

Unraveling the Molecular Pathway from Sunlight to Skin Cancer

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Skin cancer is the most prevalent form of cancer, and its incidence has been steadily rising over the past 20 years.¹ Squamous cell and basal cell carcinomas will affect about one out of four Americans in their lifetime, and the most serious form, malignant melanoma, is expected to affect one in 100 by the year 2000.² Evidence that sunlight causes skin cancer by damaging the DNA comes from a rare genetic disease, *xeroderma pigmentosum*, which is characterized by defects in the ability of cells to repair DNA photodamage.^{3,4} Patients suffering from this disease are extremely sensitive to sunlight and have an approximately 1000-fold higher chance of developing skin cancer. Insight into the type of DNA photodamage responsible for inducing skin cancer comes from the analysis of the mutations found in the p53 tumor suppressor gene of skin cancers. Unlike protooncogenes which require specific mutations to become activated, tumor suppressor genes can be inactivated by a much broader range of mutations and thus retain the signature of the carcinogen. When the p53 genes of basal and squamous cell carcinomas were sequenced, a large number of C → T mutations at dipyrimidine sites were discovered as well as the tandem CC → TT mutation.^{5,6} These mutations are highly characteristic of UVB and UVC radiation,⁷ which causes both cis-syn dimers and (6-4) products to form at these sites⁸ (Figure 1).

That many of the sites of UV-induced mutations and DNA photoproducts coincide suggests that the mutations result from error-prone DNA synthesis past the photoproducts (targeted mutations) during repair or replication. Unraveling the mechanisms by which the mutations arise becomes complicated, however, because many of the initially induced photoproducts are not stable. Saturation of the 5,6 double bond has been known for some time to facilitate tautomerization⁹ and deamination¹⁰ of C (Figure 2), whereas we only recently discovered that the (6-4) products can photoisomerize in sunlight via their long-wavelength absorption band (Figure 3) to their Dewar valence isomers.^{11,12} Even though the sites of photoproducts and mutations coincide, their frequencies do not,¹³ suggesting that the sequence flanking a photoproduct can modulate the relative rates of further chemical transformations,

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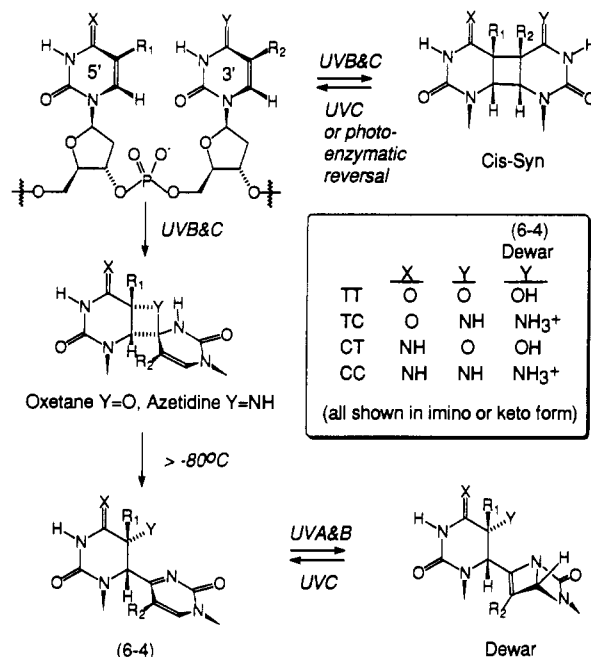


Figure 1. Photoproducts induced at dipyrimidine sites in DNA by UV radiation.

repair, and mutagenic bypass, making it difficult to assign an observed mutation to a specific photoproduct (Figure 4).

Elucidating the mechanisms of mutagenesis by sunlight takes on a certain degree of urgency, as it appears that the ozone layer which serves to shield us from the DNA-damaging solar radiation is slowly being depleted.¹⁴ The amount of ozone involved, which is distrib-

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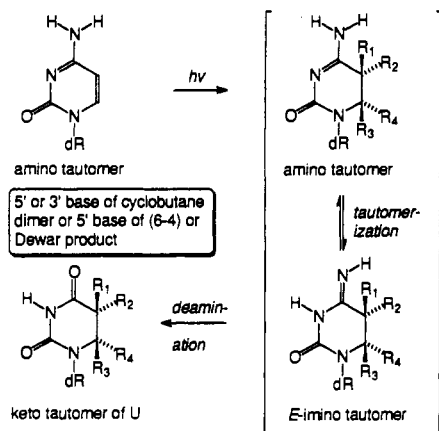


Figure 2. Tautomerization and deamination chemistry of cytosine in photoproducts of dipyrimidine sites.

uted throughout the stratosphere, is frighteningly small and, if collected, would result in a layer only 3 mm thick at standard temperature and pressure.¹⁵ Ozone's remarkable effectiveness at reducing DNA damage is due to the fact that it has an absorption spectrum almost identical to that of DNA itself (Figure 3)! To appreciate the impact of a reduction in the amount of ozone on biological systems, consider a simple calculation based solely on the solar emission spectrum,¹⁶ the absorption spectra of ozone¹⁵ and thymidine,¹⁷ and the quantum yield for cis-syn dimer formation in DNA.¹⁸ At present levels of ozone, direct exposure of DNA to sunlight for 1 h at sea level would induce approximately seven cis-syn dimers per 1000 sites. With a 50% reduction in the amount of ozone, this same amount of damage would be produced in only 10 min, and in the absence of ozone, in only 10 s! More detailed calculations have concluded that, for every 1% decrease in ozone level, there will be a 4% increase in the skin cancer rate.¹⁹

Combined Synthetic, Physical, Enzymatic, and Biological Approach to Unraveling DNA Photo-

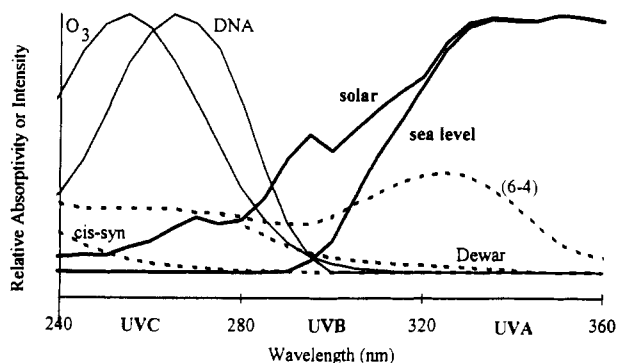


Figure 3. Absorption spectra of ozone and DNA (solid lines), and the various photoproduct classes (dashed lines), in relation to the intensity spectra of sunlight outside our atmosphere and at sea level (thick lines).

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product Structure-Activity Relationships. One of our long-range goals is to explain and predict the mutations that arise from exposure of a given organism to a specific dose and spectrum of light. On the basis of this knowledge and knowledge of which mutations cause cancer, methods could be developed for reducing the risk of skin cancer caused by sunlight. Our approach²⁰ to obtaining the required photoproduct structure-activity relationships rests on the design and synthesis of building blocks for the site-specific incorporation of DNA photoproducts into oligonucleotides of any sequence, at any site, and any number of times, by automated solid-phase DNA synthesis.²¹⁻²³ In those cases in which we are unable to construct a building block, we irradiate oligonucleotides and chromatographically isolate the photoproduct-containing products.²⁴ These uniquely photodamaged oligonucleotides are the key intermediates that we then use to construct (a) short duplexes for melting temperature, high-field

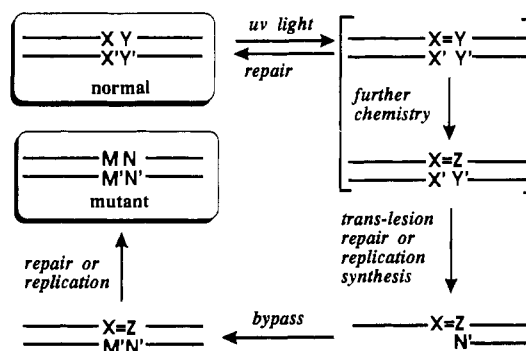


Figure 4. General scheme for the formation of UV-induced mutations by error-prone DNA synthesis past DNA photoproducts and their suppression by DNA photoproduct repair.

2D NMR,²⁵ and X-ray crystallographic studies; (b) polymers for DNA bending and unwinding studies;^{26,27} (c) long duplexes for in vitro repair and chemical probe studies,²⁸⁻³⁰ (d) template-primers for in vitro mutagenesis studies,^{31,32} and (e) viral DNA for in vivo repair and mutagenesis studies.³³

We chose to determine the structure-activity relationships of the cis-syn dimer first because, of all the photoproducts, it appears to have the highest mutagenic potential. Cis-syn dimers are induced at about 3-10 times the rate of (6-4) products^{34,35} and are repaired almost 10 times slower in both bacterial and mammalian

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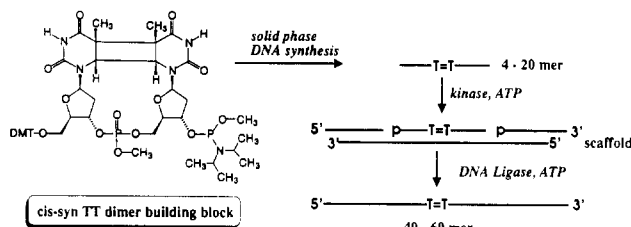


Figure 5. Building block for the construction of TT cis-syn dimer containing oligonucleotides and their incorporation into longer sequences by enzymatic ligation.

cells,³⁶ making them the most likely photoproduct to be encountered by a polymerase during repair or replication. Because cytosine-containing dimers can deaminate (Figure 2), we decided to initially study the structure–activity relationships of the cis-syn dimer of TT with the expectation that many of the structure–activity relationships should be similar. Because the biology of human cells is rather complex and poorly understood, we also decided to first focus on understanding the mechanism of mutagenesis in the much better characterized bacterium *Escherichia coli*. This bacterium appears to be a suitable model system because, like mammalian cells, the major UV-induced mutation is the C → T substitution mutation at dipyrimidine sites.^{37,38} Furthermore, many of the repair and replication enzymes of *E. coli* share homologies with those of human cells.

Structure and Repair of Cis-Syn Dimers of TT Sites. The ability of repair enzymes to locate DNA damage must depend on alterations in the structure and properties caused by the damage. To determine the effect of cis-syn dimer formation on the structure and properties of DNA we designed and synthesized a cis-syn dimer building block suitable for solid-phase automated DNA synthesis (Figure 5).²¹ This building block was then used to synthesize multimilligram amounts of a decamer containing a central cis-syn dimer which was annealed to a complementary strand to produce one turn of a DNA duplex for both 2D NMR and melting temperature studies (Figure 6).²⁵ A comparison of the chemical shifts of the dimer-containing duplex with those of the undamaged parent indicates that the dimer only perturbs the central six

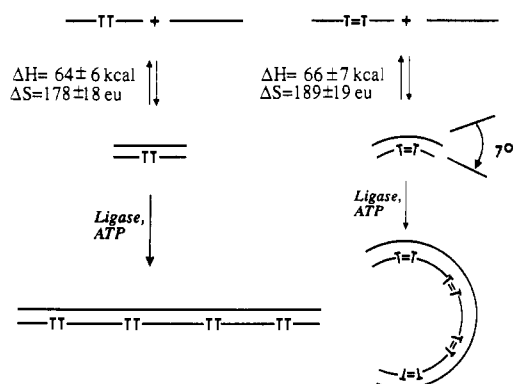


Figure 6. Melting temperature and bending studies of dimer-containing DNA.

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base pairs of the helix, while melting temperature studies indicate that this perturbation does not appreciably affect the thermodynamics of duplex formation. By synthesis and enzymatic ligation of dimer-containing duplex decamers and 11-mers with overhanging “sticky” ends, DNA polymers are produced in which the dimers are in phase and thus cause the DNA to bend and migrate slower on electrophoresis gels than the undamaged, linear counterpart (Figure 6). Analysis of the electrophoretic mobility of these polymers suggested that the dimer only caused the DNA to bend by about 7° and to unwind by 15°.^{26,27} Dimer formation was also found to be capable of reducing the native bend of a T₆ tract from 19° to 7°, suggesting that some of the toxic effects of light might be due to dimer-induced disruption of DNA bends that template the assembly of transcription and other regulatory factors.²⁶

The most general mechanism that living systems have developed for repairing DNA damage is excision of a small section of the damage-containing strand followed by synthesis of a new strand utilizing the undamaged strand as a template.³⁹ The best characterized of these systems is the uvr(A)BC excinuclease of *E. coli*, which cleaves the fourth or fifth phosphodiester on the 3′-side of the damaged nucleotide and the eighth phosphodiester on the 5′-side. To study the repair of cis-syn dimers we have ligated short dimer-containing oligonucleotides of variable sequence to other oligonucleotides in the presence of a complementary oligonucleotide scaffold to form dimer-containing oligonucleotides about 50 nucleotides in length (Figure 5). In experiments carried out in collaboration with Aziz Sancar at the University of North Carolina, the cis-syn dimer was found to be excised by the uvr(A)BC excinuclease in a relatively sequence independent manner, but at a rate 1/9 that of either the (6-4) or Dewar photoproducts in the same sequence context.²⁸ The relative rates of excision of these photoproducts also parallel their binding affinities for the uvrA subunit and the human DNA damage binding protein.²⁹ The low affinity of these damage recognition proteins for the cis-syn dimer is consistent with our findings that the cis-syn dimer only causes a minor perturbation of the structure and properties of DNA.

DNA damage can usually be repaired in an error-free manner by excision because the undamaged strand complementary to the excised section serves as a template for gap-filling by a polymerase. To investigate what would happen in the rare event that both strands were damaged, we constructed substrates containing closely opposed dimers with either a 5′- or a 3′-staggered relationship (Figure 7).²⁸ In collaboration with Aziz Sancar we found that the uvr(A)BC enzyme was able to excise either dimer in the 5′-staggered orientation nonselectively, but not both simultaneously. The resulting gapped duplex would contain a dimer that would lead to a mutation if it were bypassed in an error-prone manner by a repair polymerase. In contrast, we found that the dimer in a 3′-staggered orientation was refractory to excision by the uvr(A)BC enzyme, suggesting that, in the absence of any other repair pathways, survival of the cell line would depend on bypassing at least one of the dimers during replication, and if bypass

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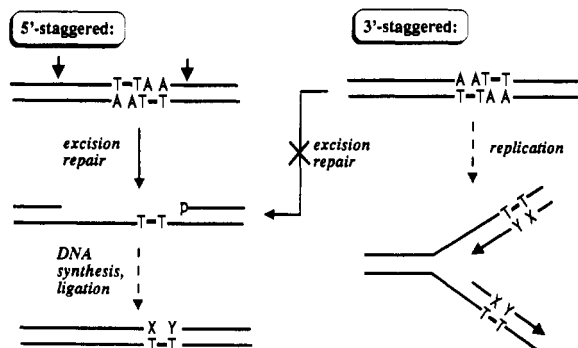


Figure 7. Excision repair of closely opposed dimers and the potential consequences.

were to occur in an error-prone manner, a mutation would result.

In Vitro DNA Synthesis Past Cis-Syn Dimers of TT Sites. The principal mechanism by which mutations are produced is thought to involve error-prone synthesis past the damage site by a repair or replication polymerase. We first chose to study DNA synthesis by *E. coli* DNA polymerase I on photodamaged templates because this enzyme is thought to play a role in repair and because the kinetic mechanism whereby it replicates DNA is well understood.^{40,41} Like most polymerases, pol I replicates DNA by a series of elongation steps in which a dNTP that is complementary to the nucleotide in the template strand is coupled to the 3'-OH of the primer DNA strand with release of pyrophosphate ($P_2O_7^{4-}$) (Figure 8).⁴² When the elongation step is slowed down because the polymerase incorporates an incorrect nucleotide, or because the polymerase encounters damaged DNA, there is sufficient time for the 3' → 5' exonuclease to hydrolytically remove the nucleotide attached to the 3'-end of the primer, a step referred to as proofreading.

To determine the ability and mutagenicity of DNA synthesis past the cis-syn dimer of TT by pol I in vitro, we annealed a 5'-radiolabeled primer to the 3'-end of the same photoproduct-containing oligonucleotides used in the repair studies and then added the polymerase and dNTPs (Figure 9). When incubated with our dimer-containing template, pol I led to time, dNTP, and polymerase concentration dependent formation of

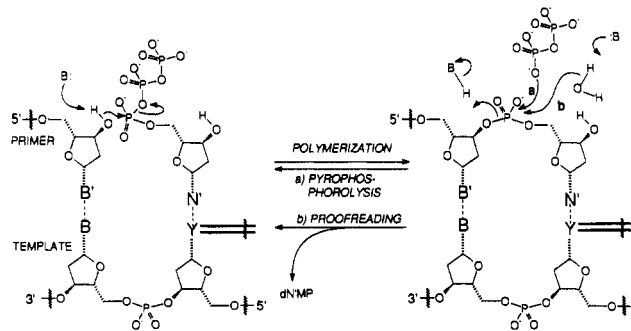


Figure 8. Basic steps in the bypass of photodamaged DNA by a DNA polymerase.

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both termination and bypass products. The ability of pol I to bypass the dimer was quite unexpected and exciting given that previous studies with inhomogeneous substrates had concluded that it could not.^{43,44} The increase in bypass rate with increasing polymerase concentration suggested that dissociation of the polymerase from the primer-template was competing with elongation past the dimer and that the binding affinity of a polymerase for the template-primer, and hence its processivity, plays an important role in the bypass of DNA damage. In support of this idea, we have recently shown in collaboration with Peter Burgers of the Washington University Medical School that the processivity factor known as PCNA dramatically enhances the ability of calf thymus polymerase δ to bypass the cis-syn dimer.⁴⁵ Polymerase δ in association with PCNA is thought to be involved in both replication and repair synthesis in mammalian cells, and the ability of this complex to bypass the cis-syn dimer suggests that it could play a role in UV-induced mutagenesis in human cells.

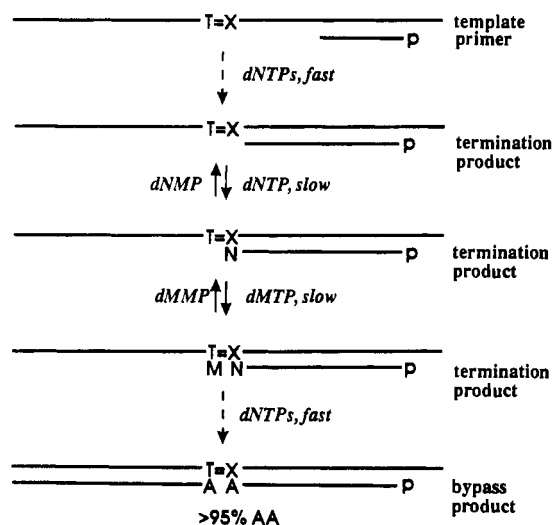


Figure 9. Scheme used to study the kinetics and mutagenic consequences of the bypass of DNA photoproducts by polymerases in vitro ($X = T$ or U).

When the bypass product from the pol I reaction was sequenced, we discovered that A was the only nucleotide incorporated opposite each T of the dimer within the limits of detection (>5%) (Figure 9). The origin of this highly specific incorporation of A's opposite the dimer is not yet understood with certainty. Some have argued that the specificity reflects a built-in bias of polymerases for incorporating A's opposite noninstructional damage, i.e., damage that is not capable of forming base pairs by hydrogen bonding (the "A rule").⁴⁶ NMR studies of the imino hydrogens in a DNA octamer containing a central cis-syn dimer of TpT indicate, however, that the cis-syn dimer is capable of hydrogen bonding to the A's in the opposite strand,⁴⁷ suggesting that the A's are preferentially incorporated by way of complementary H-bonding interactions (Figure 10). We also discovered

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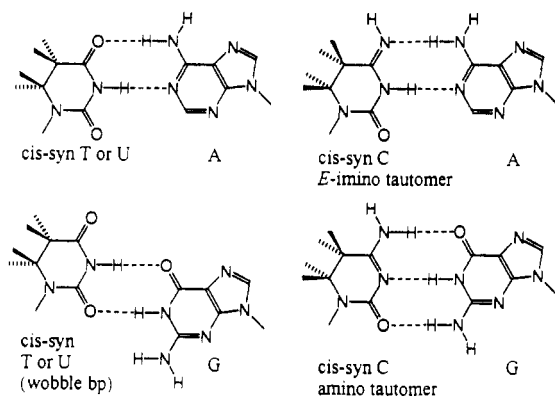


Figure 10. Possible base-pairing properties of T-, U-, and C-containing cis-syn dimers.

that G was incorporated opposite the 3'-T of the dimer in the primer terminating opposite that site, perhaps by wobble base-pairing. The fact that no G was detected at this site in the bypass product suggested to us that, in addition to preferential incorporation of nucleotides opposite a damage site, preferential elongation of primers terminating opposite damage may also contribute to the overall fidelity of bypass.

Mutation Spectrum of the Cis-Syn Dimer in Vivo. To determine the mutagenicity of the cis-syn TT dimer in vivo we have adopted a general strategy pioneered by John Essigmann of MIT that involves site-specifically incorporating DNA damage into a virus and then sequencing the mutant progeny.⁴⁸ In our laboratory, we incorporate the dimer into a bacteriophage by a general and efficient technique for site-specific mutagenesis of DNA sequences⁴⁹ in which we use the same dimer-containing template used in the in vitro studies to prime the synthesis of the (-) strand of a bacteriophage DNA containing some U's in place of T's in the (+) strand (Figure 11).³³ To distinguish the progeny resulting from replication of the two strands, two nucleotides of the (+) strand were chosen not to be complementary to the dimer-containing primer. The dimer-containing bacteriophage was then transfected (inserted) into a repair deficient, uracil glycosylase proficient, *E. coli* host which degrades the (+) strand and thereby enhances replication of the dimer-containing (-) strand. The transfected cells were then spread out on agar plates to allow the formation of

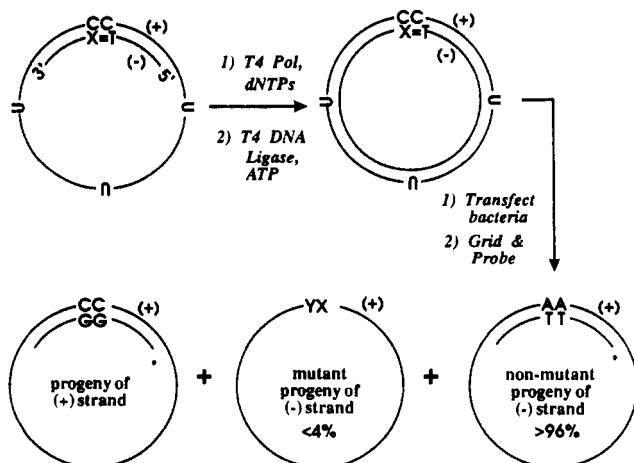


Figure 11. Scheme used to determine the mutation spectra of DNA photoproducts in vivo (X = T or U).

individual bacterial colonies that are derived from single cells. The bacterial colonies were screened for the progeny derived from replication of the (+) strand or the photoproduct-containing (-) strand by probing with complementary radiolabeled oligonucleotides. Mutant progeny were identified by their failure to hybridize well to either probe, and then they were grown in sufficient quantity for DNA sequencing. In normal cells, <1% of the progeny were derived from the photoproduct-containing strand, indicating that the cis-syn dimer is a substantial block to DNA replication. Irradiation of cells induces what is known as the SOS response, which causes the expression of a number of repair and replication proteins. Some of these proteins are thought to enable DNA polymerase III to bypass DNA damage,⁵⁰ presumably by enhancing the processivity of the enzyme much in the way that we observed PCNA to do with calf thymus pol δ . When we briefly irradiated the cells with 254-nm light prior to transfection, about 50% of the progeny were derived from the dimer-containing strand and >96% of the bypass occurred by incorporation of two A's opposite the dimer, just as we had found to be the case in vitro with pol I. A similar specificity has also been reported by Lawrence and co-workers at the University of Rochester in both *E. coli* and yeast.⁵¹

Cis-Syn Dimer Induced Deletion and Substitution Mutations in T Tracts. Though cis-syn dimers of TT appear to direct the incorporation of A's during bypass, there is evidence to suggest that they can cause mutations by mechanisms other than direct misincorporation. T tracts in the *lacI* gene of *E. coli* are hotspots for both UV-induced -1 deletion mutations⁵² and cis-syn dimer formation,³⁴ suggesting that the deletion mutation results from the bypass of a cis-syn dimer by some sort of slippage mechanism.⁵³ Unfortunately, it is impossible to deduce from mutation spectra alone which, if any, of the $n - 1$ possible dimers of a T_n tract are responsible for the observed mutations.

To determine whether cis-syn dimers can cause deletion mutations in T tracts, we used our cis-syn dimer building block to synthesize each of the five possible adjacent cis-syn dimers of a T_5 tract²⁶ and incorporated them into templates 59 nucleotides long.³² Of all the dimers, only the cis-syn dimer between positions 2 and 3 from the 5'-end of the T tract led to both -1 and -2 deletion mutations, as well as a totally unexpected A \rightarrow C transversion mutation opposite the 5'-T of the T tract when incubated with a 3' \rightarrow 5' exonuclease deficient T7 polymerase. The formation of these mutations and their specificity for one dimer site can be readily explained by a slippage mechanism (Figure 12) and two important features of the bypass of cis-syn dimers of TT that we had determined in our previous studies. The first is that cis-syn TT dimers direct the incorporation of A's, and the second is that the rate of polymerase-catalyzed primer-extension reactions is much slower opposite cis-syn dimers than it is opposite undamaged DNA. Thus, in the first step leading to a

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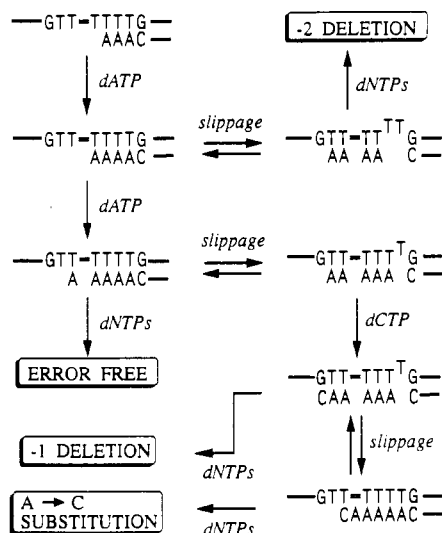


Figure 12. Misalignment-mediated bypass mechanism for the origin of cis-syn dimer induced deletion and substitution mutations in a T_6 tract.

-2 deletion mutation A is incorporated opposite the 3'-T of the dimer, but further elongation opposite the dimer is slow, giving the DNA ample opportunity to slip and misalign the A tract with the T tract. Elongation of the misaligned intermediate leading to the -2 deletion mutation takes place in competition with realignment because the primer terminus of the misaligned structure forms a normal base pair and the intermediate bulge loop is sufficiently distant from the polymerase site. Formation of the -1 mutation follows a similar course.

In support of the slippage mechanism, the A \rightarrow C mutation can be seen to arise by realignment of a misaligned intermediate in the pathway leading to the -1 deletion mutation (Figure 12). This type of UV-induced $A_nX \rightarrow A_{n-1}XX$ mutation had not been described before, but a subsequent survey of the literature revealed three instances of UV-induced $A_nC \rightarrow A_{n-1}CC$ mutations.^{52,54,56} When the same dimer-containing sequence used in the *in vitro* experiments was replicated in *E. coli* under SOS conditions, we detected about 6% of the substitution mutation, only about 1% of the -1 mutation, and none of the -2 mutation.⁵⁶ Whether the -1 deletion produced by UV *in vivo* results from one of the other cis-syn dimer containing T_6 tracts or from another photoproduct remains to be determined and is currently under study.

On the Origin of UV-Induced C \rightarrow T Mutations. The C in a cyclobutane dimer has been estimated to deaminate to U with a half-life of 12 h *in vivo*⁵⁷ compared to 30 000 years for cytosine itself.¹⁶ On the basis of the propensity of C-containing cis-syn dimers to deaminate to U-containing dimers (Figure 2), we have proposed what we call the deamination-bypass mechanism for the origin of UV-induced C \rightarrow T mutations.^{23,31,33} Because U is identical to T except for the absence of a methyl group, a U in a dimer would be expected to direct the incorporation of A by a polymerase just as we determined a T in a dimer to do (Figure 11). Thus

in systems where cell division is slow, as would be the case in human cells, there would be plenty of time for a C-containing dimer to deaminate to a U-containing dimer prior to replication. In rapidly replicating systems, a tautomer-bypass mechanism proposed by Bockrath and co-workers may apply in which the (*E*)-imino tautomer of the C ring of a cis-syn dimer (Figure 10) directs the incorporation of A by the polymerase.^{58,59} Evidence that the C of a cis-syn dimer can adopt this tautomeric form comes from theoretical calculations on 5,6-dihydrocytosine⁶⁰ and from the observation that 5,6-dihydro-CTP can substitute for either CTP or UTP in a transcription system.⁶¹

To determine whether or not replicative bypass of U or the structurally equivalent (*E*)-imino tautomer of C in a cis-syn dimer would result in the introduction of A as proposed, we incorporated the cis-syn dimer of TU site-specifically into an oligonucleotide 49-mer and then into bacteriophage DNA.³³ *In vitro*, replication by either the Klenow fragment of *E. coli* DNA polymerase I or exo-free T7 polymerase led to >95% incorporation of A opposite both the T and the U of the dimer,⁶² and in *E. coli* under SOS conditions, >97% incorporation of A was observed.³³ Because the (*E*)-imino tautomer of the cis-syn dimer of TC has base-pairing properties equivalent to those of the cis-syn dimer of TU, it seems likely the same sequence specificity of bypass would be observed. One could also propose that A would be incorporated opposite the (*E*)-imino tautomer of C's in the cis-syn dimers of CT and CC, as well as the U's in their deamination products, cis-syn dimers of UT and UU, and could explain the origin of CT \rightarrow TT and CC \rightarrow TT mutations (Figure 13). Recently, Lawrence and co-workers at the University of Rochester have reported that A's are indeed almost exclusively incorporated opposite the cis-syn UU dimer in *E. coli*.⁶³ What is not known at the moment is the mechanism by which the A's are selected for incorporation opposite T- and U-containing cis-syn dimer, nor is it known what nucleotides are in fact incorporated opposite C's in a dimer, questions that we are currently investigating.

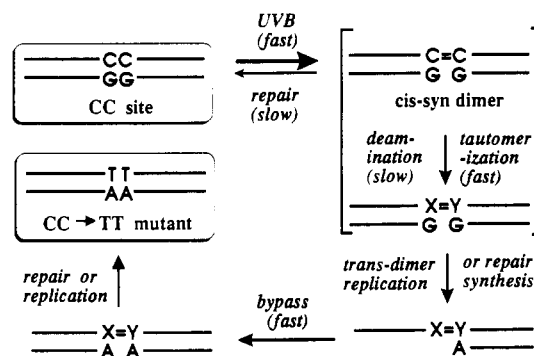


Figure 13. Tautomerization and deamination bypass mechanisms for the origin of UV-induced CC \rightarrow TT mutations.

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Conclusion. The ability to prepare site-specific photoproduct-containing DNA has greatly enhanced the ability to unravel the structure-activity relationships in UV-induced mutagenesis and carcinogenesis. Whereas we and others have found that A's are incorporated opposite the cis-syn dimer of TT, and thus it is inherently nonmutagenic, we have found that when a dimer is embedded in a T tract, it can cause both deletion and substitution mutations via a strand slippage mechanism. Because A's are also incorporated opposite the cis-syn dimer of TU, deamination of the TC dimer, or the structurally equivalent isomerization to its (*E*)-imino tautomer, is predicted to be highly mutagenic process that could explain the origin of UV-

induced CT, TC, and CC→TT mutations. These initial results with simple model systems are compelling, but there is still much to be learned about the mechanisms of mutagenesis by sunlight, especially in mammalian systems. There are many proteins and enzymes involved in the repair and replication of damaged DNA that have yet to be isolated and their interactions with DNA photoproducts fully characterized. Likewise, the rates at which photoproducts form and undergo further chemical transformation, the mechanism by which nucleotides are selected for incorporation opposite these photoproducts, and the influence of sequence, conformation, and DNA packaging on the chemical and enzymatic processes also need to be explored.