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NON-RADIOACTIVE MISMATCH ANALYSIS TO DETECT SMALL MUTATIONS IN HUMAN HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE cDNA

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SUMMARY: We have combined a cDNA-driven PCR technique and a non-radioactive chemical-cleavage mismatch method, followed by a direct sequencing for detecting small mutations in the human hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene. HPRT cDNA was synthesized by RT-PCR from 1,000 wild-type or HPRT(−) mutant cells. Wild-type cDNA was hybridized with mutant cDNA to form heteroduplexes. The resultant mismatched bases were modified and cleaved by base-specific chemicals, followed by analysis by denaturing polyacrylamide gel electrophoresis. Cleaved fragments were detected without using radioactive materials. Finally, direct sequencing of the PCR products was performed with a focus on a small limited region indicated by the mismatch analysis (focused sequencing). In this study, three small mutations in exon-3 of HPRT cDNA were detected and characterized completely with this system. As compared with the radioactive method, this system was shown to be very simple and efficient.

INTRODUCTION

The X-linked hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene has been utilized extensively as a marker for the analysis of mutations induced by physical and chemical carcinogens in both human and rodent cells. One

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concern in the analysis of the HPRT gene is detecting point mutations to identify "hot-spots". Although various direct nucleotide sequencing approaches have been attempted (1-5), it seemed difficult to locate single point mutations within the more than 700 bases that constitute the coding region (cDNA) of the HPRT gene. Under these circumstances, it would be desirable to have a simple prescreening strategy that could detect and locate the position of mutations before direct nucleotide sequencing is attempted. In this report we describe an approach that couples an RT-PCR (reverse transcription - polymerase chain reaction) amplification method to a non-radioactive DNA-DNA mismatch hybridization technique. Final characterization of the detected mutations was performed by direct nucleotide sequencing with a focus on a small limited region indicated by the prior mismatch analysis (focused sequencing).

MATERIALS AND METHODS

Cell culture: Primary human skin fibroblasts (HSF) obtained from newborn foreskins were prepared as reported (6) and used as wild-type controls. Three HPRT-deficient mutants isolated in our institute from X-ray irradiated populations of the human B-lymphoblastoid cell line, TK6, were used to validate our present technical approach. These mutants were known to have non-characterized small mutations in exon-3 of the HPRT gene. Both the mutant and HSF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 µg/ml streptomycin, 100 units/ml penicillin and 10% fetal calf serum (FCS). The cultures were maintained at 37 C in a humidified incubator with 5% CO₂.

RT-PCR: First strand cDNA synthesis and subsequent PCR amplification were performed by the method of Yang et al (4). Briefly, 1,000 HSF or mutant lymphoblast cells were microfuged at 15,000 rpm for 1 min. These cell-pellets were directly incubated at 37 C for 1 hr in 5 µl of the first strand cDNA synthesis cocktail to be used as cDNA templates in the subsequent PCR reaction. The first PCR was performed in 50-µl volume with a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) under the conditions of Yang et al. (4) using an Ampli-Taq (Perkin-Elmer Cetus). After the first pair of primers (primers 1 and 2) were removed on Sepharose columns (CL-6B, Pharmacia, Uppsala, Sweden), 1 µl of the product was used as a template for the second series of PCR. The conditions were identical to those of the initial PCR reaction except for the use of a nested pair of primers (see primers 3 and 4 below). The sequences of the primers were as follows: [the num-

bering system is as described by Jolly et al. (7) for HPRT cDNA with the AUG start codon numbered at bp 86].

Primer 1: (38) 5' CTCTGCTCCGCCACCGGCTTCCT 3' (60)

Primer 2: (975) 5' CCGCCCAAAGGGAAGTATGATAGTC 3' (953)

Primer 3: (64) 5' CCTGAGCAGTCAGCCCGCGC 3' (83)

Primer 4: (821) 5' GATAATTTTACTGGCGATGT 3' (801)

These primers were synthesized based on the information provided by Yang et al. (4) and Jolly et al (7). The final products were fractionated by 0.8% agarose gel electrophoresis and visualized by ethidium bromide staining. Appropriate fragments were eluted from the gel matrix with Prep-A-Gene (Bio-Rad, Hercules, CA) at a final concentration of 50 ng/ μ l.

Non-radioactive chemical mismatch analysis: Wild-type and mutant cDNAs (500 ng each) were mixed in 50 μ l of the hybridization mix containing 0.3 M NaCl, 3.5 mM MgCl₂, and 3 mM Tris-HCl, pH 7.7. Mixtures in tubes were heated to 100 C for 5 min and incubated at 42 C for more than 3 hr. Based on the method of Cotton et al. (11), one-half of each heteroduplex solution (25 μ l) was treated with 20 μ l of 2.5% pyridine (Sigma Chemical Co., St. Louis, MO) in water and 5 μ l of 1% osmium tetroxide (OT) (Aldrich Chemical Co., Inc., Milwaukee, WI) in water. The final concentration of OT was 0.1%. The mixture was vortexed and incubated at 37 C for 15 min. The remaining heteroduplex solution (25 μ l) was treated with 100 μ l of 5 M hydroxylamine hydrochloride (HH) (Sigma Chemical Co.) titrated to pH 6.0 by diethylamine (Sigma Chemical Co.). The mixture was vortexed and incubated at 37 C for 20 min. Then, 150 μ l of ice-cold water was added to stop the reactions and the resultant chemically modified DNAs were purified with Prep-A-Gene (Bio Rad) in a final volume of 20 μ l. Ten microliters of freshly prepared 30% piperidine (Sigma Chemical Co.) was then added to each tube (final concentration 10%) and incubated at 90 C for 20 min. After ethanol precipitation in the presence of 0.8 M ammonium acetate and 20 μ g of mussel glycogen (GIBCO BRL, Gaithersburg, MO), the pellets were rinsed once with 70% ethanol and dried completely. The samples were analyzed by electrophoresis on a denaturing polyacrylamide gel, and the DNA was visualized by staining with 0.5 μ g/ml ethidium bromide.

Radioactive chemical mismatch analysis: The ordinary radioactive technique was used for comparison with our non-radioactive mismatch technique. The preparation of radioactive wild type-probe and the subsequent chemical reactions were identical to the method mentioned by Cotton et al. (8) and Gormpe et al (9). Briefly, the individual nested primers (primer-3 and -4) were end-labeled according to the standard protocols with T4 polynucleotide kinase (21), in order to generate strand specifically radiolabeled wild-type DNA probes. The labeled primers were purified on spin columns (Bio-Gel P-30, Bio Rad) and each "hot" primer was used to drive individual PCR reactions to produce DNA probes labeled in either the "plus" or "minus" strands. Wild-type/mutant heteroduplexes were formed by mixing 10 ng of the radiolabeled probe DNA with 100 ng of target DNA

in the same reactions mentioned above. Each target DNA was melted and reannealed to both strand-specific probes in separate reactions. The following chemical modification and cleavage of mismatched bases are identical to those described in the non-radioactive method. Ten thousand counts per minute of each final product was fractionated by electrophoresis on sequencing gels followed by detection of specific products by autoradiography.

Direct sequencing: Sequencing was performed by a modified Sanger's dideoxy method using a Bst sequencing kit obtained from Bio Rad. Purified PCR products (0.5-0.7 pmoles) were mixed with 2-5 pmoles of sequencing primer (268-ATGGGAGGCCATC-280) and 2 μ l of buffer supplied by the manufacturer in 15 μ l of a reaction volume. The mixtures were heated to 95 C for 5 min and snap-cooled immediately in an ethanol-dry-ice bath for 5 min. One microliter of [α -³²P]-dATP (NEN Dupont, Wilmington, DE) and 1 unit of Bst polymerase (Bio Rad) were added to this annealing mix on ice. The extension-termination reaction was performed according to the manufacturer's standard protocol with their dNTP-ddNTP mix. After the chase reaction, 2 units of terminal deoxynucleotidyl transferase (TdT) (USB, Cleveland, OH) in 1 μ l of a solution containing 0.8 μ l of 1 mM dNTPs in 1 $\times$ TdT buffer was added to each tube and incubated at 37 C for 30 min according to the method of Fawcett et al (10). After adding the formamide-loading solution, samples were analyzed by sequencing gel (5%) electrophoresis followed by autoradiography.

RESULTS

RT-PCR (Fig. 1)

Products formed by the initial PCR amplification show faint bands at approximately 920 bp in the wild-type and one of the mutant lines. After a second 30 rounds of PCR amplification with "nested" primers, a large amount of the 740 bp product was observed in all of the samples. The amount of the 740 bp fragment in mutant-3, however, was substantially reduced and a <600 bp major product appeared. The 740 bp fragments of all samples, including mutant-3, were purified on preparative gels for further analysis. In addition, the smaller fragment (<600 bp) of mutant-3 was also purified for characterization.

Non-radioactive Chemical Mismatch Analysis (Fig. 2)

Cleavage products were detected by ethidium bromide staining when the initial amount of DNA for heteroduplex formation was greater than 500 ng. No signal was detected when the initial amount of DNA was under 300 ng (data not

Fig. 1. RT-PCR of the HPRT gene. M: Φ X-Hae III digest. Lanes 1-5: products after initial 30 cycles of PCR. 1: control (no cell), 2: wild-type, 3: mutant-1, 4: mutant-2, 5: mutant-3. Lanes 6-10: products after second run of PCR. Materials in lanes 6-10 correspond to lanes 1-5.

Fig. 2. Non-radioactive chemical mismatch in mutant-1, -2, and -3. While no cleavage occurred in wild-type homoduplex, cleaved fragments are observed in both HH (hydroxylamine hydrochloride treatment) and OT (osmium tetroxide treatment) lanes in mutant-1 (transversion of "T:A" to "G:C"). Cleaved fragments are seen only in the HH lane in mutant-2 (deletion of "C:G"). In mutant-3, cleaved fragments are observed in both HH and OT lanes ("G:C" and "T:A" deletion).

Fig. 3. Radioactive chemical mismatch analysis. There are four lanes for each sample. From left to right, plus-strand (+) with hydroxylamine hydrochloride treatment (HH), plus-strand (+) with osmium tetroxide treatment (OT), minus-strand (-) with HH treatment, and minus-strand (-) with OT treatment. Wild type: homoduplex formed between differently prepared wild-type cDNAs. Mutants 1-3: Heteroduplexes formed between wild-type and mutant-1, -2 and -3, respectively. No cleavage occurred in wild-type homoduplexes. In mutant-1, one cleaved fragment appeared in the (+)-strand OT lane. Also, one cleaved fragment showed up in the (+)-strand HH lane in mutant-2, and two cleaved fragments appeared in the (+)-strand OT and (-)-strand HH lanes of mutant-3.

shown). Several homoduplexes formed between independently amplified wild-type cDNA's were tested without yielding cleavage. One example of mismatch analysis with wild-type homoduplexes is shown in Fig. 2. In mutant-1, two cleaved fragments (410 and 330 bp) were seen with both HH and OT treatments, which indicates that the heteroduplexes between wild-type and mutant-1 have mismatching involving "T" and "C", and their location is approximately 330 bp from the annealing site of primer-3 or 4 (see Fig. 4). Similarly, mutant-2 has cleaved fragments only in the HH lane, while mutant-3 has cleaved fragments in both lanes. These indicate that mutant-2 has a mismatched "C" without involvement of "T", while mutant-3 has a mismatched "T" and "C". Unexpectedly, the size of resulting fragments turned out to be very similar, which indicates that

1) Non-radioactive method

2) Radioactive method

Fig. 4. Schematic illustration of mismatch analysis between wild-type and mutant-1. In the non-radioactive method, three different-sized fragments appear in both hydroxylamine hydrochloride (HH) and osmium tetroxide (OT) lanes, since all cleaved fragments are visualized by ethidium bromide in this method. When strand-specifically radiolabeled wild-type probes are used, however, only one cleaved fragment appears in the (+)-strand OT lane on autoradiography.

these mutants have point mutations or small deletions in closely located regions of exon 3 of HPRT cDNA.

Fig. 5. Modified direct sequencing of the focused region. A "T" to "G" transversion is observed in mutant-1. A deletion of "C" is observed in mutant-2. A two-base deletion of "TG" is observed in mutant-3.

Radioactive Mismatch Analysis (Fig. 3)

Cleavage products were not observed in the control homoduplex where the radiolabeled wild-type was melted and re-annealed to a variety of "cold" wild-type PCR products. Cleavage products appeared in heteroduplexes formed between the wild-type and each of the mutant cDNAs. A single 330 bp fragment was detected in plus-strand OT lane of mutant-1, which indicates the presence of a mismatched "T" in the plus-strand located approximately 330 bp from the annealing site of primer-3. Similarly, treatment of the heteroduplex formed between plus-strand wild-type and minus-strand mutant-2 with HH produced a cleavage product, which indicates the presence of a mismatched "C" in the same region of the plus-strand. Finally, analysis of mutant-3 indicated the presence of both a mismatched "T" in the plus-strand and "C" in the minus-strand. These results indicate that mutant-3 has mutations involving two bases.

Schematic explanation of non-radioactive and radioactive mismatch analyses between wild-type and mutant-1 is shown in Fig. 4.

Sequencing (Figs. 5 and 6)

The PCR products purified by the method mentioned above were sufficient to consistently yield "clean" sequencing information by the dideoxy method. Snap-cooling (11) was effective in preventing reassociation of the denatured PCR product. Treatment of the reaction with terminal deoxynucleotidyl transferase

Fig. 6. Direct sequencing of the small fragment generated from mutant-3. In addition to the two base-pair deletion in exon 3, exons 4, 5, 6, 7, and 8 are deleted.

(USB) worked dramatically to eliminate banding artifacts which frequently occur when PCR products are sequenced (10). One of three primers designed to span the cDNA for sequencing purposes was used to sequence the exon 3 region of mutants -1, -2, and -3 (Fig. 5). Mutant-1 has a T:A to G:C transversion at position of 381. Mutant-2 has a deletion of G:C at position of 372, while mutant-3 has deletion of G:C and T:A at position of 373-374. These results are consistent with our interpretation of the data obtained by the mismatch analysis. In addition, sequencing data of the smaller fragment of mutant-3 indicated that in addition to a two-base pair deletion in exon 3, this small fragment was devoid of sequences for exons 4, 5, 6, 7, and 8 (Fig. 6).

DISCUSSION

The idea of amplifying the first strand of cDNA generated by reverse transcription of mRNA (12) made possible the mutational analysis of cDNA from extremely small quantities of starting materials, such as a crude lysate equivalent to a few cells (13) or the precipitated cells themselves (4). This is especially important in studies involving primary cells with a finite life span, such as HSF cells. In this one-step first strand cDNA synthesis from cell pellets of mutant-3, an altered size of cDNA appeared unexpectedly. The nature of this "new" fragment is not yet clear, but it has been reported that point mutations near intron-exon boundaries can often cause aberrations in the splicing processes resulting in deletions by exon skipping (14). As a consequence, the splice site mutations can alter the size of the processed mRNA despite normal genomic Southern blot patterns (5).

The use of mismatch analysis to detect point mutations was first reported by Myers et al. (15,16) using DNA:RNA hybrid molecules. Cotton et al. (8) improved this method considerably by using DNA:DNA hybrids and osmium tetroxide and hydroxylamine hydrochloride to modify specific bases prior to chemical cleavage. In addition to its high sensitivity, mismatch analysis has the capacity to precisely locate the position of mutations, thus enabling one to focus the sequencing to a smaller region. There are two other frequently used methods to detect point mutations in a given DNA fragment. One is single-strand conformation polymorphism (SSCP) (17) and the other is denaturing gradient gel electrophoresis (DGGE) (18). Although SSCP and DGGE can also readily detect point mutations, both of them lack positional information on the detected mutations. As a consequence, when one analyzes DNA sequences by SSCP or DGGE, one must sequence the entire length of the fragment to determine the precise molecular basis of each mutation. In addition, it has been claimed that SSCP and DGGE can be applied to fragments up to 400 bp and 1,000 bp in length, respectively (17,18). In contrast, fragments larger than 1 kb can be analyzed by chemical mismatch analysis. According to Condie et al. (19), the sensitivity of each technique to detect point mutations is 90% for SSCP, 88% for constant denaturant gel electrophoresis (CDGE) and 100% for chemical mismatch analysis.

Although our PCR/mismatch protocol appears quite straight forward, this technique must cope with potential sequence changes that may result from the error-rate of the Taq DNA polymerase (20,21). Krawczak et al. (22) have attempted to calculate the replication errors induced by extensive PCR amplifica-

tion with the Taq DNA polymerase. Reiss et al. (23) tried to estimate the effect of replication errors in the mismatch analysis of PCR-amplified DNA. If their calculations were applied to our PCR scheme, approximately 57% of the final 740 bp PCR product would not contain replication errors, which is adequate to maintain the sensitivity of our mismatch protocol, despite the tendency of the replication errors induced by the Taq enzyme to cluster at specific positions in a given DNA sequence (21). Actually, no false-positive signals were detected in our experiments, although several homoduplexes formed between independently amplified wild-type cDNA's were used for mismatch analysis.

The mismatch technique has relied primarily on a variety of radiolabeling techniques (8,24-26). Radiolabeling techniques (1) are time-consuming, (2) require special protocols and facilities, and (3) are constantly ongoing processes because of the short half-lives of radioactive probes. Our non-radioactive method is not only simple but also is as sensitive as the radioactive method if more than 500 ng of DNA is used. Furthermore, the non-radioactive method can potentially detect all types of mutation (deletion, base change, and insertion), while the radioactive technique cannot detect insertion mutations if only wild-type DNA is labeled. We believe that this approach is particularly useful as a screening tool to rapidly localize and identify small mutations when a large number of mutants are being analyzed.

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