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Transcriptional Perturbations of 2,6-Diaminopurine and 2-Aminopurine

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Abstract

2,6-Diaminopurine (Z) is a naturally occurring adenine (A) analog that bacteriophages employ in place of A in their genetic alphabet. Recent discoveries of biogenesis pathways of Z in bacteriophages have stimulated substantial research interest in this DNA modification. Here, we systematically examined the effects of Z on the efficiency and fidelity of DNA transcription. Our results showed that Z exhibited no mutagenic yet substantial inhibitory effects on transcription mediated by purified T7 RNA polymerase and by human RNA polymerase II in HeLa nuclear

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.2c00369.
Materials and cell lines used, Sanger sequencing and Western-blot for verifying the successful knockout of *XPC* and *CSB* genes, plasmid construction, *in vitro* and cellular transcription assay, supplementary mass spectrum and gel images (PDF)

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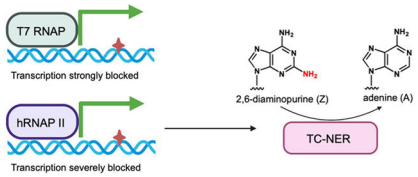
The authors declare no competing financial interest.

extracts and in human cells. A structurally related adenine analog, 2-aminopurine (2AP), strongly blocked T7 RNA polymerase but did not impede human RNA polymerase II *in vitro* or in human cells, where no mutant transcript could be detected. The lack of mutagenic consequence and the presence of a strong blockage effect of Z on transcription suggest a role of Z in transcriptional regulation. Z is also subjected to removal by transcription-coupled nucleotide-excision repair (TC-NER), but not global-genome NER in human cells. Our findings provide new insight into the effects of Z on transcription and its potential biological functions.

Graphical Abstract



2,6-Diaminopurine (Z), or 2-aminoadenine, is a naturally occurring adenine (A) analog (Figure 1). In the S-2L cyanophage genome, A is completely substituted with Z.[1] The Z-containing genome is resistant to bacterial restriction endonucleases, providing S-2L cyanophage a selective advantage.[2] The biogenesis pathways of base Z were recently elucidated,[3–6] and it was found that the Z-containing genome is more widespread in bacteriophages than previously thought.[3]

Multiple DNA modifications discovered in bacteriophages were also detected in bacterial or human genomes, albeit with different biological functions. For example, to dodge the host defense system, some bacteriophages replace cytosine (C) with 5-methylcytosine (5mC) in their genomes.[7] On the other hand, 5mC is an important epigenetic regulator in human cells.[7] Interestingly, while bacteriophages benefit from Z-substituted genomes, introducing Z into the genome is toxic to bacteria,[8] and Z has not yet been detected in the human genome. Z can readily base pair with thymine (T) or uracil (U) by forming three hydrogen bonds.[9] However, not much is known about how Z substitution modulates the efficiency or fidelity of transcription and whether Z is subjected to repair in mammalian cells.

To shed light on the above questions, we first explored whether Z in template DNA affects the efficiency and fidelity of transcription by a bacteriophage RNA polymerase *in vitro*, and by human RNA polymerase II *in vitro* and in human cells. We also investigated whether Z could be repaired in human cells and compared the transcriptional outcomes of Z with those of another structurally related adenine analog, 2-aminopurine (2AP).

We employed a competitive transcription and adduct bypass (CTAB) assay[10,11] to assess the effects of Z and 2AP on transcription *in vitro* and in human cells. The modified nucleoside-containing plasmids were prepared by inserting a single Z or 2-AP on the template strand of a nonreplicative double-stranded plasmid downstream of cytomegalovirus (CMV) and T7 promoters (Figure 1). A normal adenine base was placed at the Z or 2AP site in the control plasmid. The competitor plasmid is also modification-free but contains three additional nucleobases next to the modification site not present in the control plasmid

yielding a transcription product with a distinct length. The Z-, 2AP-, or control A-containing plasmid were premixed with the competitor plasmid and used as templates for transcription. The runoff transcripts were subjected to reverse-transcription PCR (RT-PCR), restriction digestion, and polyacrylamide gel electrophoresis (PAGE) as well as liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses (Figure 1).

Frist, we quantitatively assessed the effects of Z and 2AP on transcription elongation *in vitro* catalyzed by purified T7 bacteriophage RNA polymerase (T7 RNAP) or human RNA polymerase II (hRNAPII) in a HeLa cell nuclear extract. Linearized plasmids were used as templates and the transcription reactions were terminated after a 1-h incubation. PAGE analysis showed that base Z posed a strong blocking effect on hRNAPII with a relative transcriptional bypass efficiency (RBE) of around 1.3%, compared to the normal base A. Z also strongly blocked T7 RNAP, with an RBE of 18.4% (Figure 2). On the other hand, 2AP only modestly impeded transcription elongation by hRNAPII (with a 65.9% RBE) but strongly impaired transcription mediated by T7 RNAP (with a 12.3% RBE; Figure 3). In addition, our LC-MS/MS analysis results revealed that the full-length transcripts from *in vitro* transcription of Z- or 2AP-containing template did not elicit any detectable mutations (Figures S1 and S2).

We further asked how Z and 2AP influence transcription in human cells. To this end, we premixed Z-, 2AP-, and A-containing plasmids individually with the competitor plasmid and cotransfected them into HEK293T and HeLa cells (Figure 1). After 24 h, the transcripts were extracted and analyzed as described above. Our results showed that base Z strongly blocks transcription machinery in human cells, with the RBE values being ~18.6% and ~8.1% in HEK293T and HeLa cells, respectively (Figure 2). By contrast, 2AP was well tolerated by the human transcription machinery, where the RBE values were comparable to that of the unmodified adenine in both HEK293T and HeLa cells (Figure 3). There was no detectable mutant transcript arising from either substrate in human cells, as revealed by PAGE and LC-MS/MS results (Figures 2 and 3 and S1 and S2).

Considering that base Z markedly impedes cellular transcription, we next examined whether it is subjected to repair by the nucleotide excision repair (NER) pathway in human cells. XPA and ERCC1 are essential protein factors in NER.[12,13] We performed a CTAB assay in isogenic human skin fibroblasts that are XPA-deficient (GM04312) or XPA-complemented (GM15876).[14] The RBE value for Z was significantly lower in the XPA-deficient cells than the XPA-complemented cells at 24 h following transfection (Figure 4A), indicating that Z is an NER substrate in human cells. Likewise, the RBE value for Z was markedly lower in ERCC1-deficient CHO-7-27 cells[15] (3.6%) than in repair-competent CHO-AA8 cells (13.2%) at 24 h following transfection (Figure 4B), again supporting the role of NER in removing base Z in mammalian cells.

There are two major subpathways in NER, namely, global-genome NER (GG-NER) and transcription-coupled NER (TC-NER), which require XPC and CSB, respectively, early in the damage recognition process.[12,13] To determine the influence of the two subpathways on the transcriptional alteration induced by Z, we generated *XPC* and *CSB* knockout HEK293T cells by using CRISPR/Cas9. The successful ablations of these two genes were verified

by Sanger sequencing and Western blot analyses (Figure S4). The RBE of Z decreased significantly in *CSB*-knockout cells compared to that in parental HEK293T cells, while genetic ablation of *XPC* did not exert any significant effect on RBE values of Z in human cells (Figure 2). The transcriptional fidelity of Z was not compromised by genetic depletion of *XPC* or *CSB*, as no mutant transcripts were detectable from either PAGE or LC-MS/MS analyses (Figures 2, S1, and S2).

To further delineate the contributions of the two subpathways of NER in the removal of Z from human cells, we conducted the CTAB assay at different time points for HEK293T cells and the isogenic *XPC*- and *CSB*-knockout cells. Our time-dependent results showed that the RBE values of Z, relative to that of normal base A, displayed time-dependent increases in HEK293T and the isogenic *XPC*-knockout cells (Figure 4C). The magnitude of increase in RBE values for base Z, however, was much smaller in *CSB*-knockout cells (Figure 4C). These results are consistent with the active repair of Z in HEK293T and the isogenic XPC-deficient cells and the ensuing formation of modification-free template DNA for transcription. By contrast, this repair is impaired in CSB-deficient HEK293T cells, indicating that the repair occurs by TC-NER. Moreover, *in vitro* experiments with the incubation of recombinant XPC-RAD23B protein complex with 24-merZ-containing double-stranded oligonucleotides (dsODNs) in the same sequence context as cellular experiments showed significantly less efficient binding of the Z-containing dsODN compared to the corresponding unmodified A-containing dsODN (Figure S3). Previous studies have demonstrated that XPC-RAD23B binding requires thermodynamic destabilization of duplex DNA, and that DNA lesions such as adducts formed by aristolochic acid do not destabilize DNA duplexes, fail to specifically bind XPC-RAD23B, and thus escape GG-NER.[16] Together, these results indicated that base Z is not a substrate for GG-NER; however, when situated on the template strand of an actively transcribed gene, Z can be readily repaired by TC-NER.

The discovery of a third purine base as a genetic code in place of A in bacteriophages has attracted substantial interest recently;[3–6] however, it is unclear how this replacement affects transcription. In this study, we systematically investigated the effects of this adenine analog on the process of DNA transcription *in vitro* and in human cells and found that Z poses a significant impediment to transcription by RNAPII in human cells.

Sturla and co-workers[17] showed that altered minor-groove hydrogen bonding in DNA can block transcription elongation by T7 RNA polymerase and that the 2AP-U wobble base pair constitutes stronger blockage to transcription elongation than the Z−U base pair, which maintains Watson−Crick-type base pairing. In agreement with this previous study, our results showed that, when located on the transcribed strand, Z conferred less blockage than 2AP to transcription mediated by T7 RNA polymerase, though the Z−U base pair displays better thermal stability.[17] It is worth noting that the aforementioned study[17] used about a 16-fold higher T7 RNA polymerase to template ratio than in this study, which resulted in different absolute transcript yields between the two studies; nevertheless, the magnitude of change was similar, i.e., T7 RNA polymerase transcribed through Z about 1.5-fold more efficiently than 2AP. Contrary to single-subunit T7 RNA polymerase, the multisubunit human RNA polymerase II in HeLa nuclear extract and cultured human cells

efficiently transcribed 2AP-containing templates but was severely blocked by Z, indicating differential recognition mechanisms by RNA polymerases from different organisms.

Apart from inducing mutations during DNA replication, modified nucleosides in template DNA can give rise to mutations during transcription, a process called transcriptional mutagenesis (TM).[18,19] Several lines of evidence showed that TM of simple DNA modifications such as 8-oxo-7,8-dihydroguanine (8-oxoG) or O^6-methylguanine (O^6-meG) could lead to pathogenic protein production and contribute to neurodegenerative disease and cancer.[20,21] We did not observe any mutations arising from transcription across Z or 2AP *in vitro* or in human cells. In theory, Z or 2AP can not only pair with T and U via three hydrogen bonds but also form a wobble base pair with C. A previous study showed that, in the absence of UTP, T7 RNA polymerase can misincorporate CTP opposite Z or 2AP; however, the ensuing wobble base pair hampered transcription elongation, resulting in truncated transcripts.[17] On the grounds that our experiments were conducted in the presence of all four natural ribonucleoside triphosphates, the absence of mutations is not surprising. Similarly, DNA replication across 2AP mediated by wild-type T4 DNA polymerase did not yield any mutations.[22] With mutant bacteriophage DNA polymerase, 2AP was marginally mutagenic, pairing with T about 100 times more frequently than with C, and it has been shown that these mutagenic properties preferentially occur in AT-rich regions.[23] In addition, the imino tautomer formation is a primary mechanism for base mispairing. However, solution-phase NMR studies revealed that both bases in the 2AP-T base pair adopt the canonical amino tautomeric forms, ruling out mutagenic base pairing.[24]

We also found that the transcription efficiency of Z was lower in NER-deficient cells than in NER-competent cells. Moreover, genetic depletion of CSB, but not XPC, strongly impedes transcription across Z. Our previous observations from the CTAB assay revealed similar findings for N^2-(1-carboxyethyl)-2′-deoxyguanosine located on the template strand, the transcription efficiency of which is exacerbated by a loss of TC-NER.[25] Similarly, aristolochic acid-induced aristolactam (AL)-DNA adducts are efficiently repaired by TC-NER but are resistant to GG-NER, resulting in a mutation spectrum almost exclusively on the nontranscribed strand.[16,26] Recent structural studies showed that CSB could rescue stalled RNA polymerase II in two ways, i.e., to push Pol II forward to bypass less bulky adducts or to recruit TFIIH to the damage sites and initiate TC-NER,[27,28] a NER subpathway that selectively repairs DNA lesions on the transcribed strand of active genes.[12] Our results showed that depletion of CSB or downstream NER factors (XPA or ERCC1) substantially attenuated the transcription bypass efficiency of Z, suggesting a role of CSB in initiating TC-NER to rescue the Z-induced RNPII stalling in human cells. Along this line, TC-NER has been shown to sense transcription blockage rather than specific structural features of a lesion itself.[13] A number of other nonbulky, yet potent transcription stalling DNA modifications, including abasic sites and 5-guanidinohydantoin (Gh), are efficient substrates for TC-NER.[29–31]

In conclusion, we demonstrated that base Z did not forge transcriptional mutagenesis, but it strongly attenuated the efficiencies of transcription mediated by single-subunit T7 RNA polymerase *in vitro* and multisubunit human RNA polymerase II *in vitro* and in human cells. This result indicates that, like 5mC, the noncanonical DNA base Z, first

discovered in bacteriophages as a defense mechanism and later found to be an important epigenetic regulator, could potentially play a role in transcriptional regulation, albeit through different mechanisms. In this context, 5mC recruits methyl-CpG-binding proteins to block transcription,[32] whereas Z attenuates transcription directly by impeding RNA polymerase-mediated transcription elongation. In addition, Z was discovered in meteorites among other nucleobases[33] and was often formed during prebiotic synthesis reactions that generate adenine and guanine.[33,34] It was suggested that Z could have existed on early earth alongside A and G.[35] Furthermore, Z exhibits excellent capacities in nonenzymatic oligomerization,[36] template-directed ribonucleotide incorporation[37] and damage repair.[35] The discovery that Z strongly impedes transcription and is readily repaired by TC-NER in human cells expands our knowledge of this naturally occurring nucleobase.

Together, our study provides important insights into the impact of Z and 2AP on the efficiency and fidelity of DNA transcription. In the future, it will be pivotal to examine the structural basis underlying Z's substantial transcriptional blockage effect and the differential recognition of Z and 2AP by different RNA polymerases. It will also be interesting to investigate how placement of Z and 2-AP on nontemplate strand affects transcription *in vitro* and in cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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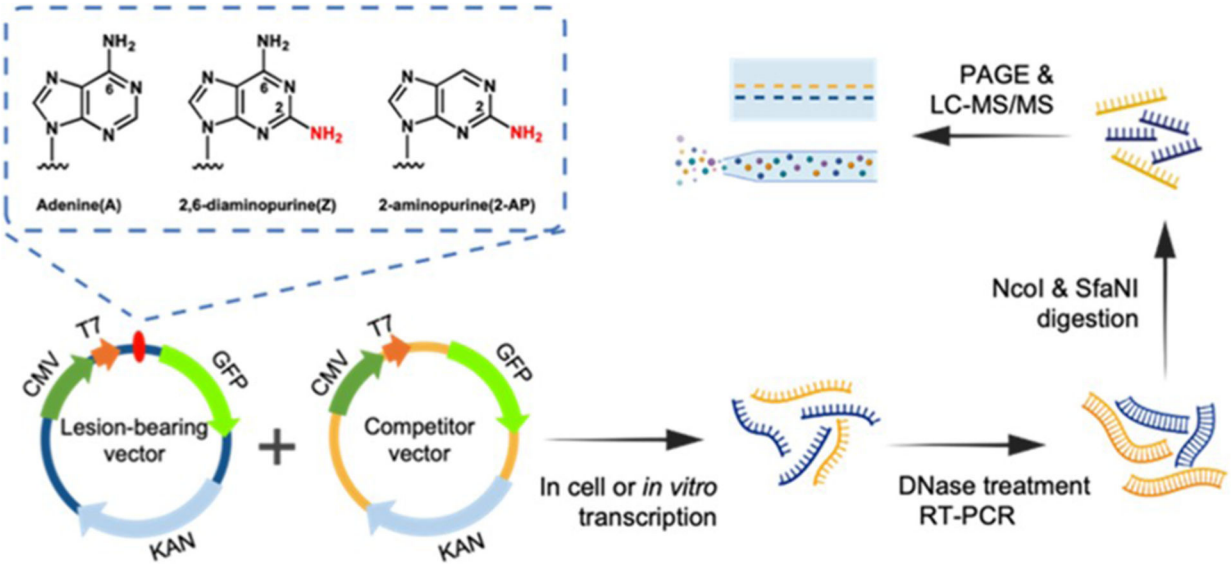

Figure 1.

A schematic diagram depicting the CTAB assay and the chemical structures of adenine and its analogs investigated in this study.

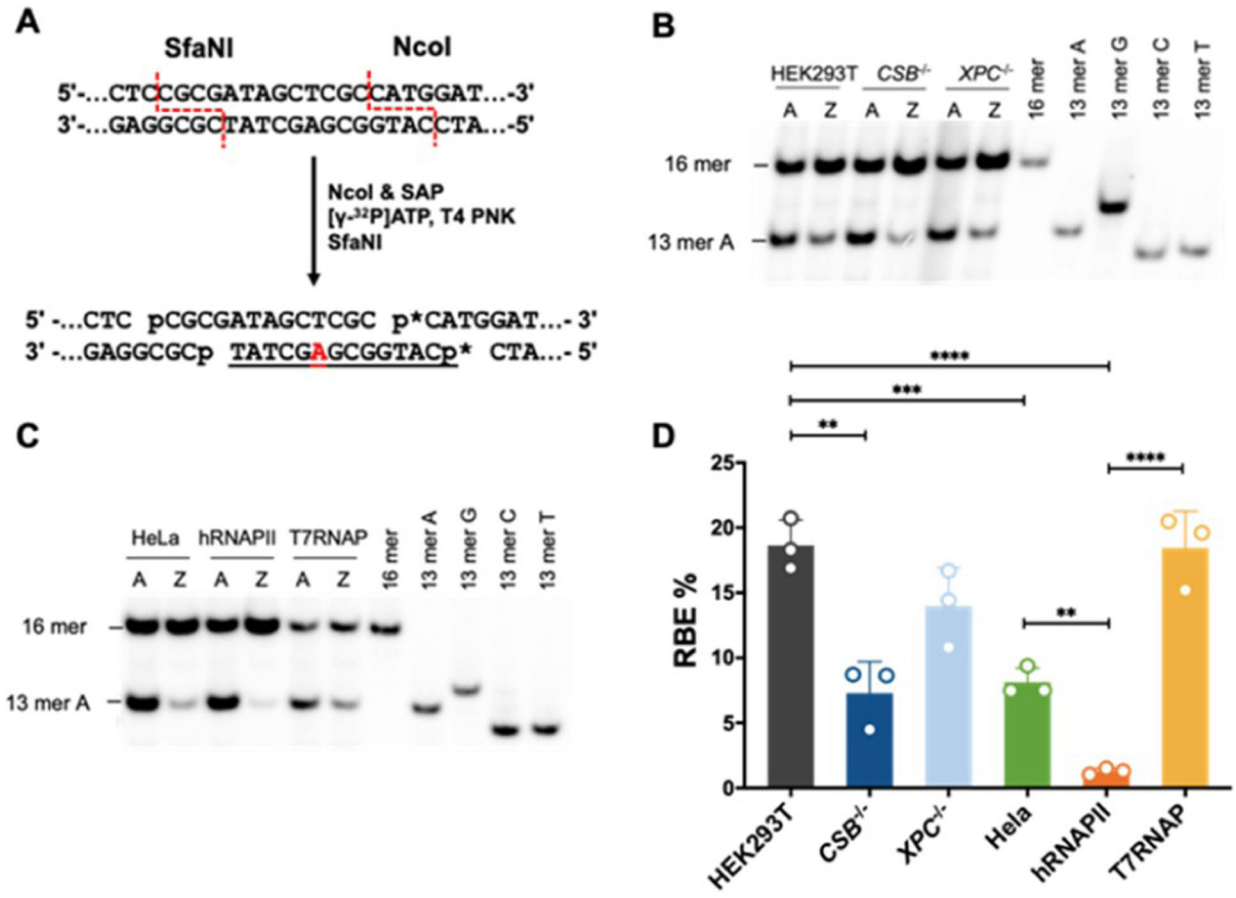

Figure 2.

Restriction digestion and postlabeling method for determining the transcriptional efficiencies of base Z *in vitro* and in human cells. (A) A schematic diagram depicting the selective labeling of the template strand via sequential digestion of the RT-PCR products. A "p*" denotes a ^{32}P-labeled phosphate group. A red colored "A" represents the site where base Z was initially installed. Representative gel images for monitoring the restriction fragments of interest in parental HEK293T cells and the isogenic cells where the *CSB* or *XPC* gene is knocked out (B), in HeLa cells and *in vitro* reactions with human RNAPII or T7 RNA polymerase (C). For all of the transfection experiments, the Z- and control A-containing plasmids were premixed with the competitor plasmid at molar ratios of 2:1 and 1:1, respectively. The transcripts were isolated from cells at 24 h following transfection. In this and other figures, the synthetic ODNs representing the restriction fragment arising from the competitor vector, i.e., d(CATGGCGATAGGCTAT), is designated as "16mer"; "13 mer G", "13 mer A", "13 mer C", and "13 mer T" represent the standard synthetic ODNs d(CATGGCGXGCTAT), where "X" is G, A, C, and T, respectively. (D) Quantitative results of RBE values of Z in different cell lines and *in vitro*. The data represent the mean ± SD of results from three independent experiments. **0.001 < P < 0.01; ***0.0001 < P < 0.001; **** P < 0.0001; ns, not significant. The P values in this and other figures were calculated by using one-way ANOVA and Tukey's multiple comparisons test.

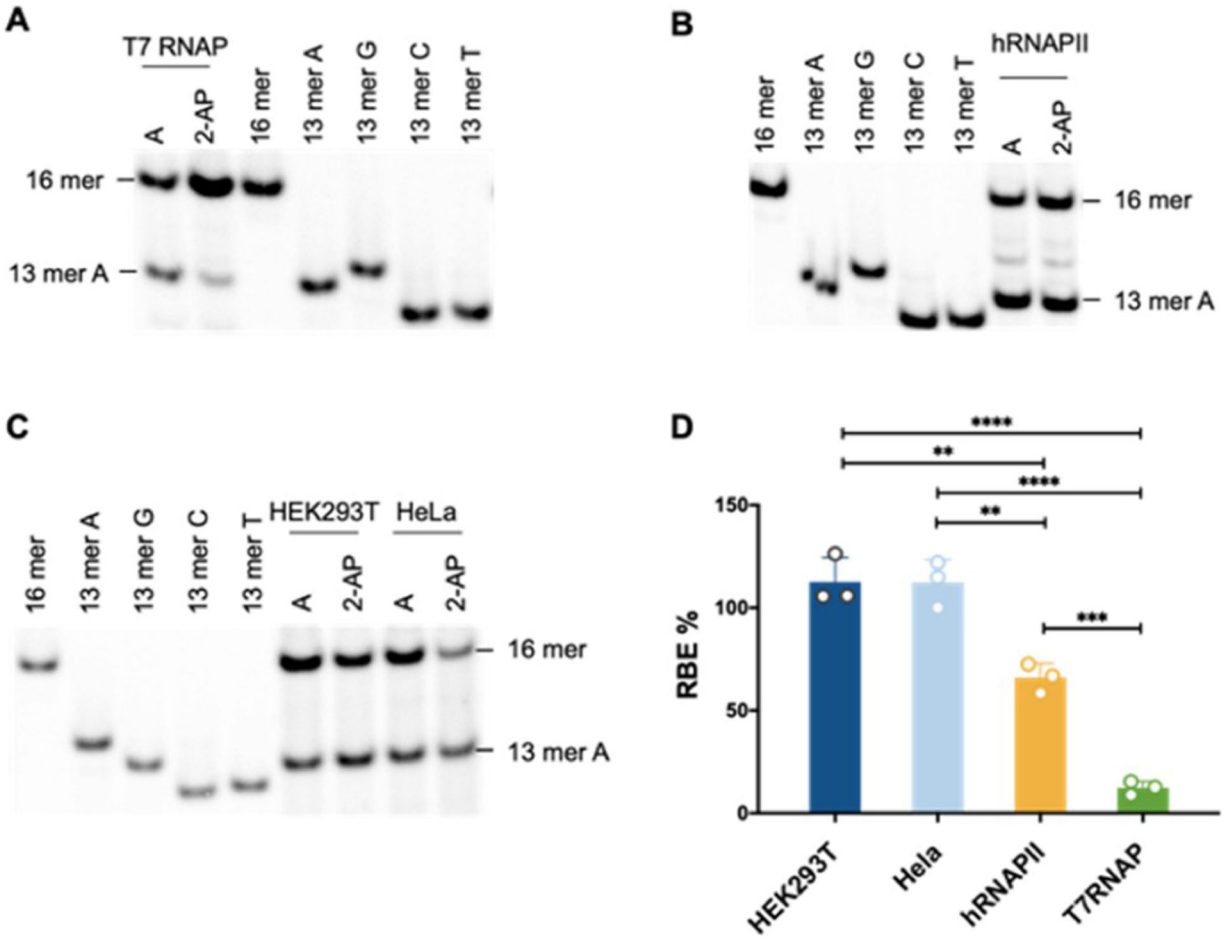

Figure 3.

Restriction digestion and postlabeling method for determining the transcriptional efficiencies of 2AP in human cells and *in vitro*. (A−C) Representative gel images showing the 16 mer (from the competitor genome) and 13 mer (from the control or lesion-containing genome) digestion products from the original template strand. For all of the transfections, the 2AP- and control A-containing plasmids were premixed with the competitor plasmid at molar ratios of 2:1 and 1:1, respectively. The transcripts were isolated from cells at 24 h following transfection. (D) The RBE values of 2AP in HEK293T cells, HeLa cells, and in *in vitro* transcription mediated by hRNAPII and T7 RNAP. The data represent the mean ± SD of results from three independent transcription experiments. **0.01 < P < 0.001; ***0.0001 < P < 0.001; **** P < 0.0001.

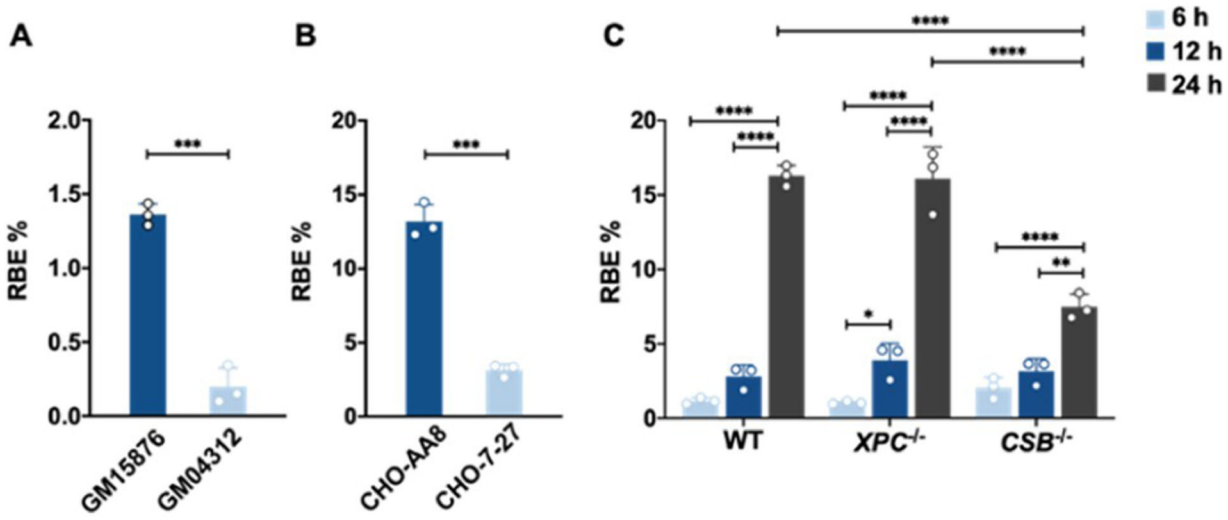

Figure 4.

Transcriptional bypass efficiencies of base Z in NER-deficient and proficient cells. The RBE values of base Z in XPA-deficient cells (GM04312) and the isogenic cells complemented with XPA cDNA (GM15876) (A) and in ERCC1-deficient (CHO-7-27) and wild-type (CHO-AA8) CHO cells (B). The RBE values in parental HEK293T cells and the isogenic CSB- and XPC-knockout cells at 6, 12, and 24 h after transfection (C). The corresponding gel images are shown in Supporting Information Figure S5. The data represent the mean ± SD of results from three independent experiments. *0.01 < P < 0.05; **0.001 < P < 0.01; ***0.0001 < P < 0.001; **** P < 0.0001.