Cyclobutane thymine dimers in a *ras* proto-oncogene hot spot activate the gene by point mutation

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ABSTRACT

ras proto-oncogenes with a cyclobutane-type thymine photodimer (*cis-syn* or *trans-syn* isomer) were constructed by replacement of a portion of the gene with a chemically synthesized fragment. When the genes were transfected by the calcium phosphate method into mouse NIH3T3 cells, they induced focus-formation, indicating that both photoproducts were mutagenic in mammalian cells. Sequence analysis of the *ras* gene fragments derived from the transformed cells showed that the genes were activated by a point mutation. The mutations detected most frequently were 3'-T → A for the *cis-syn* isomer and 5'-T → A for the *trans-syn* isomer. In contrast, a different trend of mutations was observed when a primer on a DNA template with a *cis-syn* dimer was extended *in vitro* by either DNA polymerase β or α .

INTRODUCTION

Ultraviolet (UV) light damages biologically important macromolecules, including DNA, and the damage of DNA is thought to be involved in mutagenesis. It is well known that exposure to UV light induces mutations in prokaryotes (1–5). Patients with xeroderma pigmentosum (XP) syndrome frequently develop skin cancer in areas exposed to sunlight (6) and cells derived from XP patients cannot effectively repair UV-induced DNA damage (7), indicating that the DNA damage induced by UV light may be directly related to carcinogenesis, at least in the case of XP patients. Activation of the *ras* gene in skin cancers, such as melanomas, has been reported (8–11). Keijzer and colleagues have demonstrated that a melanoma cell line from an XP patient (complementation group C) has an activated N-*ras* gene with a point mutation at the third position of codon 61 (12). *In vitro* UV irradiation of an N-*ras* gene and analysis transfection assay showed that UV light activated the *ras* gene by a point mutation at codons 61 and 12 (13). Therefore, UV light may

be involved in skin cancer development through *ras* gene activation.

Among the various types of DNA damage, cyclobutane-type pyrimidine photodimers and (6–4) pyrimidine-pyrimidone photoproducts are the major DNA lesions induced by UV light. It has been reported that the cyclobutane-type dimer is mutagenic in prokaryotes. Site-directed mutagenesis experiments using oligonucleotides with a cyclobutane-type thymine dimer (either *cis-syn* thymine dimer (csTD) or *trans-syn* thymine dimer (tsTD)) at a predetermined site showed that the dimers induced a point mutation in the modified positions when transfected into *Escherichia coli* (14, 15). We are interested in studying the mutation-inducibility of thymine dimers in eukaryotic cells. It is also important to investigate whether the *ras* gene can be activated by the production of a thymine dimer in the gene.

We have studied the inducibility and the spectra of mutations by DNA lesions in mammalian cells (*O*⁶-methylguanine, hypoxanthine, xanthine, 8-hydroxyguanine, and an abasic site analogue) (16–20). We introduced the lesions into either codon 12 or codon 61 of a synthetic c-Ha-*ras* gene with several unique restriction enzyme sites, which is very useful for DNA cassette techniques. This system can also be employed for other DNA lesions.

We report the construction of *ras* genes with a thymine dimer, csTD and tsTD (Fig. 1), at codon 61 in the antisense strand and the detection of genes activated by point mutations at the dimer site. We also report nucleotide incorporation opposite the dimers during the *in vitro* DNA synthesis by DNA polymerases β and α .

MATERIALS AND METHODS

Enzymes

*Bcl*I, *Msc*I, *Pvu*I, and *Csp*45I were obtained from TOYOBO. *Rsa*I and *Taq*I were purchased from Nippon Gene. *Eag*I was obtained from New England Biolabs. Other restriction enzymes,

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Figure 1. Structures of *cis-syn* and *trans-syn* thymine dimers (represented by csTD and tsTD, respectively).

T4 polynucleotide kinase, and T4 DNA ligase were from Takara. λ exonuclease was purchased from BRL.

Recombinant rat DNA polymerase β was purified as previously described (21). Mouse DNA polymerase α -primase complex was isolated by the procedure described previously (22).

Synthesis of oligonucleotides with a thymine dimer

A building block of csTD for DNA synthesis by the solid phase phosphoramidite method was prepared according to the method described previously (23). An oligonucleotide with csTD was synthesized as described previously (23). A tsTD-containing oligonucleotide was also synthesized using a building block (Murata *et al.*, unpublished results). The oligonucleotides were extensively purified by reverse phase HPLC and anion exchange HPLC as described previously (18).

Preparation of DNA cassettes and construction of vectors

DNA cassettes with csTD or tsTD were prepared as described previously (17). Namely, U8', an L12 oligonucleotide that contains either csTD or tsTD, and the L13 oligonucleotide were phosphorylated with T4 polynucleotide kinase and then were mixed with the oligonucleotides U7 and L11' (Fig. 2A). The mixtures were annealed, ligated with T4 DNA ligase, and then treated with T4 polynucleotide kinase to obtain the DNA cassettes. Vectors for transfection were constructed as described (17) except that pEB (20) was used instead of pRSVras. Control vectors (Gln-61 and Lys-61) were prepared by the same procedure.

DNA transfection assay

DNA transfection assays were done according to the method described (24, 25). Various amounts of vector DNA (20, 30, 50, and 150 ng) together with 30 μ g of genomic DNA from NIH3T3 cells were used.

Analysis of *ras* genes in transformants

The *ras* gene fragments around codon 61 (204 bp) were amplified from the genomic DNA of transformed cells by the polymerase chain reaction (PCR) method (26), using the oligonucleotides U5 (5' dGAAGACTCTTACCGTAAGC 3', corresponding to the region from codon 37 to codon 43 of the *ras* gene) and L9 (5' dTTTAACGCGTTTGATCTGTTACGGTATTGATGG-ATGTCTTC 3', complementary to the sense strand of the *ras* gene from codon 91 to codon 104) (27) as primers, and the products were analyzed by the polymerase chain reaction-

restriction enzyme (PCR-RE) method (16). To analyze the second position of codon 61, the mutagenic primers 5' dGATATCC-TTGATACCGCNGGC 3' (which corresponds to the *ras* gene from codon 54 to codon 60, except for the bold N) and L9 were used in the second PCR. The amplified DNA (153 bp) was then incubated with *Sna*I (N=A), *Apa*I (N=G), *Eag*I (N=C), or *Msc*I (N=T). For *Taq*I digestion, a mutagenic primer, 5' dGATAT-CCTTGATACCGCAGGTC 3' (the bold T is the mismatch base), was used in the second PCR. To analyze the third position of codon 61, the mutagenic primers 5' dATCACGCATCGC-AGAGTATNGATC 3' (which is complementary to the sense strand of the *ras* gene from codon 62 to codon 69, except for the bold NGA) and U5 were used in the second PCR, and *Bam*HI (N=G), *Bcl*II (N=T), *Bgl*III (N=A) and *Pvu*I (N=C) were used for the restriction enzyme analysis. The PCR products (99 bp) were treated with an appropriate restriction enzyme and were analyzed by 10% polyacrylamide gel electrophoresis (PAGE).

Extension of a primer on a DNA template with a thymine dimer *in vitro*

DNA templates with a thymine dimer were prepared by enzymatic joining of L11, L12, and L13 (Fig. 2B) using U8' (see Fig. 2A) as a splint. The ligation products were purified by denaturing (8M urea) 20% PAGE. The templates were annealed with a primer, U7, (Fig. 2B) and were incubated with rat DNA polymerase β or mouse DNA polymerase α -primase using the conditions described previously (28, 29). The duplex DNA thus obtained was purified by PAGE and digested with *Rsa*I. After purification by PAGE, the DNA was treated with λ exonuclease as described (30). Incorporated nucleotides were analyzed by the PCR-RE method. To analyze the second position of codon 61, mutagenic primers and L11 (see Fig. 2B) instead of L9 were used in the PCR. To analyze the third positions of codon 61, mutagenic primers and U7 (Fig. 2B) instead of U5 were used. The PCR-RE method was also used to determine whether a deletion occurred during *in vitro* DNA synthesis. Mutagenic primers, 5' dGATATCCTTGATACCGCAGTCT 3' and 5' dGATATCCTTGATACCGCAGTTC 3', were used in the PCR for detection of the -1 and -2 deleted products, respectively. For restriction enzyme analysis, *Xba*I and *Csp*45I were used for the -1 and -2 deletions, respectively.

RESULTS

Oligonucleotide synthesis and vector construction

In the present study, we focused on the mutation-inducibility of thymine dimers in mammalian cells, and introduced these DNA lesions into codon 61 of the *ras* gene, a hot spot for mutations detected in the gene in human tumor cells (31, 32). Although codon 61 of the human c-Ha-*ras* gene has a CAG sequence, hence, a 5'-CT-3' site rather than a 5'-TT-3' site, we introduced the csTD and tsTD lesions because the TT dimer is the major product of the cyclobutane-type pyrimidine dimers (33, 34), and because the TT site exists in codon 61 of the human c-Ki- and N-*ras* genes. We also planned to compare results from mammalian cells with those from bacteria (14, 15, 35).

We previously reported the synthesis of an oligonucleotide with csTD using a dimer block (23). We have also succeeded in synthesizing an oligonucleotide with tsTD (Murata *et al.*, unpublished results). Each isomer was characterized by NMR analysis using the published data (23, 36). In this study, we synthesized *ras* gene fragments that contain either csTD or tsTD

Figure 2. (A) Generation of a synthetic *ras* gene with a thymine dimer at codon 61. DNA cassettes with a thymine dimer were inserted into pEB, which was digested with *EcoRV* and *BclI*. Positions of the thymine dimer and the restriction enzyme sites are indicated by asterisks and underlines, respectively. (B) Nucleotide sequences of the DNA template and the primer for *in vitro* DNA synthesis study. A DNA template corresponding to the *ras* fragment around codon 61 and the primer, U7 are shown. The positions of a thymine dimer and an *RsaI* sequence are indicated by asterisks and underlines, respectively.

by block coupling. The oligonucleotides were purified extensively by reverse phase HPLC and anion-exchange HPLC as described (18). The purified oligonucleotides were eluted as a sharp single peak by reverse phase HPLC and by anion exchange HPLC, and the retention times of the oligonucleotides were distinctively different from that of the oligonucleotide with a normal 5' TT 3' sequence instead of either csTD or tsTD (data not shown). The presence of csTD or tsTD in the isolated oligonucleotides was confirmed by HPLC analysis after UV-irradiation, as described previously (23), although other products were generated, which were probably due to the pyrimidine cluster in the oligonucleotides (data not shown).

DNA cassettes with either csTD or tsTD (Fig. 2A) were prepared and inserted into the vector, pEB (20), which contains the Rous sarcoma virus long terminal repeats as a promoter and a *ras* gene lacking the coding sequence between the *EcoRV* and *BclI* sites (see Fig. 2A). Control vectors with either a normal (Gln-61; codon 61 in the antisense strand is 3' GTT 5') or an activated (Lys-61; codon 61 in the antisense strand is 3' TTT 5') *ras* gene were constructed simultaneously. Ligation mixtures were used for transfection. The amount of covalently closed circular DNA was estimated to be about 10% of the ligation mixture when analyzed by agarose gel electrophoresis and the ligation efficiencies were nearly equal in each case (data not shown).

Focus-formation by *ras* genes with a thymine dimer

The vector constructs carrying a *ras* gene with either csTD or tsTD and the control vectors were transfected into NIH3T3 cells. Table 1 shows the number of foci induced by the various *ras* genes. Both modified *ras* genes induced focus-formation, indicating that the thymine dimers activated the gene by virtue of their mutations, although the transforming efficiencies of the csTD- and tsTD-*ras* genes were 4–10% and 11–43%, respectively, of those of the Lys-61 gene. It seems that the tsTD-*ras* gene induced more foci than the csTD-*ras* gene. It is not clear whether the introduced csTD and tsTD were repaired in the transfected NIH3T3 cells. The relatively low transforming efficiencies may correlate with the efficiency of repair. Another

Table 1. Number of foci induced by *ras* genes

<i>ras</i> gene	Experiment No.			
	1 ^{a)}	2 ^{b)}	3 ^{c)}	4 ^{d)}
Gln (normal)	0	5	0	4
csTD	2	49	6	15
tsTD	16	93	11	41
Lys (activated)	37	512	88	360

^a 30 ng of DNA was used.

^b 150 ng of DNA was used.

^c 20 ng of DNA was used.

^d 50 ng of DNA was used.

possible explanation is that the lesions may partially block replication.

Analysis of mutations induced by thymine dimers in NIH3T3 cells

The *ras* genes present in transformed cells were analyzed by the PCR-RE method (16). The method is very useful to detect point mutations at specified sites (16, 18–20, 37). The names and the recognition sequences of the enzymes used in this study, and the point mutations detectable by the enzymes are listed in Fig. 3. We preferentially used *TaqI* rather than *EagI*, because the primer for *EagI* often generated a byproduct that has a similar size to a cleaved product. *EagI* was used when a mutation at the third position was detected, because the recognition site of *TaqI* includes the third position of codon 61 (the A in the TCGA sequence). It was also used when the PCR product was digested with *TaqI*, to confirm that the first position of codon 61 was not mutated (the primer for *TaqI* covers the first position). Fig. 4A shows the results of the analysis of a clone obtained by transfection of the csTD-*ras* gene. Cleavage was observed when the PCR product was digested with *SnaI* recognizing T in the second position of codon 61 or *BglII* recognizing A in the third

Figure 3. Primers and restriction enzymes used for the PCR-RE method. The position of a thymine dimer is indicated by asterisks.

Figure 4. Sequence analysis of *ras* genes present in the transformed NIH3T3 cells (Ethidium bromide-stained 10% polyacrylamide gel). (A) Analysis of the clone obtained with *ras* containing csTD. (B) Analysis of the clone obtained with *ras* containing tsTD. Lanes 1–4, analysis of the second position of codon 61: *StuI* for T (lane 1), *ApaI* for C (lane 2), *MscI* for A (lane 3), and *TaqI* for G (lane 4). Uncleaved and cleaved products are 153 bp and 133 bp, respectively. Lanes 5–8, analysis of the third position of codon 61: *BamHI* for G (lane 5), *BclI* for T (lane 6), *BglII* for A (lane 7), and *PvuI* for C (lane 8). Uncleaved and cleaved products are 99 bp and 77 bp, respectively. M1, M2, and M3 correspond to *MspI* digested pBR322, a marker for the second position (153 bp and 133 bp), and a marker for the third position (99 bp and 77 bp), respectively.

position (lanes 1 and 7). This result indicates that dTMP and dAMP were incorporated into the sites opposite the 3'-T and the 5'-T, respectively, of the introduced csTD. The clone has a 3'-T → A mutation, therefore, has an activated *ras* gene encoding a Leu-61 mutant. Similarly, analysis of a clone obtained by transfection of the tsTD-*ras* gene revealed that dAMP and dTMP were incorporated into the sites opposite the 3'-T and the 5'-T, respectively, of the introduced tsTD (Fig. 4B). This clone has a 5'-T → A mutation and His-61 *ras* gene. Table 2 summarizes the results of the analysis. Fifteen clones derived from the transfection of the csTD-*ras* gene, and nineteen clones originating from the tsTD-*ras* gene transfection were found to have mutation(s) among sixteen and twenty analyzed clones, respectively. The point mutations detected most frequently were 3'-T → A transversions (10 clones/16 clones analyzed) in the cases of csTD. In the case of tsTD, 5'-T → A transversions (18

Table 2. Mutations detected in transformed cells

	sequences of the modified positions	codon 61
csTD	5' TA 3' 8/16	CTA(Leu)
	GT 2/16	CAC(His)
	AT 1/16	CAT(His)
	CA 1/16	CTG(Leu)
	TC 1/16	CGA(Arg)
	TA+TG 1/16	CTA+CCA(Leu+Pro)
	CT+GT+AT 1/16	CAG+CAC+CAT(Gln+His)
tsTD	5' AT 3' 9/20	CAT(His)
	AA 7/20	CTT(Leu)
	TC 1/20	CGA(Arg)
	AA+TA+GA 1/20	CTT+CTA+CTC(Leu)
	other* 1/20	

* Both sites were mixtures of T and A.

clones/20 clones analyzed) and 3'-T → A transversions (9 clones/20 clones analyzed) were major mutations that occurred. Various other mutations were also detected in the both cases (Table 2). It is likely that the first position of codon 61 (3'-flanking site of the dimer) was not mutated, because the PCR products were digested by *StuI*, *ApaI*, *MscI*, and *EagI* which require a normal sequence at this position (see Fig. 3).

Nucleotides incorporated opposite thymine dimers *in vitro*

The nucleotide sequences of the templates with csTD or tsTD and the primer, U7, are shown in Fig. 2B. The templates were prepared by enzymatic joining of three oligonucleotides. As shown in Fig. 5, the template-primer complexes were elongated either by recombinant rat DNA polymerase β or by mouse DNA polymerase α -primase complex. The elongated products were digested with *RsaI* to generate 5'-phosphorylated blunt ends (Fig. 5). After separation from the uncleaved DNA, they were treated with λ exonuclease, which preferentially recognizes a 5'-phosphorylated blunt end and hydrolyzes the strand possessing a 5'-phosphate. The single strands that did not contain the thymine dimer were analyzed by the PCR-RE method (Fig. 5).

It is likely that almost all of the primers annealed with the csTD-template were elongated to the full-length product by DNA polymerase β , because the product was digested by *RsaI*, which recognizes a site downstream from the dimer site (data not shown). On the other hand, PAGE analysis revealed two products from the DNA polymerase α reaction. It was likely that half of the template was elongated to the full-length and that the other half was blocked during replication. The fact that the putative blocked product was resistant to *RsaI* supports this idea. No band on PAGE corresponding to the full-length replicated product was observed when the primed tsTD-template was incubated with either DNA polymerase β or DNA polymerase α . The putatively blocked DNA was also resistant to *RsaI* digestion (data not shown).

The results of the analysis of nucleotide incorporation opposite csTD by rat DNA polymerase β and by mouse DNA polymerase α are shown in Fig. 6. In the case of DNA polymerase β , the PCR products were cleaved when incubated with *MscI*, *TaqI*, *ApaI*, *BglII*, or *PvuI*. It was determined that the nucleotides were incorporated in the order of dAMP > dGMP > dCMP into the site opposite the 3'-T, and dAMP was incorporated preferentially

Figure 5. Schemes for primer-extension on a DNA template with a thymine dimer, and analysis of the product. A DNA template was annealed with a primer (U7) and replicated by either DNA polymerase β or α . Replicated DNA was digested with *RsaI*. After purification by PAGE, it was treated with λ exonuclease and analyzed by the PCR-RE method. Open circles and closed circles indicate modified positions and the opposite positions, respectively. P indicates 5'-phosphate groups.

over dCMP opposite the 5'-T (Fig. 6A). In the case of DNA polymerase α , the PCR products were cut by *MscI*, *BglII*, and *BclI*. This indicates that dAMP was incorporated opposite both sites, and that dTMP was also inserted, to a lesser extent, into the site opposite the 5'-T (Fig. 6B). The same results were obtained when *EagI* was used instead of *TaqI* (data not shown). We used the PCR-RE method with *XbaI* and *Csp45I* to determine whether -1 and -2 deleted DNAs were produced during *in vitro* DNA synthesis. The former enzyme and the latter enzyme recognize and cut the -1 and the -2 deleted DNAs, respectively. The products by DNA polymerase β and DNA polymerase α were not digested by the enzymes, indicating that few, if any, deletion events occurred (data not shown).

DISCUSSION

UV light damages DNA, and produces various photoproducts. Among these, cyclobutane-type pyrimidine photodimers are the major type of DNA lesion. Two isomers of the dimer can be generated in flanking thymine residues in DNA: csTD and tsTD (Fig. 1). It has been demonstrated that tsTD is not produced in duplex DNA (38), but it is, however, generated when a single-stranded region of DNA is exposed to UV light (39). Single-stranded regions exist in DNA at the sites of replication and transcription. Both the tsTD and the csTD lesions may contribute to UV-induced mutagenesis because replicated or transcribed regions have unprotected, naked DNA, and because genes that are frequently transcribed have more chances to produce the tsTD lesion. We therefore investigated mutations induced by both csTD and tsTD.

We have reported that synthetic *ras* genes with a DNA lesion at either codon 12 or codon 61 induced transformation of NIH3T3

Figure 6. Sequence analysis of extended products of the primer on the csTD-template by rat DNA polymerase β (A) and by mouse DNA polymerase α (B). Ethidium bromide-stained 20% polyacrylamide gel. Incorporation of dTMP, dCMP, dAMP, or dGMP opposite 3'-T was indicated by cleavage of the PCR products by *StuI*, *ApaI*, *MscI* and *TaqI*, respectively. Uncleaved and cleaved products are 75 bp and 55 bp, respectively. Incorporation of dGMP, dTMP, dAMP, or dCMP opposite 5'-T was indicated by cleavage of the PCR products by *BamHI*, *BclI*, *BglII*, and *PvuI*, respectively. Uncleaved and cleaved products are 63 bp and 41 bp, respectively. To confirm the cleavage, the non-digested controls are also shown (samples with restriction enzyme treatment are indicated as *StuI* +, and those without treatment are shown as *StuI* -, for example). Lane 1, *StuI* +; lane 2, *StuI* -; lane 3, *ApaI* +; lane 4, *ApaI* -; lane 5, *MscI* +; lane 6, *MscI* -; lane 7, *TaqI* +; lane 8, *TaqI* -; lane 9, *BamHI* +; lane 10, *BamHI* -; lane 11, *BclI* +; lane 12, *BclI* -; lane 13, *BglII* +; lane 14, *BglII* -; lane 15, *PvuI* +; lane 16, *PvuI* -; M, *MspI* digested pBR322.

cells when transfected into the cells, and that the genes in cells from the foci had a point mutation (16-20). In the present study, we introduced csTD and tsTD into the second and the third position of codon 61 of the *ras* gene in the antisense strand.

In this study we showed that both types of thymine dimers induced foci when transfected, indicating that the dimer can activate the *ras* gene by point mutation(s). Sequence analysis of the *ras* genes present in transformed cells confirmed that they had a point mutation. The csTD mainly induced a 3'-T $\rightarrow$ A transversion, while the tsTD mainly induced a 5'-T $\rightarrow$ A transversion (Table 2). Other types of mutations (5'-T $\rightarrow$ G, A, and C and 3'-T $\rightarrow$ C and G for csTD; 5'-T $\rightarrow$ G and 5'-T $\rightarrow$ A and C for tsTD) and targeted double mutations (TT $\rightarrow$ CA for csTD and TT $\rightarrow$ AA and TT $\rightarrow$ GA for tsTD) were also found. In the system used in this study, all of the point mutations cannot be detected. We utilized focus-formation by the activated *ras* gene as a selection marker. Therefore, we could not detect the mutations that do not change the 61st amino acid or that alter the amino acid to that of the transforming activity-deficient *ras* gene (40). Considering all the combinations of the second and the third bases, the possible 61st amino acids are Gln (normal, CAR), Arg (CGN), Leu (CTN), Pro (CCN), and His (CAY), where R, Y, and N denote purine, pyrimidine, and any base, respectively. Arg-61 and Leu-61 *ras* genes have high transforming activities and a His-61 mutant has a lower activity

(40). A Pro-61 *ras* gene has as a low activity as a normal gene (40). A·T → C·G mutations at the second position and A·T → G·C mutations at the third position, hence, cannot be detected unless other mutations in another, and/or in the same gene, generate an activated *ras* gene (see Table 2). In addition, the His-61 mutant has a lower transforming activity as compared to the Leu- and Arg-61 mutants (40). More A·T → C·G mutations at the second position and more A·T → G·C, T·A, and C·G mutations at the third position must occur.

In *Escherichia coli*, the mutations induced by csTD were 3'-T → A transversions (78.8% of the total mutations) and 3'-T → C (17.0%) and 5'-T → C (3.6%) transitions (14, 39). On the other hand, a 5'-T → A transversion (23.5%), 5'-T → C (5.9%) and 3'-T → C (1.5%) transitions, and a -1 deletion (48.5%) were the mutations induced by tsTD in bacteria (15). The spectra of mutations that we found were similar to those observed in *E. coli* (3'-T → A by csTD and 5'-T → A by tsTD), considering that the 5'-T → C transition and a -1 deletion do not activate the *ras* gene. Different trends, however, were found. In mammalian cells, various kinds of point mutations were induced by both csTD and tsTD. The 3'-T of tsTD was also a hot spot for point mutations in NIH3T3 cells. Targetted double substitutions were found in NIH3T3 cells, while none were detected in bacteria. These differences may be due to the DNA polymerases involved in replication, and/or the proteins involved in replication, proof-reading, and repair.

In vitro DNA synthesis of primed-templates with csTD or tsTD shows different mutation-spectra than those found in NIH3T3 cells. Various nucleotides were inserted, in the order of dAMP > dGMP > dCMP, into the site opposite the 3'-T, and dAMP was inserted in preference to dCMP opposite the 5'-T by DNA polymerase β (Fig. 6A). dAMP was inserted opposite both the thymines and some dTMP was incorporated into the site opposite the 5'-T by DNA polymerase α (Fig. 6B). Taylor and O'Day reported that the large fragment of *E. coli* DNA polymerase I (Klenow fragment) inserted various nucleotides, in the order of dAMP > dTMP ~ dGMP > dCMP, at the site opposite the 3'-T of csTD in the terminated products, and that bypass occurred when dAMP was incorporated (41). Our results using DNA polymerase β are similar to those of Taylor and O'Day, except for dTMP, although we analyzed bypassed products, whereas they studied terminated products. Our findings are also similar to those of Rabkin *et al.* (42) although they used UV-irradiated, partially-characterized DNA. DNA polymerase β may be able to bypass the products in which dGMP and dCMP were incorporated at the 3'-T site, although Klenow fragment cannot elongate such products. The elongated product of DNA polymerase α contained only a dAMP residue at the 3'-T site. As mentioned above, it was likely that half of the primed template was blocked and the other half was bypassed by DNA polymerase α . At present, the termination position (prior to and/or opposite the dimer site) and the kind(s) of inserted nucleotide are unclear. DNA polymerase α , like Klenow fragment, may bypass a product when dAMP is inserted at the 3'-T site. On the other hand, neither DNA polymerase β nor α bypasses the tsTD-template (data not shown). Taylor and O'Day reported similar results (41). Further study is necessary to clarify the terminated positions and the nucleotides inserted in the terminated products.

The results using DNA with the same nucleotide sequence in mammalian and *in vitro* systems are very different. In the case of csTD, a preferential 3'-T → A transversion, that is, an incorporation of dTMP, was observed in NIH3T3 cells, while

only dAMP was incorporated by mouse DNA polymerase α *in vitro*. DNA polymerase α did not bypass a DNA template with tsTD *in vitro*, although focus-formation of NIH3T3 cells was observed when a *ras* gene with tsTD was transfected. Three explanations are possible for these discrepancies. First, DNA polymerase(s) other than DNA polymerase α , which is actually involved in replication, may preferentially insert dTMP at the 3'-T of csTD, and may be able to bypass the tsTD. Second, other proteins involved in replication may alter the selectivity of DNA polymerase(s) and may help the polymerase(s) bypass the tsTD. Indeed, it was reported that proliferating cell nuclear antigen promoted DNA synthesis by calf thymus DNA polymerase δ past csTD and tsTD (43). Lastly, the proof-reading mechanism may participate in the reaction.

In this study, it was demonstrated that the *ras* gene with the tsTD induced more foci than the csTD-*ras* gene. Four explanations are possible for this observation. First, the csTD lesion may have been repaired more frequently than the tsTD lesion. Secondly, the csTD lesion may have blocked DNA replication more than the tsTD lesion. Thirdly, the tsTD lesion could have been misread more frequently than the csTD lesion. Lastly, mutations induced by tsTD could have altered the 61st amino acid, which has more focus-forming activity. This last explanation is not likely because the amino acid that was detected most frequently in the cells transformed by csTD-*ras* was Leu, while His was observed most frequently in the case of tsTD (Table 2). The second possibility appears unlikely because neither DNA polymerase β nor α could bypass the tsTD. The degree of inhibition of replication, however, was higher with csTD than with tsTD in SOS-induced *E. coli* (35). The higher transforming activity of the tsTD-*ras* gene may indicate the higher replication efficiency of tsTD (that is, a more serious replication block by csTD in mammalian cells). Therefore, the first, the second, and/or the third explanation(s) may be relevant to the more focus-formation of the tsTD. It is not clear whether the introduced csTD and tsTD lesions were repaired in the transfected NIH3T3 cells. Although repair enzymes for csTD have been reported to exist in various organisms (44, 45), it is unknown whether the repair enzymes for csTD also function to repair tsTD. *E. coli* and yeast photolyases do not act on tsTD (39, 46). T4 endonuclease V neither binds nor cleaves tsTD (Murata *et al.*, unpublished result). Repair enzymes that recognize distortions of duplex DNA may remove the tsTD as well as the csTD lesions, and this type of enzyme(s) may contribute to the repair reaction(s) of tsTD, if repair does occur.

NMR studies of duplex oligonucleotides containing either csTD or tsTD revealed that the 3'-T of both dimers, and the 5'-T of csTD can form hydrogen bonds with A, and that the 5'-T of tsTD is not involved in hydrogen bonding (47). The various point mutations described in this report should reflect the misincorporation opposite csTD and tsTD. Thymine residues, except for the 5'-T of tsTD, can form hydrogen bond(s). The misincorporation of deoxynucleotides opposite csTD or tsTD may be caused by the formation of hydrogen bond(s) and the distortion of the backbone structure of the dimer-containing DNA. On the other hand, it is thought that the 5'-T of tsTD can hardly participate in hydrogen bonding because of its steric bulk. The molecular mechanism(s) of the preferential incorporation of dTMP opposite the 5'-T of tsTD, which was observed in our present study and was reported to occur in prokaryotic systems (15), is unclear. Studies on the structures of DNA duplexes with csTD or tsTD pairing with various bases would provide more insight.

In this report, we demonstrated that both the csTD and the tsTD lesions activated a *ras* gene, and that activation was due to a point mutation at the dimer site. We also showed that misincorporation of nucleotides occurs opposite the csTD *in vitro*, which is very different from the results in NIH3T3 cells. Further study will increase our understanding of the mutagenic character of cyclobutane-type thymine dimers.

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