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The remarkable stability of genes seemed a puzzling feature in the early days of molecular biology. It was even suggested that new laws of physics might emerge to explain biological paradigms such as the resilience of the genetic information. The complementary sequences in the DNA double-helical structure provided a partial answer as to how this stability is retained, but it still took more than 10 years after the Watson and Crick 1953 model to realize that radiation-damaged residues in DNA could be corrected by a local excision-repair process. In retrospect, the early concerns were fully justified; mammalian cellular DNA is a constant target of thermal "noise" in the form of spontaneous hydrolysis at 37ºC, and it is also susceptible to damage caused by active oxygen as well as reactive metabolites and coenzymes. The resulting lesions are generally removed by the base excision repair (BER) pathway, resulting in short replacement patches within one of the two DNA strands. Consequently, nonreplicating DNA is not absolutely stable but turns over at a relevant, albeit slow, rate in vivo. This endogenous repair is sufficiently accurate and efficient to explain the apparent stability of the genetic material.

ENDOGENOUS DNA LESIONS

The relative importance of the different modes of endogenous DNA degradation has been the subject of much debate. This is because reaction rates for only some of the types of DNA decay can be precisely estimated. However, the DNA depurination rate at 37ºC and pH 7.4 has been determined and corresponds to the loss of 10,000 (Lindahl and Nyberg 1972) or 9,000 (Nakamura et al. 1998) bases per day by nonenzymatic hydrolytic cleavage of glycosyl bonds in a mammalian cell. The remaining uncertainty here is whether DNA in chromatin is depurinated two to five times more slowly than naked DNA, although DNA remains fully hydrated in chromatin. The reason for a possible moderate decrease in depurination rate in vivo is that DNA charge neutralization by histones is effective in nucleosomes, and depurination rates are slower in high-salt buffers than in low-salt buffers. On the other hand, the presence or absence of divalent cations such as Mg^{++} in buffers of physiological ionic strength does not significantly affect depurination rates, despite the improved charge shielding of the DNA phosphates achieved in the presence of Mg^{++}. DNA retains its B conformation in the vicinity of an abasic site, but local structural perturbation occurs with a kink and dynamic flipping of the deoxyribose residue into the minor groove (Gelfand et al. 1998; Mol et al. 2000).

The rate of hydrolytic cytosine deamination in DNA in vivo is more difficult to estimate, because of the much higher rate of deamination of single-stranded DNA than of double-stranded DNA. However, even 100% double-stranded DNA is deaminated at a significant rate. Most likely, the range is within 100–500 deaminated cytosines per day in a mammalian cell (Lindahl and Nyberg 1974; Frederico et al. 1990; Shen et al. 1994), with the higher value dependent on temporary exposure of a significant amount of single-stranded DNA during active transcription and replication processes. The spontaneous mutator phenotype of *Saccharomyces cerevisiae* and *Escherichia coli ung* mutants defective in removal of deaminated cytosines strongly indicates that the reaction is of relevance in vivo (Duncan and Miller 1980; Impellizzeri et al. 1991). Hydrolytic deamination of 5-methylcytosine is two- to threefold faster than that of unsubstituted cytosine, and this moderate rate increase may partly account for 5-methylcytosine residues being mutational hot spots in mammalian cells, although the slower rate of repair of the deaminated form of 5-methylcytosine than of cytosine is likely to be more important in this regard (Shen et al. 1994).

S-Adenosylmethionine (SAM) is the reactive methyl donor in most cellular transmethylation reactions, and it acts like a weak alkylating agent. The most important DNA product generated by SAM is the cytotoxic lesion 3-methyladenine; mutagenic miscoding lesions are not formed to a significant extent. SAM is present both in the cell nucleus and in the cytoplasm, typically at a concentration of 30 μM, with reported values for different cell types generally being 10–80 μM. The rate of reaction of SAM with DNA under physiological solvent conditions can be determined conveniently and accurately by using ^{3}H-labeled SAM, and corresponds to the formation of 600 3-methyladenine residues in the DNA of a mammalian cell in 24 hours (Rydberg and Lindahl 1982). There is no significant protection of DNA in chromatin against methylating agents, as determined by studies on the effects of dimethylsulfate (DMS) and methylmethane sulfonate (MMS) treatment, so this value for cytotoxic DNA alkylation by SAM should be correct under in vivo conditions. It is 10–20 times lower than the rate of DNA depurination, but still high enough to make it advantageous to living cells to have the specific repair enzyme 3-methyladenine-DNA glycosylase. In contrast, 7-methylguanine, the major DNA lesion generated by simple alkylating agents, appears to be innocuous and is only very slowly removed with a half-life of about 1 day, largely by nonenzymatic cleavage of the chemically la-

bile glycosyl bond of 7-methyldeoxyguanosine residues. The generation and subsequent loss of this common base lesion contribute significantly to the total DNA depurination rate with about 3000 abasic sites formed per day. A steady state of about 4000 7-methylguanine residues per genome apparently occurs (Rydberg and Lindahl 1982). In normal repair-proficient mammalian cells, 7-methylguanine is most likely the main aberrant base residue regularly present in DNA; it seems unlikely that any potentially cytotoxic or promutagenic base would be allowed to remain at such a level. The existence of minor endogenous methylating agents other than SAM has been indicated by spontaneous mutator phenotypes of repair-deficient bacteria (Rebeck and Samson 1991). These agents may be more closely related to methylnitrosourea, induce formation of both O^6-methylguanine and 3-methyladenine in DNA, and can arise by nitrosation of endogenous metabolites (Taverna and Sedgwick 1996).

Bacteria and eukaryotic cells have specific repair enzymes to remove the various major types of oxidative damage from DNA. A defect in DNA repair of the most common mutagenic base lesion, 8-hydroxyguanine (8-oxoG), leads to a strong mutator phenotype in bacteria and yeast (Michaels and Miller 1992; Thomas et al. 1996). These data show that repair of endogenous DNA damage generated by reactive oxygen species is relevant in vivo and that significant endogenous oxidative damage to DNA occurs continuously, perhaps at a similar level as damage due to hydrolysis and endogenous alkylation. However, the relative importance of this form of DNA decay remains unclear in comparison with other types of intrinsic DNA damage. This is because the intracellular, and especially intranuclear, concentrations of the relevant reactive oxygen species have not been determined, whereas the intracellular concentration of, for example, water that accounts for hydrolytic degradation is known to be 55 M. Reactive oxygen species that damage DNA are generated by iron-mediated Fenton reactions (Henle and Linn 1997), and the frequency of such reactions in the cell nucleus is not known. Furthermore, in eukaryotic cells, most oxygen metabolism has been delegated to mitochondria, so the cell nucleus is practically anoxic (Joenje 1989). Processes such as lipid peroxidation in the nucleus and cytoplasm are clearly relevant, however, and cellular stress may increase the amount of 8-oxoG formed in DNA (Conlon et al. 2000).

Exocyclic DNA base adducts generated from lipid peroxidation by-products have been characterized. The most abundant of these appears to be the pyrimidopurinone M_1G, which is generated by reaction between a G residue in DNA and the lipid peroxidation product, malondialdehyde (Fink et al. 1997). In addition, lipid peroxidation may yield acrolein and crotonaldehyde, which are readily metabolized to epoxides that can generate exocyclic etheno modifications of DNA bases. Two such bases, etheno-A and etheno-C, are excised efficiently by DNA glycosylases (Hang et al. 1998; Saparbaev and Laval 1998), which strongly suggests that generation of such adducts occurs at sufficiently high rates in vivo to endanger genomic stability.

Oxygen free radicals generate small amounts of bulky DNA damage that require repair by the nucleotide excision repair pathway, in addition to the many base alterations corrected by BER. A particularly interesting lesion of this class is the 5′,8-cyclopurine deoxynucleoside (Brooks et al. 2000; Kuraoka et al. 2000). This cytotoxic, chemically stable form of damage could accumulate in DNA of repair-deficient cells over long time periods, ultimately leading to pathological consequences.

To evaluate the relative contribution by reactive oxygen species to endogenous DNA damage in mammalian cells, the key DNA repair enzymes and pathways involved in the correction of such damage are being characterized, and mouse gene knockouts of these activities are allowing further biochemical and pathological studies in vivo.

THE BER PATHWAYS

Eight different DNA glycosylases are known to act on various forms of endogenously damaged DNA in mammalian cells (Lindahl and Wood 1999). After cleavage of the base-sugar bond, most DNA glycosylases remain bound to the abasic site in the DNA substrate (Fig. 1). AP endonuclease (APE1, also called HAP1) displaces the DNA glycosylase and cleaves the DNA chain 5′ to the abasic site (Mol et al. 2000). APE1 interacts with DNA polymerase β (pol-β) and recruits this gap-filling enzyme to the damaged sites (Bennett et al. 1997). Finally, the DNA ligase III/XRCC1 heterodimer interacts with pol-β, displaces the polymerase, and seals the phosphodiester bond (Kubota et al. 1996). A separate editing enzyme should also be required to compensate for the lack of proofreading ability of pol-β (Höss et al. 1999).

pol-β is able to excise the 5′-terminal base-free sugar phosphate residue in a nonhydrolytic elimination process, and this trimming of an end group by the AP lyase activity of pol-β may be the rate-limiting step in the repair pathway (Sobol et al. 2000). However, repair of oxidatively damaged nucleotides has no requirement for such a pol-β-associated lyase activity, because the DNA glycosylases concerned, NTH1 and OGG1, possess endogenous AP lyase activity themselves. This strategy helps to keep the excised region restricted to a single nucleotide (Fortini et al. 1999; Klungland et al. 1999a; Dianov et al. 2000), which could be particularly important in the repair of clustered lesions on both strands generated by ionizing radiation. Another unusual feature of the repair of oxidatively damaged DNA is the secondment of the XPG protein, which has a major role in nucleotide excision repair, as a cofactor for glycosylase excision of damaged pyrimidines (Cooper et al. 1997; Klungland et al. 1999a).

The sequential transient protein-protein interactions occurring on the damaged DNA substrate during BER (Fig. 1) may aid the accuracy, speed, and specificity of the endogenous repair process (Lindahl and Wood 1999; Wilson and Kunkel 2000). A similar strategy has been observed for several heterodimeric transcription factors, where consecutive rather than simultaneous binding of the protein monomers improves the quality and accuracy

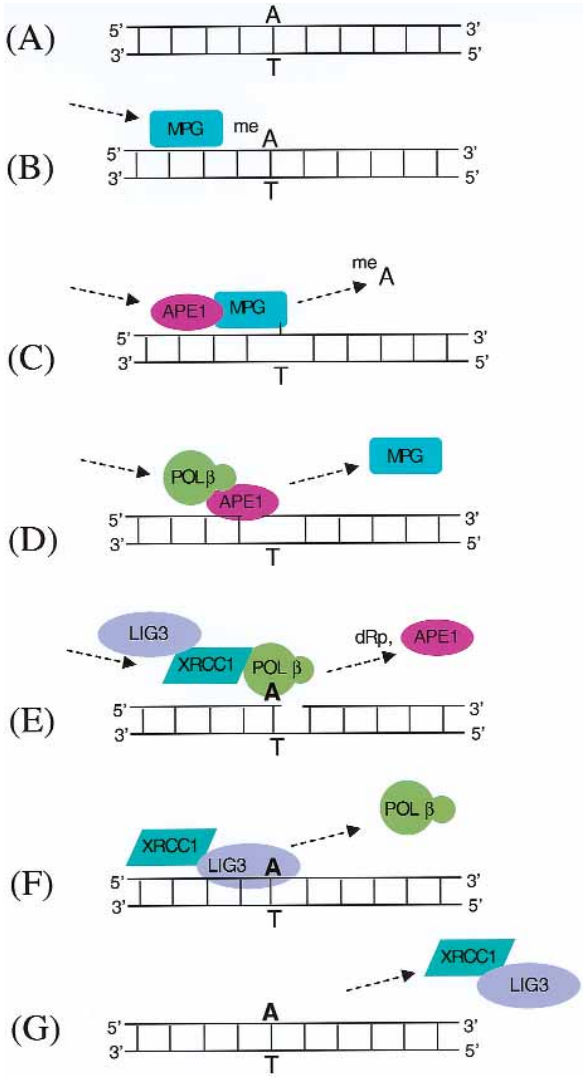

Figure 1. Main BER pathway in mammalian cells. A single aberrant nucleotide residue is replaced by consecutive pairwise interactions of the repair factors. The scheme illustrates the removal of an endogenously formed cytotoxic 3-methyladenine residue from DNA. (*A*) Intact DNA; (*B*) 3-methyladenine-DNA glycosylase (MPG) scanning DNA for lesions; (*C*) excision of the alkylated base in free form and recruitment of AP endonuclease; (*D*) displacement of MPG by APE1, incision at the abasic site, and recruitment of DNA polymerase β; (*E*) release of APE1, gap-filling and removal of the 5′-terminal sugar phosphate residue by pol-β, and recruitment of DNA ligase III using the XRCC1 scaffold protein to promote the polymerase-ligase interaction; (*F*) release of pol-β and joining of the DNA strand interruption by Lig3; (*G*) dissociation of the Lig3/XRCC1 heterodimer from the repaired DNA. For further details, see text.

of the process (Kohler et al. 1999). The only preformed protein complex in BER is that of DNA ligase III (Lig3) and the scaffolding protein, XRCC1. The link to pol-β is provided by XRCC1 (Kubota et al. 1996), since the polymerase and the ligase do not interact directly with each other. Lig3 and XRCC1 occur as a heterodimer in vivo and are bound to each other through their carboxy-terminal regions (Nash et al. 1997). Interestingly, the binding regions correspond to BRCT domains (Bork et al. 1997), a motif of approximately 95 amino acids widely distributed among proteins active in cell cycle control, recombination, and repair. In the case of Lig3/XRCC1, the BRCT sequences are clearly employed as protein-protein interaction motifs. The three-dimensional structure of the BRCT domain of XRCC1 has been solved at moderate resolution and reveals a core of four β-sheets surrounded by three α-helices as a common fold of BRCT domains (Zhang et al. 1998; Huyton et al. 2000). The interaction surfaces have been defined recently by site-specific mutagenesis experiments (A. Dulic et al., in prep.).

A minor alternative BER pathway occurs, which may be of particular importance for correction of complex lesions where both a base and the adjacent deoxyribose residue have been damaged. This subpathway employs replication factors such as FEN1, PCNA, and DNA ligase I for the final steps of the pathway, and apparently shares many features with the latter steps in lagging-strand replication. The alternative pathway has been reconstituted in vitro with purified human proteins (Klungland and Lindahl 1997; Matsumoto et al. 1999; Pascucci et al. 1999). *S. cerevisiae* employs this pathway during BER and apparently lacks the single-nucleotide pathway of mammalian cells. Thus, repair factors corresponding to pol-β with its AP lyase domain, XRCC1 and Lig3, have not been found in yeast and may only occur in higher eukaryotes.

MICE DEFICIENT IN REPAIR OF PROMUTAGENIC BASE RESIDUES

Gene knockout mice defective in BER functions such as APE1, pol-β, or XRCC1 exhibit an embryonic lethal phenotype (Friedberg and Meira 2000). These observations indicate that loss of the main pathway for repair of abasic sites in mammalian cells is a lethal event and provides an explanation for the apparent absence of inherited human syndromes associated with nonfunctional BER. These deductions are not totally conclusive at present, however, because several BER factors have accessory roles in other pathways. For example, the amino-terminal region of the mammalian APE1 endonuclease (Fig. 1) can serve as a coactivator of the hypoxia-inducible transcription factor HIF-1α (Xanthoudakis et al. 1994; Carrero et al. 2000). Additional gene-targeting experiments in mice, with site-specific mutation of the active site for endonuclease function of APE1, should serve to settle this point.

Several different mammalian DNA glycosylases act on particular subsets of DNA lesions to initiate BER, and consequently, each has fewer targets than the core enzymes in the later common steps of the pathway (Fig. 1). Mice deficient in 3-methyladenine-DNA glycosylase, with crippled ability to excise the cytotoxic 3-methyladenine lesion, have been constructed and remain viable, although hypersensitive to simple alkylating agents (Engelward et al. 1997; Elder et al. 1998). We were interested in

generating mouse mutants defective in removal of promutagenic DNA lesions such as uracil and 8-oxoG by BER, where bacterial and yeast mutants deficient in these DNA glycosylases exhibit strong mutator phenotypes.

MICE DEFICIENT IN 8-OXOG-DNA GLYCOSYLASE

The oxidized base 8-oxoG can direct incorporation of either C or A during replication and so causes transversion mutations unless repaired. Two groups have constructed mice lacking 8-oxoG-DNA glycosylase (OGG1), with concordant results (Klungland et al. 1999b; Minowa et al. 2000). Mice deficient in this enzyme show no detectable DNA glycosylase activity for 8-oxoG·C base pairs and a moderately but significantly increased (two- to threefold) spontaneous mutation frequency, largely accounted for by an increase in G·C→T·A transversions. This is a much weaker mutator phenotype than that seen in microorganisms unable to excise 8-oxoG from DNA. In contrast to mismatch-repair-deficient mice, but as expected for an accumulated endogenous lesion, increased mutagenesis in *ogg1* null mice was observed for an organ with a low level of proliferation (liver), whereas similar increased mutagenesis was not detected in rapidly proliferating testes cells. *ogg1* null mice appear to be normal and fertile, although a slight increase in tumor frequency in aging knockout mice might occur; this is being monitored. Interestingly, inactivating mutations in the human *OGG1* gene, which is located to chromosome 3p25, have now been observed as infrequently occurring events in collections of human tumor biopsies (Boiteux and Radicella 2000; Bruner et al. 2000).

A striking phenotype of *ogg1* null mice is the age-dependent accumulation of elevated levels of 8-oxoG residues in the DNA of nonproliferating tissues (Klungland et al. 1999b; Minowa et al. 2000). The amount of 8-oxoG detected is substantial, because part of the background in cellular DNA is due to trivial artificial oxidation of G residues during DNA isolation. An estimate of the correct in vivo background level of 8-oxoG in DNA can be made from the increased steady-state level of oxidized residues in an $ogg1^{-/-}$ fibroblast line, together with data on the reduced rate of elimination of the lesion from such cells (Klungland et al. 1999b). This approach yields an estimated steady-state value of only 50–100 8-oxoG residues per genome in normal repair-proficient cells, whereas livers from *ogg1* null mice contain 10,000–15,000 8-oxoG residues per cell. Greater than hundredfold increased amounts of an endogenous promutagenic DNA lesion in vivo would at first sight be expected to result in a greatly increased frequency of spontaneous mutagenesis; backups clearly exist to minimize mutagenic expression of the oxidized G residues. One such function could be the mammalian homolog of the MutY DNA glycosylase, which excises A residues base-paired with 8-oxoG, providing a second opportunity for the correct insertion of a C rather than an A by BER of the strand opposite the lesion (Slupska et al. 1996). A separate backup function was identified recently by the observation that $ogg1^{-/-}$ cells retain the ability to remove 8-oxoG from DNA by a transcription-coupled repair process (Le Page et al. 2000). The biochemical details of this process are presently unknown, because an in vitro assay for transcription-coupled repair is not available. However, a likely scenario is that the substantial accumulation of 8-oxoG residues in the DNA of *ogg1* null mice largely occurs in nontranscribed noninformational DNA, where it would do much less harm than in actively expressed genes.

MICE DEFICIENT IN URACIL-DNA GLYCOSYLASE

Uracil may appear in DNA by two separate routes: as a consequence of endogenous hydrolytic deamination of cytosine residues, and by the occasional use of dUTP instead of TTP as a precursor during DNA synthesis. In *E. coli* and *S. cerevisiae*, the UNG uracil-DNA glycosylase is required for removal of uracil from DNA. Consequently, bacterial or yeast mutants lacking UNG accumulate some uracil instead of thymine in their genomes and show a mutator phenotype with a greatly increased frequency of C→T transitions. Mammalian cells have an UNG enzyme with biochemical properties very similar to those of the microbial enzymes; in fact, the UNG sequence is one of the most highly conserved between *E. coli* and humans.

Mice deficient in UNG were constructed by standard gene knockout procedures (Nilsen et al. 2000). Nuclei from $ung^{-/-}$ cells incorporated and retained much higher levels of uracil in newly synthesized DNA than nuclei from control cells, although uracil was still slowly excised from the *ung* null nuclei. An elevated steady-state level of uracil in cellular DNA was also observed in vivo, with $ung^{-/-}$ cells containing about 2000 uracil residues per genome, whereas control cells had undetectably low (<100 per genome) levels of this nonconventional DNA base. Despite these clear differences, *ung* null mice only exhibited a marginally increased mutation frequency, and the animals appeared normal and fertile without showing any increased cancer frequency. These data strongly indicate that the increased steady-state level of uracil in the genome of $ung^{-/-}$ cells is due to an increase in the number of U·A base pairs replacing T·A pairs. Recent studies of the subcellular distribution of the UNG enzyme in wild-type cells have shown that UNG is largely or entirely confined to DNA replication factories in S-phase cells, where the protein interacts directly with the replication factors proliferating cell nuclear antigen (PCNA) and replication protein A (RPA) (Otterlei et al. 1999). It would appear that UNG has taken on a more specialized role in mammalian cells than in microorganisms and that the enzyme mainly functions in the replisome to excise uracil incorporated instead of thymine and to initiate BER of newly synthesized DNA. This postreplicational editing is reminiscent of the DNA mismatch repair pathway, which also acts on newly synthesized DNA, although substitution of uracil for thymine is not inherently mutagenic.

Table 1. Properties of Mammalian Proteins with Uracil-excising Activity

Gene product	Molecular mass (kD)	Substrate specificity				Inhibition by Ugi
		U:G	U:A	T:G	ssDNA	
UNG	34	yes	yes	no	yes	yes
TDG	46	yes	no	yes	no	no
MBD4	63	yes	?	yes	?	?
SMUG1	30	yes	yes	no	yes	no
Back-up activity in *ung*$^{-/-}$ cells	30–40	yes	yes	no	yes	no

The lack of a distinct mutator phenotype of *ung* null mice suggests that an alternative system exists to deal with the separate and important problem of cytosine deamination. Biochemical investigations of extracts from *ung*$^{-/-}$ cells and tissues revealed a second uracil-DNA glycosylase activity. The UNG enzyme activity could be blocked completely with the specific protein inhibitor, Ugi, or with UNG antibodies, whereas the second activity was refractory to these. In attempts to identify the accessory activity (Nilsen et al. 2000), its properties were compared with other enzymes that show some uracil-excising activity (Table 1). The low-abundance TDG activity, which can remove uracil or thymine base-paired with guanine (Neddermann et al. 1996), has a substrate specificity different from the backup activity observed in murine *ung*$^{-/-}$ cells and, moreover, TDG antiserum failed to inhibit the backup function. Two additional mammalian uracil-excising activities have been described recently. The MBD4 glycosylase has properties similar to those of the TDG enzyme in that it acts on deaminated methylated CpG sites (Hendrich et al. 1999), and also differs in size and specificity from the backup activity. *mbd4* null mice have been constructed (A. Bird, pers. comm.); cell-free tissue extracts from both *mbd4* and *ung* null mice showed the same level of uracil-excising activity refractory to the Ugi inhibitor, effectively ruling out MBD4 as the backup activity (our unpublished data). The SMUG1 DNA glycosylase was identified by in vitro expression cloning (Haushalter et al. 1999). The detailed properties of this nuclear activity remain to be clarified, but it appears to occur only in higher eukaryotes and is at present indistinguishable from the backup activity in *ung*$^{-/-}$ cells. It may be concluded that the backup activity is due either to the murine version of SMUG1, which appears the most likely possibility, or to yet another previously unrecognized DNA glycosylase encoded by a presently undefined gene. Considerable diversification of uracil-DNA glycosylases has clearly taken place during evolution to higher eukaryotes, in order to improve the cellular ability to correct deaminated cytosine and 5-methylcytosine residues in large genomes.

The compartmentalization of the abundant UNG activity to sites of DNA replication (Otterlei et al. 1999) may greatly facilitate the search for, and correction of, U·A base pairs in DNA. Since U·A and T·A base pairs are of similar stability, a genome-wide search for U·A pairs would presumably have to involve extrusion of very large numbers of deoxynucleoside residues into an extrahelical form during scanning by the relevant DNA glycosylase (Verdine and Bruner 1997), whereas such searching by UNG can be confined to newly replicated DNA. In contrast, most endogenous DNA lesions such as pyrimidine glycols, 3-methyladenine residues, or U residues opposite G are already destabilized in the double helix and could most likely be detected without large-scale scanning of DNA base residues in extrahelical form.

In conclusion, endogenous DNA damage is corrected by evolutionarily conserved, ubiquitous repair pathways. However, the large size of the mammalian genome necessitates increased complexity and diversification of the active factors.

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