Induction of Mutation of a Synthetic c-Ha-ras Gene Containing Hypoxanthine

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ABSTRACT

The second base of codon 61 of a synthetic c-Ha-ras gene was replaced with a hypoxanthine residue in a site-specific manner. Transformation of this gene into NIH3T3 cells by the calcium phosphate procedure resulted in increased focus formation. Total DNA was extracted from transformed cells, and the sequences of the inserted c-Ha-ras DNA were analyzed by the polymerase chain reaction-single-strand conformation polymorphism method. Mutations with A (or hypoxanthine) to G transition were detected exclusively. These results suggest that the synthetic c-Ha-ras gene can be used for investigations of mutageneses caused by DNA lesions.

INTRODUCTION

Activated ras genes have frequently been detected in tumors and tumor cells and are thought to be involved in tumor initiation and/or progression (1, 2). These activated ras genes have been found to have a point mutation at a specific site, either in codon 12, 13, or 61 or some other position that induces substitution of an amino acid in the ras-encoded protein, p21. The mutant p21s differ from normal p21 in having lower GTPase activities and altered guanine nucleotide-binding activities (1, 2). In addition, the GTPases of the mutated p21s are not activated by GTPase-activating protein (3). Thus the mutant p21s remain as GTP complexes. The fact that the regions around the 12th, 13th, and 61st amino acids contact with a phosphate group of a bound guanine nucleotide was substantiated by determination of the three-dimensional structure of p21s complexed with GDP or GTP analogues (4–10).

Point mutations are thought to be induced in many cases by modification of a base in DNA by a mutagen and subsequent misincorporation of an "incorrect" deoxynucleoside triphosphate at the site opposite to the modified base catalyzed by DNA polymerase. Hypoxanthine is produced by deamination of an adenine base either spontaneously or in the presence of nitrous acid (11). The existence of a specific repair enzyme for hypoxanthine (hypoxanthine-DNA glycosylase) (12, 13) suggests the generation of hypoxanthine in DNA in vivo. The preferential recognition of a hypoxanthine-thymine base pair by hypoxanthine-DNA glycosylase (14) indicates that hypoxanthine originates from A in double-stranded DNA. The base-pairing properties of hypoxanthine in oligodeoxyribonucleotides and oligoribonucleotides have been studied extensively (15–20), and results have shown that hypoxanthine in oligonucleotides forms a stable base pair with an adenine or cytosine residue in the opposite strand. Therefore, generation of hypoxanthine in vivo may lead to a point mutation.

We previously synthesized genes for human c-Ha-ras proteins (21, 22). Of these synthetic human c-Ha-ras genes, those with a point mutation in codon 12 or 61 and a long terminal repeat promoter in the 5'-flanking region showed transforming activity when introduced into NIH3T3 cells by transfection (23). These synthetic c-Ha-ras genes have several unique restriction enzyme sites and, therefore, can easily be modified in a site-specific manner by insertion of a modified base using a DNA cassette mutagenesis technique. We recently reported that the gene is useful for site-directed incorporation of a modified base into codon 12 (24).

We report here high transforming activity of a synthetic c-Ha-ras gene with hypoxanthine in the second position of codon 61 and mutations with A to G transition in this position.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were purchased from Takara. Taq DNA polymerase was obtained from Perkin Elmer Cetus. Oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems model 380A DNA synthesizer. The reagents necessary for oligonucleotide synthesis including deoxynucleosine phosphoramidite were also purchased from Applied Biosystems. The oligonucleotides synthesized were purified by reverse-phase and anion-exchange high-performance liquid chromatography as described previously (21). NuSieve GTG agarose was obtained from FMC BioProducts.

Construction of a Plasmid for a Normal Human c-Ha-ras Gene. A plasmid, pRSVras, which has EcoRV and BclI sites on the 5' and 3' sides, respectively, of codon 61 and encodes a normal p21 (codon 61 is CAA for glutamine), was constructed by exchanging a DNA fragment around codon 61 of pRSV-rr61 (23). The nucleotide sequence of this modified c-Ha-ras gene was confirmed by the method of Maxam and Gilbert (25) and Sangre et al. (26) after the plasmid had been transfection into, and amplified in, the Escherichia coli GW5100 strain.

Construction of a Vector having a c-Ha-ras Gene containing a Hypoxanthine Base. Phosphorylation and ligation of oligonucleotides were carried out as described previously (21, 22). A DNA cassette (Fig. 1b) was purified by NuSieve GTG agarose gel electrophoresis followed by phenol and chloroform extractions. It was then phosphorylated with T4 polynucleotide kinase and joined to a vector derived from pRSVras by digestions with EcoRV and BclI. The joined DNA was treated with EcoRV and then T4 DNA ligase (Fig. 2). Control vectors with a CAA codon (normal, Gln-61) or a CTA codon (activated, Leu-61) were constructed by the same procedure. The vectors were purified by phenol and chloroform extractions and ethanol precipitation and quantitated with DNA DIPSTICK (InVitrogen).

DNA Transfection. DNA transfection was performed by the calcium phosphate procedure as described previously (23, 27); 20 or 100 ng of DNA and 30 ng of genomic DNA isolated from NIH3T3 cells were used for each transfection assay.

Sequence Analysis of ras Genes Present in Transformed NIH3T3 Cells. Genomic DNA was extracted from transformed cells by the conventional method using proteinase K. A portion of the ras gene around codon 61 was amplified by the PCR (28) in a DNA thermal promoter in the 5'-flanking region showed transforming activity when introduced into NIH3T3 cells by transfection (23). These synthetic c-Ha-ras genes have several unique restriction enzyme sites and, therefore, can easily be modified in a site-specific manner by insertion of a modified base using a DNA cassette mutagenesis technique. We recently reported that the gene is useful for site-directed incorporation of a modified base into codon 12 (24).

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RESULTS

Synthesis of Oligonucleotides Containing Hypoxanthine. Because a hypoxanthine residue is produced by deamination of an adenine base, the adenine residue located in the second position of codon 61 of the c-Ha-ras gene was replaced by hypoxanthine. For this purpose, we introduced a hypoxanthine base at the 17th position from the 5' side of oligomer U8, which corresponds to codon 56 to codon 62 of the sense strand of synthetic c-Ha-ras genes (Fig. 1). The oligonucleotides synthesized were highly purified by high-performance liquid chromatography, as described in the “Materials and Methods,” to avoid effects of by-products.

Construction of a Vector Having a c-Ha-ras Gene containing a Hypoxanthine Base. Cassette mutagenesis was carried out by the method used previously to avoid insertion of cassettes as trimers. A similar procedure was previously used successfully for introducing an O6-methylguanine residue into codon 12 of a synthetic c-Ha-ras gene (24).

Fig. 1b shows DNA cassettes containing CIA (I, hypoxanthine), CAA (normal, Gln-61), and CTA (activated, Leu-61) codons with a BclI end and an internal EcoRV site. These DNA cassettes were synthesized as described previously (21, 22), phosphorylated, and ligated.

The plasmid pRSVras, which has EcoRV and BclI sites on the 5' and 3' sides, respectively, of codon 61 and encodes a normal p21 (Gln-61), was digested with EcoRV and BclI. When the E. coli HB101 strain was used as a host, pRSVras was not cleaved by BclI because of methylation of an adenine base at the BclI site. Therefore, the dam- GW5100 strain was used. The cleaved pRSVras was joined with the DNA cassette, and then DNA was digested with EcoRV (Fig. 2). After subsequent ligation, the vector was purified by phenol and chloroform extractions and ethanol precipitation.

Transformation of NIH3T3 Cells with the Synthetic c-Ha-ras Gene Containing Hypoxanthine. The constructed vectors were transfected into NIH3T3 cells by the calcium phosphate procedure (23, 27). After culture for 3 weeks, foci were formed in cultures of NIH3T3 cells transfected with the CIA-ras gene.

The numbers of foci induced by the CIA-ras gene were approximately half those induced by the CTA (Leu-61)-ras gene (Table 1). This high transforming activity may suggest inefficient repair of hypoxanthine residues in NIH3T3 cells. On the other hand, transfection of the normal CAA (Gln-61) ras gene resulted in scarcely any foci, as expected (Table 1).

Analysis of c-Ha-ras Genes Present in the Transformed NIH3T3 Cells. Duplex DNA corresponding to codons 49–104 of c-Ha-ras isolated from transformed cells was amplified by the PCR (28). The amplified 168-base pair fragments were isolated by agarose gel electrophoresis, and the sequence of codon 61 was determined by PCR-SSCP analysis, which is capable of detecting a single base alteration (29). Fig. 3 shows the electrophoretic patterns obtained by analyses of three clones, as examples. The amplified DNA derived from these clones showed exactly the same mobility as the standard DNA containing G in the second position of codon 61. The mobility of these amplified DNAs in the single-stranded form was quite different from those of the standard DNAs having A, C, or T in the second position of codon 61. This suggested that the mutation induced by hypoxanthine located in the second position of codon 61 of c-Ha-ras was exclusively an A (I) to G transition. This sequence was confirmed by direct sequencing.
MUTATION INDUCED BY HYPOXANTHINE IN ras GENE

Standards

Fig. 3. Analysis of the synthetic c-Ha-ras genes present in transformed NIH3T3 cells by PCR-SSCP. Amplified DNAs were analyzed by 6% polyacrylamide gel electrophoresis. The gel did not contain glycerol, and electrophoresis was performed at 4°C. All samples analyzed in experiment 2 in Table 1 (lanes 1–3) showed the same electrophoretic pattern as the "G" standard. Lanes A, G, T, and C indicate standard samples containing CAA, CGA, CTA, and CCA, respectively, at codon 61; lane ds, marker corresponding to the double strand.

Fig. 4. Maxam-Gilbert sequence analysis of the amplified c-Ha-ras gene (the same sample as for lane 2 in Fig. 3).

DISCUSSION

Hypoxanthine, produced by deamination of adenine, is structurally analogous to guanine rather than parental adenine. It has the same hydrogen donor (endocyclic imino group) and acceptor (carbonyl oxygen) sites as guanine. Therefore, hypoxanthine may induce a mutation to G, because it appears to pair with C in the Watson-Crick manner like a G-C pair (Fig. 5, a and b). Furthermore, there are reports that complementary oligonucleotides containing a hypoxanthine-adenine pair as well as a hypoxanthine-cytosine pair form stable duplexes and that pairs with G and T do not induce destabilization (15–20). These findings suggest that hypoxanthine may cause transition and transversion.

We recently reported site-directed insertion of an O-methylguanine base into codon 12 of a synthetic c-Ha-ras gene and its transforming activity in NIH3T3 cells (24). A similar strategy using DNA cassette mutagenesis should be very useful for investigating DNA lesions in other positions. Because the second base of codon 61 is A and is one of the hot spots of c-Ha-ras gene activation, using the DNA transfection assay, we examined the effect of its substitution with hypoxanthine by cassette mutagenesis. As shown in Table 1, ras genes containing hypoxanthine showed potent transforming activity. Thus, generation of hypoxanthine from adenine by deamination may be one cause of mutations.

In our system, mutations to C are thought not to lead to focus formation because it was reported that a Pro-61 (CCA at codon 61) mutant did not have transforming activity (30). However, it is possible to detect mutations to G (Arg-61) or T (Leu-61) in our system. In the present study, mutations to G were detected almost exclusively, suggesting that DNA polymerase(s) in NIH3T3 cells incorporates dCTP but not dATP as a substrate in the position opposite to hypoxanthine. In an in vitro study in which the Klenow fragment was used, the DNA polymerase was found to insert C to pair with hypoxanthine (31). This finding suggests that DNA polymerases utilize dCTP by the method of Maxam and Gilbert (25) of amplified DNAs from these clones (Fig. 4). Subsequently, 17 additional transformed NIH3T3 foci induced by transfection of c-Ha-ras containing hypoxanthine were determined by PCR-SSCP analysis. Of the 20 clones analyzed, 19 were found to have G in the second position of codon 61, while one contained both G and T in this position.

Fig. 5. Base pair characteristics of hypoxanthine. a, a normal Watson-Crick base pair (G-C); b, a hypoxanthine-C base pair; c, a hypoxanthine-A base pair (Watson-Crick type). The distance between the two bases is more than in a normal Watson-Crick base pair; d, a hypoxanthine-A base pair (Hoogsteen type).
preferentially in spite of the ability of hypoxanthine to form base pairs with C and A.

Hypoxanthine can pair with C in the normal Watson-Crick manner (17) like a G-C pair (Fig. 5, a and b). Nuclear magnetic resonance studies showed that a hypoxanthine-A base pair was a Watson-Crick type (17), although it was a Hoogsteen type in crystals (32) (Fig. 5, c and d). Although A can pair with hypoxanthine and the hypoxanthine-C pair (Fig. 5, a-c). Furthermore, the total conformation of a hypoxanthine-A pair is different from that of normal (purine-pyrimidine) base pairs and the hypoxanthine-C pair (Fig. 5, a-c). This finding may indicate that dAMP was incorporated into DNA with a thermostable DNA polymerase. Science (Washington DC), 262: 10171-10179, 1987.

In this communication, we showed that a synthetic c-Ha-ras gene is very useful for site-specific modification of codon 61 with a modified base. Studies of the focus-forming activities of ras genes containing other carcinogen-induced lesions at codon 61 are in progress.

REFERENCES

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