different, however, this eliminates the possibility of PCR contamination.

CSB affects the probability of MND transcripts

To investigate the role of other NER proteins in MND formation, we transfected a cyclo-dA construct into SV40-transformed cells from the XPA patient XP12BE, a helicase-deficient XPD patient (XP6BE) and a Cockayne syndrome B (CSB) patient (CS1BE). As shown in Table 2, both 5'A and MND transcripts were observed in all three cell lines, indicating that neither the full-length CSB protein nor the helicase activity of XPD is essential for either type of transcript. This analysis also showed another type of MND transcript containing a -19 deletion (Table 2). Importantly, the

Fig 3 | Transcripts containing stepwise multiple nucleotide deletions resulting from bypass of an 8,5'-cyclo-2'-deoxyadenosine lesion or a cyclobutane pyrimidine dimer lesion. (A) The sequences of the MND transcripts are indicated above the double-stranded DNA construct, showing the location of the cyclo-dA lesion (red A). The underlined nucleotides represent potential template nucleotides for resuming transcription (see Fig 4). Sequence electropherograms of cDNAs corresponding to the -7, -13 and -21 nucleotide (nt) deletion transcripts are below. (B) The sequence of the -12 nt deletion transcript obtained from cells transfected with a CPD is indicated above the double-stranded DNA construct showing the location of the CPD (red T-T). The underlined nucleotides represent potential template nucleotides for resuming transcription. A sequence electropherogram of the -12 nt cDNA is shown below. CPD, cyclobutane pyrimidine dimer; cyclo-dA, 8,5'-cyclo-2'-deoxyadenosine; MND, multiple nucleotide deletions.

ratio of MND to 5'A mutants was lower in the CSB cell line than in either of the other two lines, suggesting that the CSB protein increases the probability of MND transcript formation.

DISCUSSION

We have developed a new method to characterize the RNA transcripts resulting from bypass of a single cyclo-dA or CPD lesion by Pol II in human cells. We found that bypass of the cyclo-dA lesion generated two types of mutations: 5'A mutations and MND mutations. A qualitatively similar MND mutation, but no 5'A mutations, was observed by using a template containing a CPD. The different pattern of mutations observed with the two different DNA lesions eliminates the possibility that the mutations

Table 2 | Frequency of 5'A and multiple nucleotide deletion transcripts in XPA, CSB and XPD cells

Type of mutant transcript	Cell line		
	XPA	CSB*	XPD
5'A	43	48	23
−7	22	7	15
−13	3	2	2
−19 [‡]		2	1
−21		1	2

*Significantly different from XPA and XPD, $P < 0.05$ (χ^2 test). XPA and XPD distributions are not significantly different from each other. [‡]The −19 deletions observed in this analysis had the sequence ...5'-CAAT(−19)ATCGAGG-3'... and are therefore consistent with the proposed mechanism for MND formation involving the use of a downstream template dA to reinitiate transcription (see Figs 2,4). Further details about the cell lines are given in the supplementary information online. 5'A, misincorporation of adenosine opposite the template dA 5' to the lesion; CSB, Cockayne syndrome B; MND, multiple nucleotide deletion; XPA, xeroderma pigmentosum, complementation group A; XPD, xeroderma pigmentosum, complementation group D.

already existed in the plasmid, because, if that was the case the same mutations would have been observed regardless of the lesion present in the oligonucleotide used to generate the constructs. Instead, our results indicate that Pol II in living human cells has the ability to bypass helix-distorting DNA lesions *in vivo*, resulting in transcriptional mutagenesis.

We also considered other explanations for our observations than bypass of the lesions by Pol II. Wild-type transcripts could result from either constructs that do not contain a lesion, or removal of the lesion *in vivo*. The restriction digestion strategy that we used to remove contaminating lesion-free DNA constructs might not be 100% effective, and the level of unscheduled DNA synthesis—a measure of NER—in the XPA cells we used has been reported to be less than 2% of normal (Kraemer *et al*, 1975). This leaves the possibility of a very low level of lesion repair *in vivo*. However, we consistently observed that the amount of luciferase activity we obtained from the lesion-containing plasmids was between 10% and 40% of lesion-free controls (Brooks *et al*, 2000; C.M. & P.J.B., unpublished data). As shown in Table 1, more than 40% of the full-length luciferase-encoding transcripts contained the wild-type sequence. It is difficult to see how this proportion of lesion-free constructs could be generated in NER-deficient cells using the methods described here. Therefore, our results provide strong evidence that at least some of the transcripts containing wild-type sequences result from error-free bypass of the lesion by Pol II.

The mutant transcripts we observed cannot be explained by the existence of lesion-free plasmids. Therefore, aside from transcriptional mutagenesis, the only other explanation for these transcripts would be a mechanism involving mutagenic bypass of the lesion by a DNA polymerase. Although the plasmids we used do not contain any mammalian replication origins, it is possible that a DNA polymerase could encounter the lesion *in vivo* during some other process, for example, mismatch or long-patch base excision repair (BER) synthesis. However, any such mutant non-transcribed strand would have to undergo another replication event to generate a mutant transcribed strand, which would then have to

be transcribed and the RNA transcript spliced to be detected in our assay, despite the absence of mammalian replication sequences. Furthermore, the 5'(S)-cyclo-dA lesion we studied here is a complete block to both replicative and specialized DNA polymerases (Kuraoka *et al*, 2001).

Other evidence against a DNA polymerase-mediated bypass mechanism is that MNDs have not been observed in studies of DNA mutagenesis by UV light, which primarily generates CPDs (Friedberg *et al*, 2005b). Notably, Bredberg *et al* (1986) transfected a UV-irradiated plasmid shuttle vector into SV40-transformed cells from patient XP12BE—the same cells used in our work—to study mutagenesis during DNA replication. Bredberg *et al* did not detect any of the −12 deletion mutations that we observed with the CPD construct, despite the fact that the mutagenic reporter gene they used (*supF*) was well suited for detecting such mutations.

Conversely, RNA transcripts containing MNDs have been previously observed during bypass of damaged DNA templates during transcription (Zhou *et al*, 1995; Chang and Taylor, 2002). Notably, Remington *et al* (1998) detected transcripts containing 3 and 6 nt deletions—in addition to single-base mutations—as a result of bypass of different benz[a]pyrene-DNA adducts by T7 RNA polymerase *in vitro*, an experimental situation in which DNA replication cannot occur. For all these reasons, therefore, transcriptional mutagenesis is a far more parsimonious explanation for our results than a mechanism invoking a DNA polymerase.

Expanding the scope of transcriptional mutagenesis

Doetsch and colleagues (Doetsch, 2002) introduced the term transcriptional mutagenesis to describe the formation of mutant RNA transcripts resulting from bypass of DNA lesions by RNA polymerases *in vivo*. Our results show that transcriptional mutagenesis can also occur in living human cells. Furthermore, our results show that transcriptional mutagenesis is not limited to DNA lesions that are repaired by BER, but can also be stimulated by helix-distorting DNA lesions of the type that are repaired by NER as well.

Quantitative considerations

Cyclopurines are present in DNA from brain tissues at levels between 0.1 and 1 lesion/10⁷ nucleotides (J. Theruvathu, M. Dizdaroglu & P.J.B., unpublished data). A single cyclo-dA lesion on the transcribed strand of an active gene in the sequence context used here results in approximately 75% reduction of luciferase activity (Brooks *et al*, 2000; data not shown). The 5'A mutation does not result in a change in the open reading frame (Fig 2C); therefore, the luciferase activity we measured results from both wild-type and 5'A mutant transcripts. From Table 1, approximately 41% (34 out of 81) of the RNA transcripts resulting from bypass of the cyclo-dA lesion had either 5'A or MND mutations, with most (24 out of 81) being 5'A. In other sequence contexts, the spectrum of mutant transcripts might be different, although the effect on luciferase activity in XPA cells is essentially the same (Brooks *et al*, 2000; C.M. & P.J.B., unpublished data). Overall, our results show that an unrepaired cyclopurine is a strong block to transcription and, in some protein-coding sequence locations, could also cause the constitutive production of mutant proteins. Both effects could contribute to the neurological disease seen in some XP patients (Brooks *et al*, 2000; see also Doetsch, 2002).

Implications for transcription-coupled repair

Current models of transcription coupled-NER (TC-NER; Svejstrup, 2003; Andressoo & Hoeijmakers, 2005; Mellon, 2005; Laine & Egly, 2006) incorporate the idea that the stalling of Pol II at a lesion recruits Cockayne syndrome (CS) proteins and transcription factor IIH (TFIIH), ultimately resulting in NER. Although our results do not formally address the mechanism of TC-NER, as we used NER-deficient cells, our results are consistent with this aspect of the models. Indeed, as discussed below, alteration of the polymerase conformation by proteins recruited to the polymerase stalled at the lesion might be necessary for some of the bypass events we observed. Our results are also consistent with earlier proposals (Ljungman, 1999; Svejstrup, 2003) that lesion bypass is one of several possible options for Pol II after stalling at a bulky DNA lesion in a eukaryotic cell, and that CSB might facilitate mutagenic bypass of transcription-blocking DNA lesions (Svejstrup, 2003).

Mechanistic considerations

To form 5'A transcripts, we propose that after the polymerase incorporates a U opposite the lesion and translocates, the altered base stacking and/or distorted sugar-phosphate backbone resulting from the cyclo-dA lesion favours incorporation of an rA opposite the downstream template dA. In addition, the sequences downstream of the lesion might also be important (Holmes & Erie, 2003; Gong *et al*, 2005). In contrast to the 5'A mutation, the formation of MND transcripts is consistent with the polymerase incorporating a U opposite the cyclo-dA but then failing to translocate. Thus, the translocation step seems to be a mechanistic branchpoint between 5'A and MND transcripts.

Although there are several possible explanations for MND transcript formation, our working model (Fig 4) involves DNA 'scrunching', recently shown to be used by *Escherichia coli* RNA polymerase during transcription initiation (Kapanidis *et al*, 2006). In this mechanism, while tethered to the promoter, RNA polymerase pulls downstream DNA into itself and past the active centre, and the scrunched single-stranded template DNA is extruded. Zhou *et al* (1995) previously proposed a similar 'pulling-in' mechanism to explain bypass of gaps in template DNA by T7 RNA polymerase. During initiation, tethering of the RNA polymerase results from the holoenzyme binding to the promoter. After stalling at a DNA lesion, tethering could result from an accessory protein binding to the stalled polymerase.

In our model, scrunching is proposed to take place in an elongation complex where a nascent RNA transcript already exists. Therefore we propose that after stalling at the lesion, the hybrid transiently dissociates to allow the DNA to move past the transcript during scrunching. This concept is adapted from the model of Liu *et al* (1993), who showed that an elongating *E. coli* transcription complex can continue transcription and generate a full-length RNA transcript after being bypassed by a DNA replication complex using the same template strand. The hybrid dissociation step is likely to require opening of the 'clamp' that closes on the hybrid in the elongation complex (Gnatt *et al*, 2001). Opening of the clamp might be facilitated by TFIIH associating with the stalled polymerase (Sarker *et al*, 2005). After scrunching, the transcript could be re-opposed to the template strand with closure of the clamp to allow transcription to continue. Finally, in our model, the unusual pattern of MND transcripts we observed indicates that scrunching proceeds in steps of 6–7 nt.

Fig 4 | A speculative model for multiple nucleotide deletion transcript formation. The violet rounded rectangle represents polymerase II and CM indicates the clamp module (Gnatt *et al*, 2001). The RNA transcript is shown in yellow, template DNA strand in white, non-template strand in grey and the portion of the template strand that is 'skipped' in blue. The Δ represents the cyclo-dA lesion and the blue A represents the downstream template dA residue used to reinitiate transcription (underlined in Fig 3). The proposed tethering protein is not shown. After incorporating U opposite the cyclo-dA lesion and stalling (A), the CM partially opens, allowing dissociation of the hybrid, but with retention of the nascent RNA (see Liu *et al*, 1993; B). Downstream DNA is pulled into the polymerase past the RNA transcript and extruded upstream by a scrunching mechanism (C). The CM closes on the 'hybrid' and the extruded DNA rewinds, allowing transcription to continue (D). As discussed in the text, other factors in addition to the polymerase are likely to be necessary, but are not shown for clarity. Cyclo-dA, 8,5'-cyclo-2'-deoxyadenosine; U, uridine.

Speculations on the role of CSB

Although CSB is not essential for MND transcript formation, it appears to increase the probability of such events (Table 2). We speculate that CSB might facilitate scrunching, perhaps by acting as an additional polymerase tether. This idea is consistent with the proposal that CSB stabilizes Pol II stalled at a lesion (Laine & Egly, 2006), and the observations that CSB prevents Pol II from backtracking and transcript cleavage in response to TFIIIS (Selby & Sancar, 1997; Laine & Egly, 2006). A TFIIIS-mediated transcript cleavage reaction is incompatible with simultaneous MND transcript formation. However, given the known biochemical properties of CSB and its homologues (Selby & Sancar, 1997; Svejstrup, 2003), we do not exclude a more active role for CSB in promoting MND transcript formation. Future studies, using reconstituted *in vitro* systems, will be necessary to fully address this point.

Summary

We have discovered two new types of mutant RNA transcripts resulting from bypass of helix-distorting DNA lesions by Pol II in cells from patients with hereditary DNA repair diseases. Future studies will focus on the mechanistic basis of these transcripts, which might provide new insights into the molecular processes that occur at Pol II complexes stalled at DNA lesions in human cells.

METHODS

The construction and purification of plasmids containing single lesions, cell culture and transfections were carried out as described by Marietta *et al* (2002).

RT-PCR was carried out using approximately 80 ng of RNA with the One-Step RT-PCR Kit (Qiagen, Valencia, CA, USA). The 357 bp band (Fig 1C), resulting from spliced RNA transcripts, was gel-purified and cloned into plasmids that were screened for mutations using *MfeI* digestion followed by direct sequencing using automated sequencing methods.

A more detailed description of the methodology is given in the supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

Note added in proof. After our manuscript was accepted for publication, Brueckner *et al* (2007) showed that blockage of yeast Pol II transcription by a CPD lesion is due to lesion-induced misincorporation, and that placement of adenosine residues opposite both Ts of the CPD allows lesion bypass. Their findings are consistent with the interpretation that the wild-type RNA transcripts we observed in XPA cells transfected with the CPD construct result from error-free bypass of the CPD lesion by human Pol II *in vivo*.

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