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The T–T pyrimidine (6–4) pyrimidinone UV photoproduct is much less mutagenic in yeast than in *Escherichia coli*

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ABSTRACT

We have examined the mutagenic properties of the T–T pyrimidine (6–4) pyrimidinone UV photoproduct in *Saccharomyces cerevisiae*, transforming the yeast cells either with single-stranded vectors that carried this adduct at a unique site or with gapped duplex vectors in which the adduct was located within a 28 nt single-stranded region. In an earlier study with SOS-induced *Escherichia coli*, we found that this photoproduct is highly mutagenic, specifically generating 3′ T→C substitutions in >85% of replicated molecules, and ascribed this specificity to the formation of a stable guanine–pyrimidinone mispair via hydrogen bonds at N-3 and O-2. In constrast, this adduct is very much less mutagenic in yeast, with 60–70% of molecules being replicated accurately and only 12–20% of them exhibiting 3′ T→C substitutions. The enhanced accuracy may reflect the ability of a yeast DNA polymerase, but not *E.coli* DNA polymerase III, to trap the adduct in a configuration favorable for the formation of an adenine–pyrimidinone base pair.

INTRODUCTION

A comparison of the mutagenic properties of a particular DNA lesion in different organisms can provide insights into the mechanisms responsible for its mutagenicity. As first shown in a comparative study of the properties of the *cis-syn* and *trans-syn* isomers of the T–T cyclobutane dimer in yeast and *Escherichia coli* (1), these properties can differ very substantially. Based on this finding, we suggested that two of the three parameters defining mutagenic potential, namely the frequency of translesion synthesis and the error frequency of this process, were determined both by the structure of the lesion and by the particular replication proteins and conditions at play. The types of substitutions induced, however, were chiefly determined by the stucture of the lesion alone.

As part of a test of this hypothesis, we thought it interesting to examine the properties of the thymine–thymine pyrimidine (6–4) pyrimidinone UV photoproduct in the budding yeast *Saccharomyces cerevisiae* and compare these with its properties in *E.coli*, determined previously (2). In the bacterium this photoproduct is very mutagenic. Specific 3′ T→C mutations were induced in >85% of replicated vector molecules when SOS-induced cells were transfected with constructs carrying a T–T (6–4) adduct at a unique site. We suggested (2) that this high mutagenicity and specificity were the consequence of a relatively stable mispair between an incoming dGTP nucleotide and the 3′ pyrimidinone base. Although formation of the 6–4 bond eliminates O-4, co-planar hydrogen bonds could potentially form at N-3 and O-2 (Fig. 1). We obtained indirect evidence in support of this model by investigating the mutagenic properties of the T–C (6–4) adduct and also of the Dewar isomers of both this and the T–T photoproduct (2,3). Since the pyrimidinones derived from cytosine and thymine are very similar, differing only by the presence of a hydrogen or methyl group at C-5, both are expected by hypothesis to mispair with guanine, which would result in low mutagenicity for the T–C adduct. The mutagenicity of this photoproduct was indeed much lower (2), though a significant minority of 3′ C→T mutations was also observed, which was not predicted by the model. Conversion of either of the adducts to their Dewar photoisomer substantially decreases the probability of formation of the pyrimidinone–guanine mispair and the expected decrease in mutability of the T–T adduct and increase in mutability of its T–C counterpart were both observed (2,3).

We have now determined the properties of this photoproduct in yeast, using both a single-stranded vector carrying a uniquely located T–T (6–4) adduct and also a gapped duplex vector, in which the photoproduct is placed within a 28 nt single-stranded region. Methods were devised that routinely produced the gapped construct with an efficiency of 50–80%. We find that in both of these vectors the T–T (6–4) adduct induces few mutations; replication was accurate in 60–70% of molecules. The 3′ T→C mutation is still, by a small margin, the most common substitution, though it occurs in only 12–20% of replicated molecules.

MATERIALS AND METHODS

Construction of single-stranded and gapped duplex vectors carrying a site-specific T–T (6–4) adduct

Single-stranded constructs were made as described (1), using pYMV1 and an 11mer containing the T–T (6–4) lesion (2). This modified 11mer was shown to be >98% pure, using a digestion assay (2). Duplex constructs containing a 28 nt single-stranded

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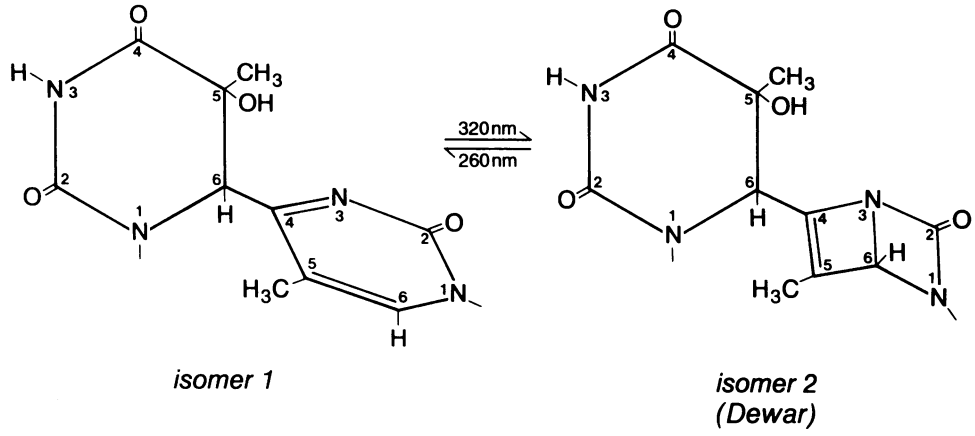

Figure 1. Structure of the normal and Dewar valence photoisomers of the T–T pyrimidine (6–4) pyrimidinone adduct (10,11).

region with the site-specific T–T (6–4) adduct were made by inserting a 36mer between the 3′ and 5′ recessed ends of *Eco*RI/*Pst*I-digested pYDV1 RF DNA. The 36mer was made by ligating a flanking 13mer to the 5′-end and a 12mer to the 3′-end of an 11mer that contained the T–T (6–4) adduct. The oligomers were annealed to a 50mer scaffold, followed by ligation, denaturation and purification by polyacrylamide gel electrophoresis. To provide an exact comparison, the sequence of the 28 nt single-stranded region was identical to that of the comparable region in the entirely single-stranded construct. pYDV1 was made by replacing the polylinker region of pYMV1 with a 24 nt sequence that created unique *Eco*RI and *Pst*I sites.

Several expedients were used to improve ligation efficiency, since direct ligation of a 36mer to the double digested vector is very inefficient. These included annealing the kinased 36mer to an unkinased complemetary 28mer (creating *Eco*RI and *Pst*I cohesive ends) and ligating each end independently in the presence of an appropriate restriction enzyme. Single-strand/double-strand junctions are ligated with only ~1% of the efficiency of normal ends. The presence of restriction enzyme during ligation reduces undesirable ligation products, but does not interfere with ligation of insert to vector because the 36mer sequence destroys the restriction site. Specifically, the method entailed digestion of pYDV1 RF DNA with *Eco*RI, dephosphorylation with calf intestinal phosphatase and ligation of a 5-fold molar excess of annealed 28mer/36mer (1:1) to the linearized vector at a DNA concentration of 700–850 ng/ml at 14°C overnight in the presence of *Mun*I (0.7 U/μg DNA). *Mun*I and *Pst*I, but not *Eco*RI, are sufficiently active in ligation buffer at 14°C to digest insert dimers. NaCl, to a final concentration of 80 mM, was added to the ligation buffer. First step ligation efficiencies of >95% can be achieved using these conditions. Increasing the molar excess of insert decreases the ligation efficiency, presumably because dimer formation is favored. Following this first ligation step, the construct was heated at 65°C for 15 min, digested with *Pst*I and the released fragment, together with excess insert, removed with the aid of a Wizard column (Promega, Madison, WI). The second, recircularization, ligation step was then carried out at a DNA concentration of 2.5 ng/ml, for 1–2 days at 14°C in the presence of *Pst*I (20 U/μg DNA). A 2-fold molar excess of 28mer was added to the mix prior to the second ligation reaction, to replace any that might have been removed from the 36mer by the Wizard column. Overall efficiency (proportion of vector molecules converted to desired construct) was 50–80%. Finally, construct preparations were concentrated by ultrafiltration through a Centricon-30 microconcentrator (Amicon, Beverly, MA).

Other methods

Yeast strain PG6-5B (MATa *rad1Δ::LEU2 phr1-1 his ura3-52*) was transformed with either single-stranded or gapped duplex vector DNA as described (1), but for the latter only 25 ng rather than 1 μg DNA was used and carrier DNA (4) was added. Lesion-free control construct, as well as construct containing a T–T (6–4) adduct, was used in each experiment. Prior to transformation, construct DNA was heated at 85°C for 5 min in the presence of a 1000-fold molar excess of the complement of the 28mer, to denature the 28mer from the gap and prevent it from re-annealing. The 28mer contained a C–C mismatch opposite the UV photoproduct, so that failure to denature or prevent re-annealing could be detected by the presence of a G–G sequence in the template strand of replicated products. Sequence analysis of a region starting ~50 nt 3′ to the abasic site and extending ~100 nt 5′ to this site was performed by extracting DNA from a random set of transformants, using the method described in Gibbs *et al.* (1), though in later experiments using twice the concentration of KAc (which improves recovery of the plasmid) and transfection of JM101 to provide single-stranded templates. All other methods are as described in Gibbs *et al.* (1).

RESULTS AND DISCUSSION

Experimental method

We investigated the mutagenic properties of the T–T (6–4) adduct in yeast using both a single-stranded construct and a double-stranded construct that possessed a 28 nt single-stranded region containing the photoproduct. The former provides an exact comparison with earlier data for other photoproducts, which were also obtained using the single-stranded vector. We used the latter because they have properties that make them particularly appropriate for work with yeast and other eukaryotes. By placing the lesion in a single-stranded region, the gapped duplex constructs share with single-stranded vectors the advantages of a

reduced susceptibility to repair and a lack of ambiguity in the sequence analysis of replicated products. However, unlike the single-stranded constructs, they possess a double-stranded origin of replication which permits normal initiation of DNA synthesis. Probably as a consequence, double-stranded vectors transform yeast ~100-fold more efficiently than single-stranded vectors. We chose a 28 nt single-stranded region for our constructs because it simulates an excision repair gap; it is likely that the majority of mutations induced by mutagen treatment of repair-proficient strains arise during the course of excision repair (5,6). Such mutations presumably occur when the single-stranded template in the excision repair gap itself contains a lesion.

Double-stranded vectors of this kind have been used previously by other investigators (7,8), but their methods were not efficient enough for repetitive use. We therefore explored methods to improve the efficiency (see Materials and Methods) and were able to routinely convert 50–80% of vector molecules into the desired construct. The remaining linear molecules transform yeast very poorly. The nucleotide sequence in replicated products of either type of contruct was determined in random sets of transformants, without use of selection or detection procedures, to avoid bias. DNA from transformants was transfected into *E.coli* strain JM101 and the resulting plaques used to provide templates for sequence analysis.

Consequences of replicating past the T–T (6–4) adduct

In contrast to *E.coli*, in which the T–T (6–4) photoproduct is highly mutagenic, replication past this lesion in yeast is much more accurate (Table 1). With the single-stranded construct, 71% (25/35) of the replication products contained an entirely normal sequence and with the gapped duplex construct the proportion was 59% (35/59). The two sets of data are not significantly different in this respect and on average 64% of molecules are replicated without error. This is very different from *E.coli*, where accurate replication occurs in only 9% of the molecules and by far the most common outcome (85%) is the insertion of guanine opposite the pyrimidinone base, giving a 3′ T→C substitution. This is the second most common event in yeast, though at 12% (7/59) and 20% (7/35) for the gapped duplex and single-stranded vectors respectively, it is much less frequent. The difference between the two sets of data cannot be ascribed to differences in purity of the samples of photoproduct-containing oligomer used or a lack of repeatability in the experiments. When tested in SOS-induced *E.coli*, the photoproduct-containing sample used for the yeast work was highly mutagenic, with 14/14 sequenced products exhibiting the 3′ T→C mutation. We also wish to emphasize that the sequence context was identical in all constructs used in the yeast and *E.coli* experiments, over a region extending 14 nt 5′ and 12 nt 3′ of the photoproduct site.

Although the photoproduct has substantially different properties in the two organisms, these results are nevertheless in reasonable agreement with the suggestion (1) that the types of nucleotide insertion are a property of the template, whereas the frequency of translesion synthesis and its accuracy are determined by both template structure and replication conditions. The two major types of insertions, of guanine and adenine, are found in both species, even though with different frequencies. The status of the remaining events is uncertain, because of their low frequency, but they too, for the most part, appear to follow the suggested rule. Both 5′ and 3′ T→A transversions were found only in yeast, however. The same is true of mutations at sites adjacent to the lesion (near-targeted mutations) and these may therefore also be an exception. None were found in yeast transformed with the single-stranded construct, however, so such events may not be characteristic of yeast. As noted above, and in keeping with the model, the frequencies of the various events are quite different in the two species and similarly the frequencies of translesion synthesis are also substantially different. Such synthesis occurs in ~40% of the construct molecules replicated in yeast and does not require the cells to be UV irradiated, but in *E.coli* the proportion is only ~20% and is almost entirely dependent on SOS-induction.

Table 1. Sequence at theT–T (6–4) photoproduct site in gapped duplex or single-stranded constructs replicated in yeast, compared with results from *E.coli*[a]

Sequence at the T–T target site	Number observed (%) Yeast Gapped duplex	Single-stranded	*E coli*[a] Single-stranded
T–T	35 (59)	25 (71)	16 (9)
T–A	4 (7)	1 (3)	0
T–C	7 (12)	7 (20)	158 (85)
T–G	2 (3)	1 (3)	1 (0.5)
A–T	4 (7)	0	0
C–T	0	0	2 (1)
G–T	2 (3)	0	2 (1)
Tandem	0	1[b]	5[c] (3)
Other	5[d] (8)	0 (3)	1[e] (0.5)
Total	59	35	185

[a]Data from LeClerc *et al.* (2).
[b]T–T→G–C.
[c]Three T–T→A–C and two T–T→C–C.
[d]One G–T–T→T–T–T and two each of G–T–T→T–T–C and T–T–G→T–T–T (photoproduct target site underlined).
[e]ΔT.

Although the results are broadly in agreement with the proposed model, the ability of yeast to avoid the high mutagenicity and specificity of the T–T (6–4) adduct must still be explained, particularly since these qualities were ascribed to an inherent property of DNA containing the photoproduct. In view of the photoproduct's properties in *E.coli*, we suggested that a stable mispair could be formed between guanine and the pyrimidinone base, by means of hydrogen bonds at N-3 and O-2. In support of this hypothesis, the same specificity is also seen with the T–C (6–4) adduct (3), where it results in accurate replication. The observation that specificity is decreased when either of these adducts is converted to its Dewar photoisomer also supports the model (2,3). These results would seem to preclude the formation of a stable adenine–pyrimidinone base pair, of the kind that might be expected to occur in yeast. Possible evidence for the existence of such a base pair, or some similar association, was however found in a study of the T–C (6–4) adduct (3); insertion of adenine, resulting in a 3′ C→T transition, occurred in 28% of the molecules analyzed. It is not known why this event is less common with the T–T (6–4) photoproduct, but it is perhaps the consequence of steric hindrance between the C-5 methyl, but not

C-5 hydrogen, and the phosphodiester backbone (3). This hindrance may impede the ability of the pyrimidinone base to assume a configuration favorable for the formation of a base pair with adenine. If this is so, it is perhaps possible that interaction of the lesion-containing template with the yeast, but not the *E.coli*, DNA polymerase traps the template in this favorable configuration. Whatever the case, it is unlikely that preferential insertion of adenine opposite the pyrimidinone base in yeast is the result of an 'A-rule' (9). We find that insertions opposite an abasic site specifically located at the same position as the photoproduct, in the same sequence context and vector are predominantly of cytosine (Gibbs *et al.*, unpublished data).

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