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Mutation-spectrum of a true abasic site in codon 12 of a c-Ha-*ras* gene in mammalian cells

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An abasic site is postulated to be a premutagenic lesion We previously reported that a c-Ha-*ras* gene with an abasic site analogue at either the first or the second position of codon 12 induced the formation of foci by point-mutational activation of the gene [Kamiya et al. (1992) Nucleic Acids Res. 20, 4409–4415]. In this study we constructed c-Ha-*ras* genes with a true abasic site in codon 12. The *ras* genes were found to be activated in NIH3T3 cells by a mutation to A at the modified and flanking positions, and the flanking mutations were detected more frequently.

True abasic site; Abasic site analogue; c-Ha-*ras* gene; Focus-formation; Point mutation

1. INTRODUCTION

An abasic (apurinic/apyrimidinic) site is a DNA lesion that is generated by hydrolysis of *N*-glycosyl bonds. The hydrolytic events can occur spontaneously, or by chemical modification of bases [1]. Enzymatic cleavage of the *N*-glycosyl bond of a nucleotide with a modified base also produces the lesion as an intermediate during DNA repair in which a DNA glycosylase is involved [2]. The abasic site is postulated to be a premutagenic lesion because misincorporation of deoxynucleotides opposite an abasic site or its analogue in vitro and in bacteria has been reported [3–9]. Using a shuttle vector, it was also shown that misinsertion occurs opposite an abasic site in COS cells [10,11].

We reported the transforming activity of c-Ha-*ras* genes with a chemically stable analogue of an abasic site at either the first or the second position of codon 12 (the 34th and the 35th positions, respectively) and point mutations induced by the lesion [12]. In the study, we demonstrated that c-Ha-*ras* genes with an abasic site analogue in codon 12 induced foci when transfected into NIH3T3 cells and that the analogue caused various point mutations, at both the modified and adjacent positions.

In order to investigate the mutation-spectrum of an abasic site in a mammalian system, it is better to use a true abasic site (D). In this study we introduced D into the same positions as studied before with the abasic site analogue. We report here that the *ras* genes with D in either the 34th or the 35th position (34D-*ras* and 35D-*ras*, respectively) induced focus-formation and that point mutations in transformed cells were detected more frequently in adjacent positions than in the modified positions.

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Abbreviations D, true abasic site, HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction, RE, restriction enzyme.

2. MATERIALS AND METHODS

2.1. *Synthesis and purification of oligonucleotides with an abasic site*

1-*O*-[(*tert*-Butyl)dimethylsilyl]-3-*O*-[(2-cyanoethoxy)(*N*,*N*''-diisopropylamino)phosphino] - 2-deoxy - 5 - *O* - (4,4'-dimethoxytriphenylmethyl)-α-D-ribofuranose was synthesized according to the method described by Groebke and Leumann [13]. Oligonucleotides with an abasic site (HRU2' 34D, 5'dGCCDGCGGTGTGGGCAAGAG3' and HRU2' 35D, 5'dGCCGDCGGTGTGGGCAAGAG3') were synthesized by the phosphoramidite method [14] with an Applied Biosystems model 394 DNA/RNA synthesizer using the trityl-on state. Amino-protecting groups were removed as described [13]. The oligonucleotides were purified by reverse-phase HPLC and anion exchange HPLC as previously described [15]. Deprotection of the silyl group was carried out by treatment with 10 mM phosphate buffer (pH 2.0) for one hour at room temperature [13] after 5'-phosphorylation. After neutralization, the phosphate was removed by gel filtration with Sephadex G-25 (Pharmacia) and the oligonucleotides were purified by reverse-phase HPLC and subsequent gel filtration.

2.2. *Construction of c-Ha-*ras *vectors*

HRL1' was annealed with HRU1 and with phosphorylated HRL2 (Fig. 1) and joined by T4 DNA ligase. The 'DNA cassette' thus obtained was purified by 5% NuSieve GTG agarose gel electrophoresis and phosphorylated. In method A, the 'DNA cassette' was joined with a vector, pCB [15], which was digested with *Cla*I and *Bss*HII, and phosphorylated HRU2' with an abasic site was annealed and ligated (Fig. 2). In method B, the cassette was first annealed with phosphorylated HRU2' and then mixed with the gapped vector and ligated (Fig. 2). Control vectors with either GGC (normal, Gly-12) or GTC (activated, Val-12) sequence at codon 12 were constructed in the same manner.

2 3 *DNA transfection*

DNA transfection was performed according to the method described previously [16,17] with the following modification Medium into which the precipitated DNA was added was buffered with 25 mM HEPES-NaOH at pH 7.5. 300, 500 and 1500 ng of DNA were used for DNA transfections.

2 4. *Analysis of the c-Ha-*ras *genes in transformed cells*

The *ras* genes present in transformed cells were analyzed by the PCR-RE method as described [12] and were directly sequenced by an Applied Biosystems model 373A DNA sequencer using the Taq DyeDeoxy Terminator Cycle Sequencing Kit.

3. RESULTS

3.1. *Synthesis and purification of oligonucleotides with an abasic site*

In our previous study we introduced an abasic site analogue into the first and second positions of codon 12 (the 34th and 35th positions, respectively) [12]. We incorporated D into the same positions. The oligonucleotides with D (HRU2′) were synthesized by the method described by Groebke and Leumann [13] with a slight modification. They deprotected the 5′-dimethoxytrityl group before cleavage from the support, whereas we removed the group after the cleavage and the first purification by reverse-phase HPLC. Although treatment with 80% acetic acid for detritylation caused partial removal of the silyl group and hence lower yields of the purified oligonucleotides, it was easier to isolate full-length oligonucleotides and to obtain the oligonucleotides with high purity for a site-directed mutagenesis study. The silyl group was deprotected after the 5′-end was phosphorylated and just before the oligonucleotides were used for vector construction. Positions where D was introduced were confirmed by 1 M piperidine treatment after 5′-labeling and subsequent analysis by polyacrylamide gel electrophoresis.

3.2. *Construction of c-Ha-*ras *vectors*

We reported the construction of c-Ha-*ras* genes with a DNA lesion including an abasic site analogue in codon 12 by a DNA cassette technique [12]. Because of the alkali-lability of D, we modified our previous method to avoid alkaline conditions as much as possible (Fig. 2). In both methods oligonucleotides with D existed only in the final step (ligation). Control vectors with a normal (Gly-12, codon 12 is GGC) or an activated (Val-12, codon 12 is GTC) gene were constructed in the same manner.

3.3. *Transforming activity of c-Ha-*ras *genes with an abasic site*

Table I shows the numbers of foci induced by c-Ha-*ras* genes with D and by the controls. Both modified genes induced more foci than the normal gene, albeit to a much lesser degree than the activated *ras* gene. These results indicate that a true abasic site was repaired in NIH3T3 cells and an activated c-Ha-*ras* gene was produced. It appears that the 34D-*ras* gene had more transforming activity than the 35D-*ras* gene.

3.4. *Analysis of the c-Ha-*ras *genes in transformed cells*

Next we analyzed the c-Ha-*ras* genes present in transformed cells by the PCR-RE method [12]. The principle of this method is that a point mutation at the site of interest forms a certain restriction enzyme site by introducing second mutation(s) by a mutagenic primer in a position other than the investigated position.

The results of the PCR-RE analysis of transformants obtained by transfection of the 35D-*ras* gene are shown in Fig. 3. In one clone (lanes 1 7), cleavage was observed only when the PCR product was treated with *Sal*I (lane 5) indicating that the 35th position was mutated to A. The results from another transformant of the 35D-*ras* gene are shown in Fig. 3 (lanes 8–14). Cleavage was observed after treatment of the PCR product with *Msc*I (lane 9). This indicates that the 34th position, which is the 5′-adjacent position, was replaced with A. These results were confirmed by direct sequencing of the PCR products with an automated DNA sequencer (data not shown). Table II summarizes the results of the analysis. Surprisingly, in the case of the transformants induced by the 34D-*ras* gene, the mutation found most frequently was to A at the 35th position (7 clones/21 clones analyzed). A clone with a mutation to T at the same position was also detected. Only five clones had mutations at the 34th position. Similarly, clones with a mutation at the 5′-adjacent (the 34th) position were found more frequently than those with a substitution at the modified (the 35th) position (Table II). The type of a mutation detected most frequently was a substitution to A in both cases.

Some transformants did not have a mutated *ras* gene and seemed to contain a normal c-Ha-*ras* gene. This conclusion was supported by the susceptibility of the PCR products to digestion with *Hap*II or *Nae*I, which recognizes CCGG or GCCGGC sequences that exist in the normal gene (data not shown). The existence of a

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      1                                                  12                  17
     Met-Thr-Glu-Tyr-Lys-Leu-Val-Val-Val-Gly-Ala-    -Gly-Val-Gly-Lys-Ser-
<--------------------HRU1--------------------> <-----------HRU2'--------->
CGAT ATG ACG GAA TAT AAG CTG GTG GTG GTG GGC GCC **C GGT GTG GGC AAG A G
  TA TAC TGC CTT ATA TTC GAC CAC CAC CAC CCG CGG CCG CCA CAC CCG TTC T CGCGC
  <---------------HRL2-------------> <---------------HRL1'----------------->
ClaI                                                                  BssHII
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Fig. 1. Nucleotide sequence of a DNA cassette with a true abasic site (D) The first and the second positions of codon 12 are indicated by asterisks. *Cla*I and *Bss*HII sites are underlined.

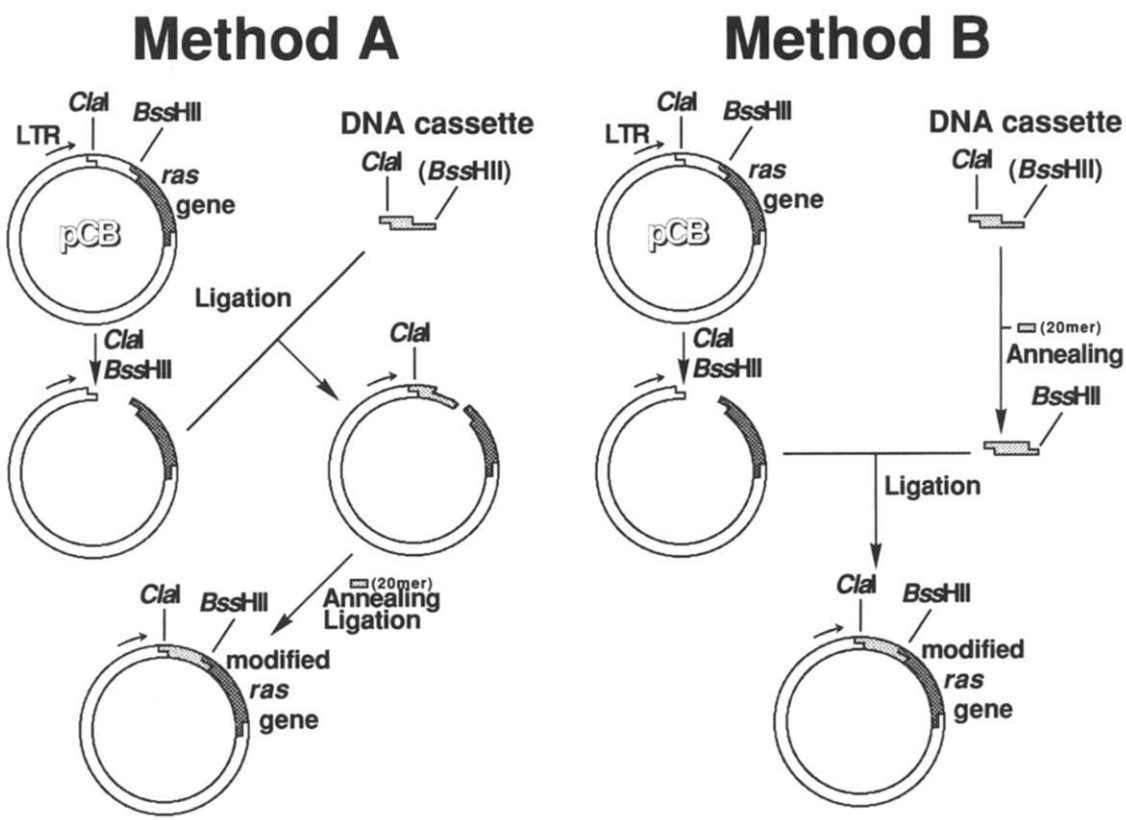

Fig. 2. Construction of vectors for transfection

normal *ras* gene in the transformants may be explained by focus-formation caused by the overproduction of normal c-Ha-*ras* proteins [18].

4. DISCUSSION

We previously reported the transforming activity of c-Ha-*ras* genes with an analogue of an abasic site in NIH3T3 cells and the spectra of point mutations induced by the DNA lesion [12]. We showed that the analogue induced mutations to A as well as flanking mutations, which are very different results from those previously obtained in in vitro, prokaryotic, and mammalian systems [3–11]. Because D was used in most of the report and because it is interesting to compare the different effects of the mutations, we modified our procedures used in the previous study [12] and introduced D into codon 12 of the c-Ha-*ras* gene.

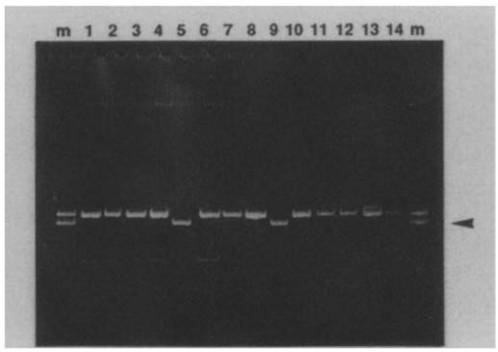

Fig. 3. Sequence analysis of c-Ha-*ras* genes present in NIH3T3 cells transformed with the gene with a true abasic site in the second position of codon 12. Lanes 1–7 = analysis of one clone. Lanes 8–14 = analysis of another clone. Lanes 1,8 = *Stu*I treatment; lanes 2,9 = *Msc*I treatment; lanes 3,10 = *Apa*I treatment; lanes 4,11 = *Aat*II treatment; lanes 5,12 = *Sal*I treatment; lanes 6,13 = *Bbe*I treatment; and lanes 7,14 = *Hap*II treatment m, marker DNA corresponding to uncleaved and cleaved PCR products (184 and 162 bp, respectively). An arrowhead indicates cleaved products.

In our previous study we demonstrated that c-Ha-*ras* genes with an abasic site analogue induced focus-formation [12]. The genes with D also induced the formation of foci (Table I). When compared to the Val-12 gene, the relative transforming efficiencies of the genes were similar to those of the *ras* genes with an analogue, although different methods were used. These results suggest that the degrees of repair were nearly equal and that the repair of D by a β-elimination mechanism may play little, if any, role in NIH3T3 cells. Other processes may primarily act on D repair [19,20].

Sequence analysis of the *ras* genes in transformed cells clearly revealed that D induced point mutations to A (Table II), consistent with our previous results [12]. These results imply that dTMP was preferentially incorporated opposite an abasic site (analogue) in NIH3T3 cells. Also, mutations at the adjacent as well as the modified positions were found, in results similar to our

Table I

Number of foci induced by c-Ha-*ras* genes

Codon 12	Experiment no.		
	1[a]	2[b]	3[c]
GGC (Gly-12)	22	11	6
DGC[d] (34D)	62	27	18
GDC[d] (35D)	48	31	14
GTC (Val-12)	356	373	169

[a] 1,500 ng of DNA was used.
[b] 500 ng of DNA was used.
[c] 300 ng of DNA was used.
[d] D, abasic site.

previous ones. However, an important difference was that mutations at adjacent positions were more frequently found than those at the modified sites (Table II).

Preferential incorporation of dAMP opposite an abasic site (analogue) by DNA polymerases in vitro has been reported [3–6,12]. Also, an abasic site induced mutations to T in *Escherichia coli* [7–9]. Our present finding that dTMP was preferentially incorporated was very different from the previous results. Gentil et al. constructed double-stranded shuttle vectors with D and analyzed plasmids replicated in COS7 cells [10,11]. They found a deficit in the incorporation of dGMP and no preferential insertion of dAMP, dTMP, or dCMP, although the ratios of the nucleotides were different depending on the base opposite D in the original shuttle vectors [10,11]. They also studied the mutation-spectrum of heat-induced abasic sites on a single-stranded shuttle vector replicated in COS7 cells and found that the order of base insertion opposite the abasic sites was G>A>T>C [21]. In this report they found that the spectra were very different and depended on the position of the lesion [21]. These results suggest that in mammalian cells dAMP is not preferentially inserted and that the incorportion of nucleotides opposite an abasic site is affected by the neighboring sequence and a base in the complementary strand. Our results showed preferential insertion of dTMP opposite D. This may be a situation in which the neighboring sequence and/or the cytosine base in the complementary strand determines the preferential nucleotide selection. Alternatively, the preferential incorporation of dTMP could be unique to mouse cells or NIH3T3 cells.

Transitions of GC to AT by an ethylating agent in Chinese hamster ovary cells were decreased by treatment with methoxyamine, which interacts with an abasic site [22]. This means that the abasic sites were formed by the agent and that the lesions are involved in the transition as well as the ethylation of the O^6-position of guanine bases. Therefore, dTMP incorporation opposite an abasic site may be common in Chinese hamster ovary cells and NIH3T3 cells.

A remarkable feature of our present study is that point mutations at 5′- or 3′-flanking sites were more frequently detected than those at the modified sites (Table II). The flanking mutation was also found in the case of an abasic site analogue, although the frequency was lower [12]. Also, dTMP was preferentially incorporated into a flanking site in the elongated strand; that is, a G-to-A transition in a neighboring position was induced (Table II). The mutation in a flanking site was not detected in COS7 cells [10,11]. However, the sequence context is very different in the COS7 system [10,11] as compared to our system. The region around codon 12 of the human c-Ha-*ras* gene is highly GC-rich, while Gentil et al. used D in pyrimidine clusters [10,11]. The frequency of flanking mutations may be very sensitive to the nucleotide sequence around the damage. Another difference may exist in the DNA polymerases participating in replication. Gentil et al. used a shuttle vector, which existed as a plasmid in COS7 cells. The vector may be replicated by a different DNA polymerase than those involved in the replication of DNA on chromosomes. Alternatively, mouse and simian cells may have different replication polymerases. It is possible that such differences between DNA polymerases and nucleotide sequences may yield distinct results.

The molecular mechanism by which mutations at adjacent positions are generated is unclear. The fact that the nucleotide incorporated most frequently in both the modified and adjacent positions was dTMP suggests a loop-out mechanism. Possible mechanisms are shown in Fig. 4. One assumption is that dTMP and dCMP are incorporated opposite D, although the insertion of dCMP cannot be detected in our assay. In the case of 34D-*ras*, the 3′-flanking G residue is looped out and dTMP is inserted opposite D (Fig. 4a). Recovery from the loop-out structure brings the 3′-T opposite G in the 35th position, the mismatched terminus is elongated by a DNA polymerase, and dCMP is inserted opposite D. In the case of 35D-*ras*, dCMP is incorporated opposite D and the C residue is looped out, then dTMP is in-

Table II

Mutations induced by *ras* genes

ras gene		34D-*ras*	35D-*ras*
Mutation in the 34th position	A	4/21[a]	9/23[b,c]
	T	1/21	0/23
	C	0/21	0/23
Mutation in the 35th position	A	7/21[a]	5/23[b]
	T	1/21	2/23[c]
	C	0/21	0/23
Normal gene		9/21	10/23

[a] 34A + 35A, 1/21.
[b] 34A + 35A, 2/23.
[c] 34A + 35T, 2/23.

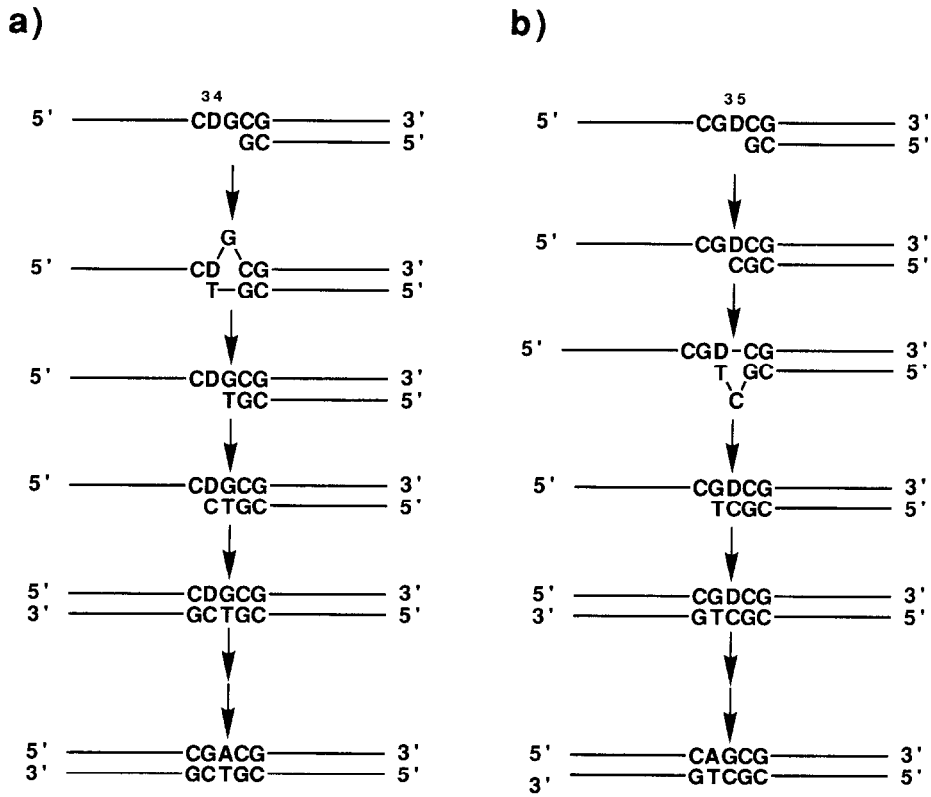

Fig. 4. Proposed mechanism of flanking mutation of (a) 34D-*ras* and (b) 35D-*ras*.

serted opposite D (Fig. 4b). Regression to the stacked structure creates a G-T mismatch at the 3′-terminus and the 3′-end is elongated by a DNA polymerase. The G-T end is a mismatch that is known to be elongated by DNA polymerase α [23]. The high frequency of flanking mutations by D in the sequence may be explained by the generation of the relatively stable G-T mismatch.

In this paper we described that true abasic sites at the first and second positions induced point mutations to A at the modified and adjacent positions. Further studies are needed to reveal the molecular mechanism of the generation of mutations at adjacent positions.

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