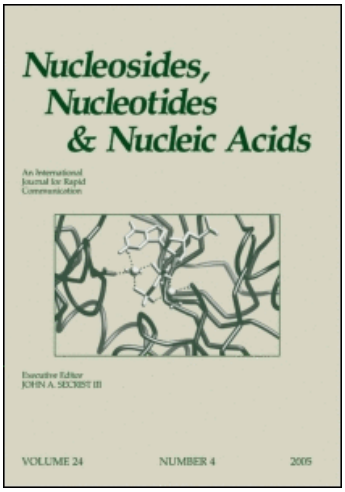

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Mutation Induced by Deoxyxanthosine in Codon 12 of A Synthetic c-Ha-ras Gene

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MUTATION INDUCED BY DEOXYXANTHOSINE IN CODON 12 OF A SYNTHETIC c-Ha-*ras* GENE§

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ABSTRACT : To evaluate the base-pairing properties and mutagenicity of deoxyxanthosine in DNA, the modified base was incorporated into a synthetic c-Ha-*ras* gene and a DNA transfection experiment was done. The *ras* gene containing deoxyxanthosine showed very high focus-forming activity. Analysis of the genes from transformants showed almost exclusively a transition of G to A. These results indicate that dTMP was preferentially incorporated at the site opposite to deoxyxanthosine, and deoxyxanthosine can induce G to A transitions in mammalian cells.

INTRODUCTION

DNA is a macromolecule which contains genetic information. The genetic information is the nucleotide sequence itself, and conservation (replication) and expression (transcription) are mediated by interaction between bases, components of nucleotides. Hydrogen bonds and base-stacking are important in interaction between bases. Thermal stability experiments and structural studies by NMR or X-ray crystallography are potent tools in analyzing these interactions. Nucleotide incorporation studies with DNA or RNA polymerases of nucleotide analogues, which include unnatural nucleotides and DNA lesions derived from modification of natural nucleotides by chemical compounds and irradiation, are also important to study the base interactions and provide various informations on properties of bases. Site-directed

§ This paper is dedicated to the memory of Professor Tohru Ueda.

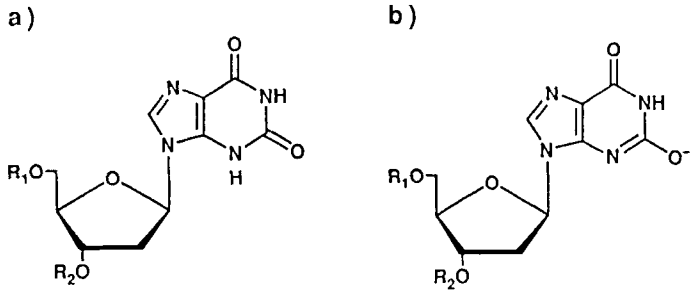

FIG. 1. Structure of deoxyxanthosine. a) Deoxyxanthosine with 2,6-diketo form (neutral form) of a xanthine base. b) Deoxyxanthosine with 6-keto-2-enolate form (ionized form) of a xanthine base. Deoxyxanthosine in DNA is believed to exist in an ionized form shown in b) under physiological conditions (see DISCUSSION).

mutagenesis experiments *in vitro* and *in vivo* have been done to evaluate base-pairing properties and the mutation-inducibility of a variety of DNA lesions. For example, O^6-methylguanine (O^6-MeG), which is derived from selective *O*-methylation of guanine by methylating agents such as *N*-nitroso-*N*-methylurea (NMU)[1], have been studied in many *in vitro*[2,3] and *in vivo*[4-7] experiments. Combined with the results obtained in the thermal stability experiments[8] and structural studies[9,10] of oligonucleotides containing O^6-MeG, it is suggested that incorporation of nucleotides by DNA polymerases is related to the pattern of hydrogen bonds between bases.

Xanthine (X) is a base analogue the structure of which is shown in Figure 1. Eritja *et al.* reported on the synthesis and properties of oligonucleotides containing 2'-deoxyxanthosine (dX)[11]. They measured the melting temperature of duplex oligonucleotides containing X:A, X:G, X:C, and X:T pairs at neutral pH and reported that the thermodynamic stabilities of the mispairs were in the order of T > G > A and C. They also reported that deoxynucleotides were incorporated at the site opposite to X in the order of T > C >> A and G by *Drosophila* DNA polymerase α *in vitro*. They were interesting results because dCMP was incorporated in spite of the very low stability of the X:C pair. Therefore, it is of great interest to know whether a deoxynucleotide is incorporated in mammalian cells. Furthermore, it is quite attractive to investigate mutation-inducibility of dX because dX may be present in DNA as a result of spontaneous deamination of dG residues and modification by nitrous acid[12], which may be present in the human stomach, and may be concerned with carcinogenesis.

We have previously reported on site-directed incorporation of O^6-MeG into codon 12 of a synthetic c-Ha-*ras* gene and transfection of the gene into NIH3T3 cells, and showed that the DNA lesion can activate the *ras* gene by mutating at the modified position[4]. Because any point mutations at the second position of codon 12 of *ras* gene results in activation of the gene, it is a very useful system to detect a point mutation induced by a modified base[4,13].

In this paper, we describe the introduction of dX into the second position of codon 12 of the synthetic c-Ha-*ras* gene and a DNA transfection experiment of the gene. The gene containing dX showed high transforming activity and almost exclusive mutation to A by the base analogue. These results clearly indicate that dTMP was incorporated at the site opposite a dX residue in NIH3T3 cells.

MATERIALS AND METHODS

Enzymes

*Bss*HII and *Aat*II were purchased from Toyobo Co. Snake venom phosphodiesterase was obtained from Boeringer Mannheim. *Taq* DNA polymerase was purchased from Cetus Co. Other enzymes were from Takara Shuzo Co.

Synthesis of oligonucleotide containing dX

A deoxyxanthosine phosphoramidite derivative was prepared by the method described by Eritja *et al.*[11] with modifications and details will be reported elsewhere. Briefly, 6-*O*-[2-(4-nitrophenyl)ethyl]-3',5'-di-*O*-acetyl-2'-deoxyguanosine was synthesized from dG without protection of the amino group[14] and converted to 5'-*O*-(4,4'-dimethoxytrityl)-6-*O*-[2-(4-nitrophenyl)ethyl]-2'-deoxyxanthosine as described[11]. The nucleoside was phosphitylated using 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (Sigma Chemical Co.).

HRU2 35X, which contains dX at the site corresponding to the second position of codon 12 of a synthetic c-Ha-*ras* gene (5' dGCCGXCGGTGTG 3', Figure 2) was synthesized by the phosphoramidite method[15] using an Applied Biosystems model 380A DNA synthesizer. A dX-containing oligonucleotide was purified by the method described below.

Purification of oligonucleotides

The oligonucleotides for construction of a vector for transfection other than HRU2 35X were previously prepared[4]. Oligonucleotides for pCB construction were synthesized and purified as described[16,17]. Namely, purification by reverse phase high-performance liquid chromatography (HPLC) was done before and after detritylation with 80% acetic acid using a YMC AM-324 column (10 mm X 300 mm). The products were purified further by aion exchange HPLC using a TSK gel DEAE-2SW column (4.6 mm X 250 mm, TOSOH) when necessary.

HRU2 35X was purified by the same procedure although it was treated with 0.5 M 1, 8-diazabicyclo[5. 4. 0]undec-7-ene (DBU) in pyridine (r.t., 75 min.) just before the first reverse phase column. The purity and base composition of the oligonucleotide containing dX were confirmed by reverse phase HPLC after complete digestion with snake venom phosphodiesterase and *E. coli* alkaline phosphatase.

Construction of pCB

A new plasmid, pCB, was constructed by exchanging a DNA fragment of pRSV-rg12[18]. Oligonucleotides (5' dCGATATGGTGATGACCTAATAG 3' and 5' dCGCGCTATTAGGTCATCACCATAT 3') were annealed and joined by T4 DNA ligase with the larger fragment of pRSV-rg12/*Cla*I-*Bss*HII, which was purified by agarose gel electrophoresis. The ligated DNA was transduced into *E. coli* HB101 and the plasmid was isolated and purified.

Construction of vectors for DNA transfection

Phosphorylation and ligation for DNA cassette construction were done by methods described previously[4,16,17]. The DNA cassettes (Figure 2) thus obtained were phosphorylated, and joined with a plasmid pCB which was previously digested with *Cla*I and *Bss*HII. The DNAs were then treated with *Bss*HII and ligated to obtain the vectors for transfection (Figure 3). Aqueous solutions of the vectors were treated with phenol and chloroform. The vectors were then precipitated with ethanol, and measured with a DNA DipStick (InVitrogen).

DNA transfection

The vectors were transfected into NIH3T3 cells by the calcium phosphate procedure as described previously[18,19] : 50 or 150 ng of the vector DNA and 30 μg of genomic DNA isolated from NIH3T3 cells were used for each transfection assay.

Analysis of the mutation found in the synthetic c-Ha-*ras*

The sequence around codon 12 of the synthetic human c-Ha-*ras* present in the transformed NIH3T3 cells was amplified by the polymerase chain reaction (PCR)[20] in a DNA Thermal Cycler (Perkin Elmer Cetus). The primers used for the PCR were Seq 1 (5' dTTTTTTATCGATATGACGGAATATAAG 3'), which corresponds to *ras* sequence from a *Cla*I site to codon 5 except the first 6 T, and L10[17] (5' dAAAAGATTTGGTGTTGTTGATAGCGAAAACGCACAG 3') (Figure 4). The following reaction conditions were used for the PCR : 94°C 0.5 min, 55°C 1 min and 72°C 2 min; 45 cycles. The amplified PCR products were purified by agarose gel electrophoresis and their nucleotides were sequenced. A mutagenic primer, 5' dAAGCTGGTGGTGGTGGGCG*N*CG 3' [4], which corresponds to the human c-Ha-*ras* sequence from codon 5 to codon 11 except that the second position of codon 11 (N) was replaced by T, A, or G was used in the second PCR together with L12[17] (5' dCAGAGTATTCTTCTTGGCCTG 3') (Figure 4). The products were then incubated with *Sal*I (for detection of a mutation to A), *Aat*II (for a mutation to T) or *Bbe*I (for a mutation to C), and analyzed by 8% polyacrylamide gel electrophoresis (PAGE) [4].

RESULTS

Synthesis and purification of an oligonucleotide containing dX for cassette mutagenesis

The oligonucleotide containing a dX residue was synthesized by the phosphoramidite method[15]. The oligonucleotide was purified extensively as described in MATERIALS AND METHODS because oligonucleotides of interest should be as pure as possible for use in mutational analysis. When analyzed by reverse phase HPLC after detritylation, two peaks were observed (data not shown). Complete digestion of the isolated oligonucleotides and subsequent HPLC analysis showed that the faster oligonucleotide contained dX while another included not dX but an unknown component. Retention time of the oligonucleotide containing dX was later than a control oligonucleotide containing dG instead of dX due to ionization by loss of proton at N-3 of X[21] in anion exchange HPLC. Purity and base composition of the desired product were analyzed by complete digestion as mentioned above. It was confirmed that the oligonucleotide did not contain any components other than dG, dC, dT, and dX (data not shown). Furthermore, when the oligonucleotide was checked by reverse

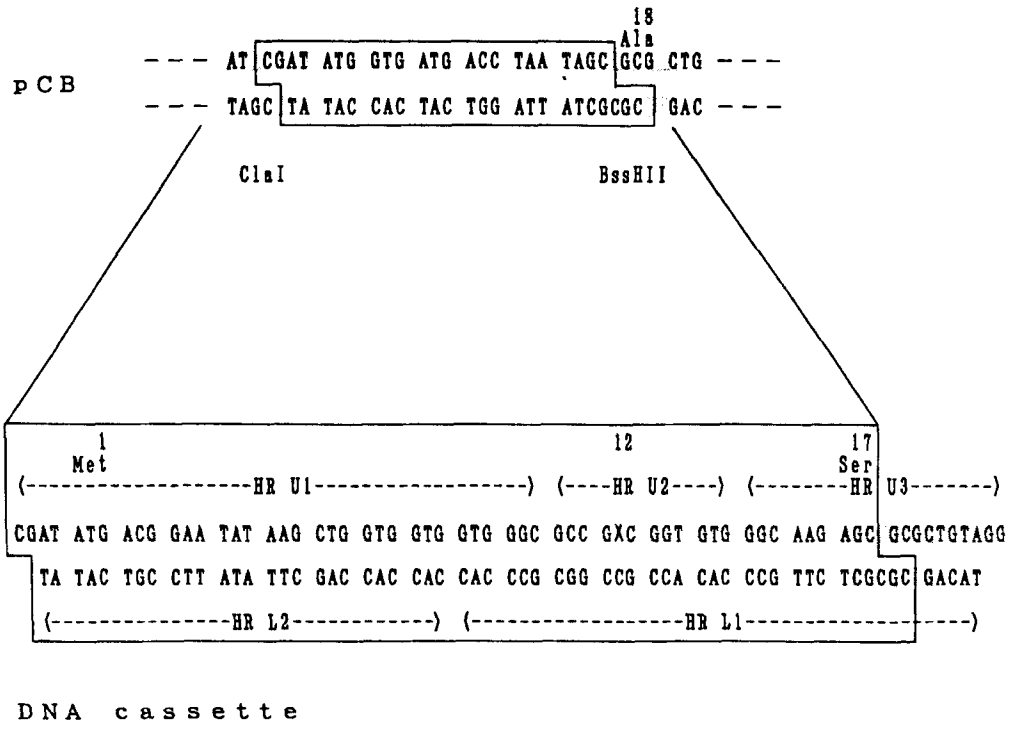

FIG. 2. Nucleotide sequences of a DNA cassette for construction of a c-Ha-*ras* gene containing deoxyxanthine in the second position of codon 12 and a corresponding part of the plasmid pCB. The nucleotide sequence of the cassette is that of a human c-Ha-*ras*-1 gene. *Cla*I and *Bss*HII sites are indicated with shading. X means deoxyxanthosine.

phase HPLC after incubation in buffers used in DNA cassette construction, no changes were observed, indicating that the modifdied base was stable under the conditions in which the DNA cassette was constructed.

Construction of vector containing dX

The DNA cassette (Figure 2) and the vector containing dX for DNA transfection assay was constructed by the procedure outlined in Figure 3 to avoid inserting the DNA cassette as a trimer. A similar strategy was used when O^6-MeG was incorporated into a synthetic c-Ha-*ras* gene[4]. Vectors with the sequence GGC in codon 12 (normal, Gly) or GAC (activated, Asp) were also constructed by the same procedure.

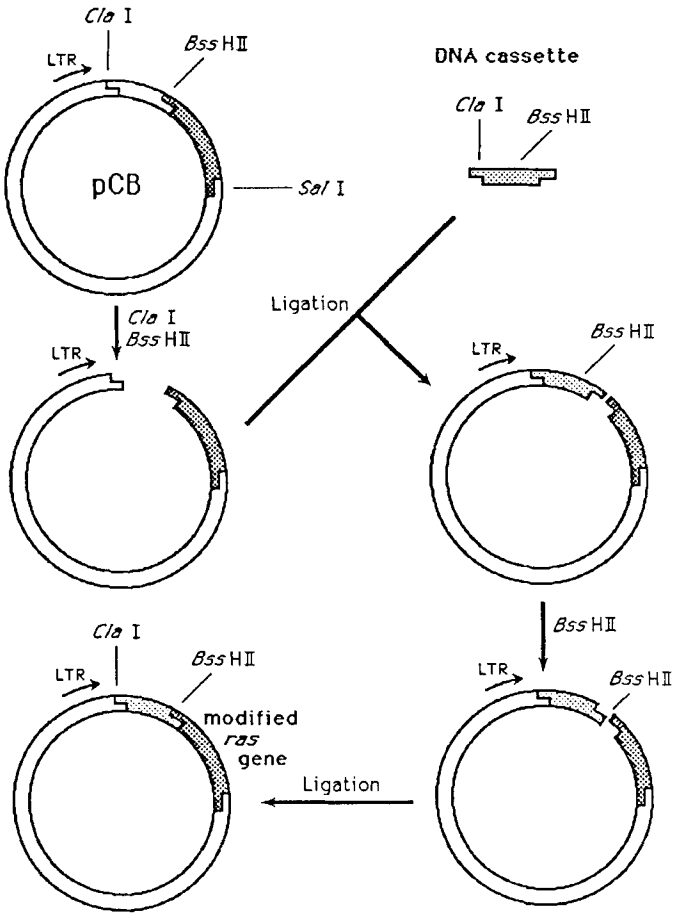

FIG. 3. Construction of a vector for transfection. pCB was digested with *Cla*I and *Bss*HII and joined with a phosphorylated DNA cassette. The DNA thus obtained was then treated with *Bss*HII and joined with T4 DNA ligase.

Transformation of NIH3T3 cells by dX-containing c-Ha-*ras* genes

DNA transfection was done by the calcium phosphate procedure.[18,19] As shown in Table 1, the synthetic human c-Ha-*ras* gene with dX at the second position of codon 12 showed very high transforming activity. The efficiency was 30 - 50 percent of that of an activated (Asp) c-Ha-*ras* gene. It may suggest that DNA repair mechanism in NIH3T3 cells did not work well against dX.

TABLE 1. Number of foci induced by c-Ha-*ras* genes.

	Experiment 1[a)]	Experiment 2[b)]
Gly-12 (normal)	0	0
X	12	27
Asp-12 (activated)	27	97

a) 50 ng of DNA was used

b) 150 ng of DNA was used

Analysis of mutations in codon 12 of the c-Ha-*ras* present in transformed cells

The sequence in the region of codon 12 of the c-Ha-*ras* present in the transformed cells was analyzed as previously described[4]. A mutagenic primer dAAGCTGGTGGTGGTGGGCG*T*CG (italicized T was not complementary to the human c-Ha-*ras* sequence) was used as a primer in the second PCR reaction (Figure 4). When the second position of codon 12 is replaced by A, the PCR product should contain thesequence of ---G*T*CG*A*C--- (the italicized A corresponds to the second position of codon 12) and, therefore, should be susceptible to cleavage by *Sal*I. To detect other types of mutations in the second position of codon 12, we used other combinations of mutagenic primers, containing an appropriate base instead of an italicized T, and restriction enzymes, *Aat*II (for a mutation to T) and *Bbe*I (for a mutation to C) as described in MATERIALS AND METHODS. *Hap*II digestion of a PCR product, which was amplified by non-mutagenic primers, was done to detect a normal c-Ha-*ras* gene (*Hap*II recognizes a sequence of ---CCGG---, corresponding to codon 11 and 12).

PCR products were made from the transformed cells derived from 17 clones which were isolated after transfection with c-Ha-*ras* containing dX. Figure 4 shows the results when 3 clones were analyzed. Cleavage was observed only when they were

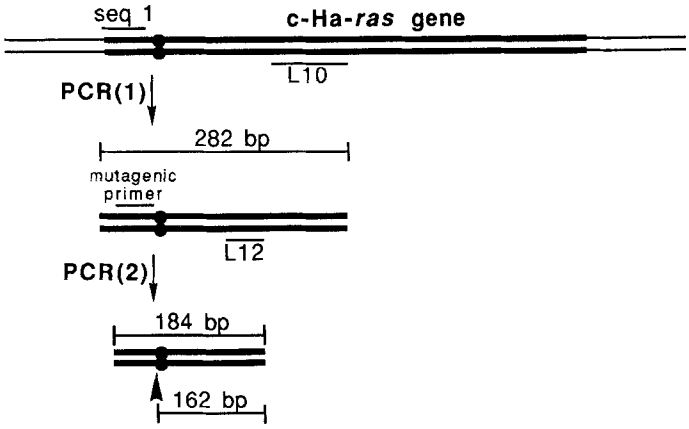

FIG. 4. Primers used in the PCR reactions. Nucleotide sequences of the primers were described in MATERIALS AND METHODS. Closed cicles and an arrow head indicate the position of codon 12 and the possible cutting position by a restriction enzyme, respectively.

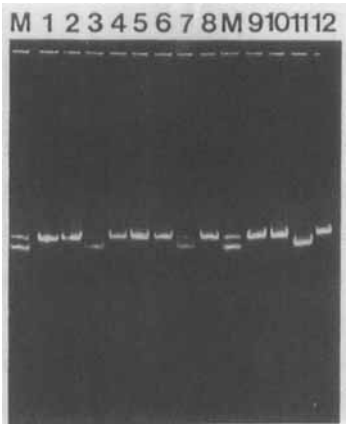

FIG. 5. PCR-restriction enzyme digestion analysis of the PCR products. *Hap*II digestion, lanes 1, 5 and 9; *Aat*II digestion, lanes 2, 6 and 10; *Sal*I digestion, lanes 3, 7 and 11; *Bbe*I digestion, lanes 4, 8 and 12. Lanes 1 - 4, clone 1; lanes 2 - 8, clone 2; Lanes 9 - 12, clone 3. M : marker DNA corresponding to uncleaved (184 bp) and cleaved (*Hap*II product, 162 bp) PCR products.

digested with *Sal*I (lane 3, 7, and 11), and not other enzymes (*Hap*II, *Aat*II, or *Bbe*I), indicating a mutation from G to A. Sixteen of the analyzed 17 clones had a G to A mutation in the second position of codon 12 in the c-Ha-*ras* gene. DNA from one clone was digested by *Aat*II, indicating that a G to T transversion was generated (data not shown). Four clones contained a normal c-Ha-*ras* gene in addition to a mutant gene with A.

DISCUSSION

Xanthine (X) is a purine base with 2,6 - diketo groups (Figure 1a). It was demonstrated that loss of a proton at N-3 gives a 6-keto-2-enolate anion (Figure 1b)[21]. Because the pK of dX is about 5.7, by analogy with the riboside, it should exist in the 6-keto-2-enolate form under physiological conditions. It may have an important effect on the base pairing properties of dX. Eritja *et al.* reported on the thermodynamic stability of DNA duplexes containing dX and on nucleotide incorporation against a X residue by *Drosophila* DNA polymerase α *in vitro*[11]. They showed that the order of thermostability (X:T > X:G > X:A and X:C) and that of incorporation by *Drosophila* DNA polymerase α (T > C >> A and G) did not correlate. These results indicated that dCTP is a good substrate for DNA polymerases against X albeit the X:C pair is less stable. Therefore, it is interesting to know how DNA polymerase reads an X residue in DNA in mammalian cells.

It is of added interest to investigate the mutagenecity of dX concerned with mutagenesis and carcinogenesis because deamination of G generates X. Although deamination of G can occur spontaneously, it has been known that nitrous acid generates dX when DNA is treated with it[12]. Hirose *et al.* treated DNA with sodium nitrite at pH 4.3 and transfected the DNA into *E. coli*, and observed a G to A transition although the frequency was low[22]. It was suggested that formation of X derived from deamination of G causes such a mutation. Nitrous acid exists only at low pH and the condition can be present in the highly acidic human stomach. Therefore, it is possible that dX in DNA is involved in mutagenesis and carcinogenesis.

This study showed that a c-Ha-*ras* gene with dX in the second position of codon 12 induced focus formation (Table 1). Transforming efficiency of a dX-containing *ras* gene was 30 - 50 percent of that of an activated (Asp-12) *ras* gene. This indicated that dX is a highly mutagenic lesion. Furthermore, sequence analysis of the c-Ha-*ras* present in the transformants showed that almost all of the mutations found

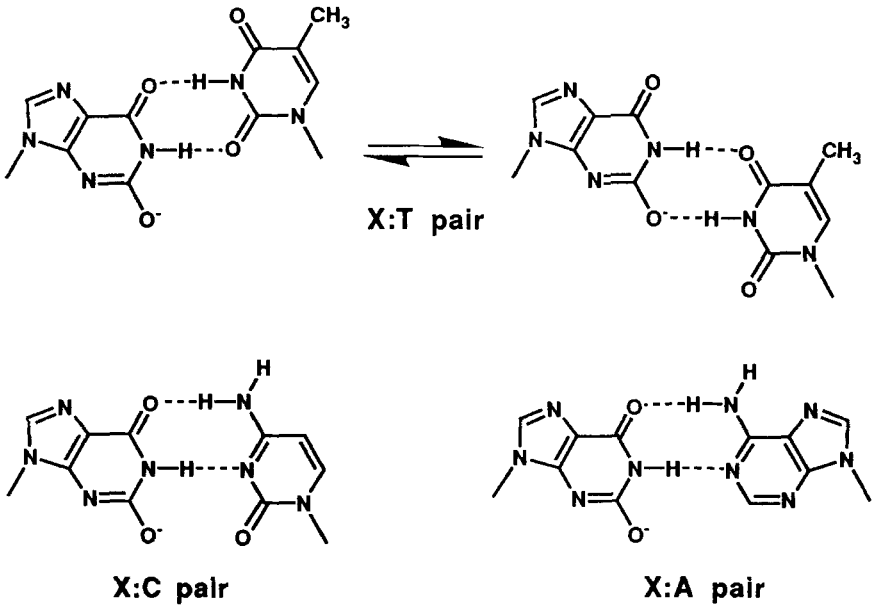

FIG. 6. Postulated base pairs containing xanthine (X:T pair, X:C pair and X:A pair).

were transitions from G to A in the position of dX. It is likely that the DNA repair mechanism in NIH3T3 cells did not work well against dX. Because repair enzymes for dX are still unknown, they may not be present in cells. If it is true, the transforming efficiency may correlate with ratio of dTMP/dCMP which were incorporated by DNA polymerase in NIH3T3 cells. Although the ratio of incorporated dCMP is unclear because repair activity for dX is unknown, it was evident that dTTP was a good substrate at sites opposite dX.

Many *in vitro* and *in vivo* replication experiments using DNA with a nucleotide analogue have been reported. In these studies, combined with the results on thermal stability of oligonucleotides containing the analogue, the hydrogen bond pattern has an important effect on the incorporation of deoxinucleotide at sites opposite the analogue. In the case of O^6-MeG, the O^6-MeG:C pair is much more stable than the O^6-MeG:T pair[8]. In spite of that, it was demonstrated that DNA polymerases inserted dTMP with preference for dCMP into sites opposite O^6-MeG *in vitro*[2,3]. The results of experiments using mammalian cells[4-6] or *Eschericia coli*[7] showed dTMP incorporation. It was reported that the O^6-MeG:C pair is a wobble base pair consisting

of two hydrogen bonds[9] and the O^6-MeG:T pair is a Watson-Crick base pair[10]. It is explained that a wobble base pair or a non-Watson-Crick pair, which distorts the DNA duplex, is not suitable for polymerization. Previous *in vitro* study by Eritja *et al.* indicated that dTTP was a good substrate and dCTP was also a relatively good substrate opposite dX in spite of the lower stability of the X:C pair[11]. The frequency of inserted dCMP in NIH3T3 cells was not clear in this study because incorporation of dCMP did not lead to activation of a c-Ha-*ras* gene and because it is unknown whether repair of dX occurred in the cells. It is postulated that the X:C pair is a Watson-Crick pair and the X:T pair is a wobble pair (Figure 6). Albeit dCTP is thought to be a good substrate because the X:C pair is a Watson-Crick pair, dTMP was also incorporated in NIH3T3 cells, which was indicated in this study, and by DNA polymerase in a previous *in vitro* study[11]. It may be due to destabilization of the X:C pair originated from the unsolvated negative charge of dX, which will be shielded in the X:T pair[11] and/or from repulsion of the ionized oxygen of dX and the negatively polarized carbonyl oxygen of dC.

One clone contained a mutated gene with a G to T transversion. This result may indicate that dAMP, in addition to dTMP, was also incorporated opposite X in NIH3T3 cells. Eritja *et al.* also reported dAMP incorporation into the site opposite to X[11]. A can form a base pair with X in a similar way to X:C pair although distance between C1'-C1' is longer than a Watson-Crick pair (Figure 6). DNA polymerase may recognize the distance of the base pair and incoporate dAMP in low frequency.

Four clones contained both of a normal c-Ha-*ras* and a mutated c-Ha-*ras* gene. As several copies of the c-Ha-*ras* genes are known to be inserted into the cells on transfection, mutational activation of one of the c-Ha-*ras* genes inserted into the cells was probably sufficient for transformation of these clones.

This study clearly indicated that dTMP was incorporated at the site opposite dX in the second position of codon 12 of the c-Ha-*ras* gene. Therefore, a mutation with a G to A transition can occur when dX is generated by deamination of dG, and the mutation may be involved in activation of a *ras* gene.

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