Technical Advance

Development of a Yeast Stop Codon Assay Readily and Generally Applicable to Human Genes

Akihiko Kataoka,^{**‡} Mitsuhiro Tada,[‡]
Masahiro Yano,[‡] Keiji Furuuchi,[‡]
Santoso Cornain,[§] Jun-ichi Hamada,[‡]
Gaku Suzuki,^{†‡} Hidehisa Yamada,[‡] Satoru Todo,^{*}
and Tetsuya Moriuchi[‡]

From the First Department of Surgery^{*} and the Third Department of Internal Medicine,[†] Hokkaido University School of Medicine, Sapporo; and the Divisions of Cancer-Related Genes[‡] and Gene Therapy Development,[§] Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan

We established a yeast-based method to screen chain-terminating mutations that is readily applicable to any gene of interest. Based on the finding that 18- to 24-base-long homologous sequences are sufficient for gap repair *in vivo* in yeast, we used a strategy to amplify a test-gene fragment with addition of 24-bp sequences homologous to both cut-ends of a yeast expression vector, pMT18. After co-transformation with the amplified fragment and the linearized pMT18, each yeast (*Saccharomyces cerevisiae*) cell automatically forms a single-copy circular plasmid (because of CEN/ARS), which expresses a test-gene::ADE2 chimera protein. When the reading frame of the test-gene contains a nonsense or frameshift mutation, truncation of the chimera protein results in lack of ADE2 activity, leading to formation of a red colony. By using a nested polymerase chain reaction using proofreading *Pfu* polymerase to ensure specificity of the product, the assay achieved a low background (false positivity). We applied the assay to *BRCA1*, *APC*, *bMSH6*, and E-cadherin genes, and successfully detected mutations in mRNA and genomic DNA. Because this method—universal stop codon assay—requires only 4 to 5 days to screen a number of samples for any target gene, it may serve as a high-throughput screening system of general utility for chain-terminating mutations that are most prevalent in human genetic diseases. (Am J Pathol 2001, 159:1239–1245)

and molecular oncology. Because DNA sequencing primarily defines the location and nature of the change, it is considered to be the ultimate method of mutation detection. A long sequence of a gene of interest (GOI), however, often precludes the direct analysis of many samples in a limited period of time. Therefore an appropriate screening method to make up the drawback is necessary. To date, various screening methods based on primary DNA structure have been reported;¹ denaturing gradient gel electrophoresis, heteroduplex analysis, chemical mismatch cleavage, and single-strand conformational polymorphism analysis. These, especially the last one, have widely been used as practically useful techniques. However, because of the limit in DNA length examinable by these techniques, we must divide a GOI into many parts, for each of which an optimization of experimental condition is required. Another drawback is that the sensitivity of mutation detection is primarily affected by the physical properties of the DNA fragment, often falling below 100% even in the best hands.²

After Ishioka and colleagues³ first reported an efficient screening method using yeast prototrophy (URA3 marker) for uracil, namely yeast-based stop codon assay, a modification using yeast color selection (ADE2 marker) has been reported for detection of chain-terminating mutations in some specific genes.^{4–6} Taking advantage of gap repair and production of GOI::ADE2 (EC 4.1.1.21) chimera protein as a visible reporter in yeast, this method permits a quantitative, efficient detection of chain-terminating mutations as red colonies because of accumulation of an ADE2 substrate *in vivo* in yeast.⁷ This method requires, however, construction of an expression vector that is specific to each tested GOI (cloning of GOI into the vector), and thus is not immediately applicable to a newly interested gene. To remove this drawback without compromising the reliability of assay, we made two modifications: 1) amplification of GOI by a nested polymerase chain reaction (PCR) procedure with addition of short

Accepted for publication July 10, 2001.

Yeast strain and vectors used in this study are available by request for research purposes.

Address reprint requests to Mitsuhiro Tada, M.D., Division of Cancer-Related Genes, Institute for Genetic Medicine, Hokkaido University, N-15 W-7, Kitaku, Sapporo 060-0815 Japan. E-mail. m_tada@med.hokudai.ac.jp.

The task of identifying mutation in nucleic acid sequences is a crucial part of research in human genetics

terminal sequences for gap repair, and 2) utilization of a universal vector for the acceptor of the amplified GOI. We determined optimal conditions of the assay in regard to the specificity and efficiency of gap repair, and confirmed quantitiveness of the mutation detection and its general applicability to a variety of GOI, minimizing the assay background (false positivity). We here present the establishment of the assay named "universal stop codon assay." Taking the prevalence of chain-terminating mutations in the whole mutation spectrum⁸ into account, we suggest that the assay is able to cover the majority of human genetic diseases including cancers.

Materials and Methods

Principle of Universal Stop Codon Assay

This assay utilizes a YCp type yeast expression vector, which is held in a single copy in a yeast cell and expresses a chimera protein of the reading frame of a tested gene fragment and *ADE2* gene (phosphoribosylaminoimidazole carboxylase, E.C. 4.1.1.21). To permit an automatic integration of the gene fragment in-frame to *ADE2* gene by homologous recombination in yeast, short sequences homologous to the vector cut-ends are added to the fragment during PCR amplification. Failure in producing a complete chimera protein because of the presence of a chain-terminating mutation in the tested fragment leads to accumulation of phosphoribosylaminoimidazole in yeast cells, resulting in red color of a yeast colony (Figure 1, A and B).

Vector Construction

The *S. cerevisiae*/*Escherichia coli* shuttle expression vector pLS381, an immediate origin of pCA57,⁴ which has *URA3* marker, *CEN/ARS* sequence, Amp-resistant gene, *ColE1* Ori, and *ADE2* driven by *CYC* promoter, was used for construction of vectors as follows. pLS381 was linearized at the *Bam*HI site between *CYC* promoter and *ADE2*, and a double-strand oligonucleotide (5'-CACTAAATT AATA-ATGACC GGCGCC ATGGATTCTA GAACAGTTG-3') was integrated into the vector in yeast to replace a *Sfo*I site with a *Bam*HI site. The resultant new vector, pMT18 (Figure 1a), was linearized with *Sfo*I and dephosphorylated with calf intestinal alkaline phosphatase (Gibco-BRL, Tokyo, Japan). Complete digestion of pLS381 and pMT18 was confirmed by an additional agarose gel electrophoresis after gel purification and calf intestinal alkaline-phosphatase treatment. Yeast strain yPH857 was transformed with the linearized 50-ng pMT18 or pLS381 in a manner described below for comparison of self-ligation of the vectors.

To insert a spacer sequence, a double-strand oligonucleotide, 5'-tta ata atg acc *gpc* GCC. . . GGA TCC gcc atg gat tct aga-3', flanking a tag sequence (FLAG, 5'-ATG GAT TAC AAG GAT GAC GAC GAT AAG ATC-3') was integrated into the *Sfo*I-linearized pMT18, yielding pMT18flag. This was also linearized with *Sfo*I, treated with

Figure 1. Vector structure and principle of the assay. **A:** Structure of pMT18 *S. cerevisiae*/*E. coli* shuttle expression vector for universal stop codon assay. Gap repair of the linearized vector at *Sfo*I site with a GOI occurs *in vivo* in yeast by homologous recombination with 24-bp sequences added to the both ends of the GOI. *CEN/ARS* and *URA3* allow a single-copy presence of the vector in a yeast cell in uracil-deficient medium. **B:** A schematic view of the universal stop codon assay. The test gene fragment is amplified by a nested PCR adding gap repair sequences and co-transfected into a haploid yeast strain, yPH857, lacking *URA3* and *ADE2* genes. Gap-repaired plasmids permit growth of the transfected cell and forms GOI: ADE2 chimera protein. The GOI: ADE2 protein provides ADE2 activity when GOI does not contain a nonsense mutation; otherwise truncation of the chimera protein results in the absence of ADE2 activity, making the colony color red because of accumulation of the ADE2 substrate—phosphoribosylaminoimidazole.

calf intestinal alkaline phosphatase, and tested for complete digestion.

Primer Design for Nested PCR

For nested PCR to amplify GOI with addition of sequences for gap repair, two primer sets (external and internal) were designed by using a computer program OLIGO ver. 4.1 (MedProbe, Oslo, Norway). External primers were made in both outer arms encompassing the target site, giving a special priority to the specificity and the absence of a secondary structure. Gene-specific parts of internal primers were designed as their 5'-termini

Table 1. Primers Used in this Study

Gene*	Accession no.*	Use†	Forward primer: name, sequence, position‡	Reverse primer: name, sequence, position‡	Size Tm§
BRCA1 exon 11 middle 1/3	L78833	External	BR1EX11F2, aaaagaataggctgaggaggaagtc, nt. 35021–35045	BR1EX11R2, ctcatccccatttctcttcaggt, nt. 36228–36252	1232 bp 58°C
		Internal	gBREX11F2, (vs)+agaaatctaagcccacctaata, nt. 35086–35106 from codon 629	gBREX11R2, (vs)+agtaatgagtcagtttcgtt, nt. 36082–36102 to codon 967	1017 bp 58°C
BRCA1 exon 11 upper 2/3	L78833	External	BR1EX11Fn, gtacctgttattttgtatatttcag, nt. 33844–33871	BR1EX11R2	2409 bp 58°C
		Internal	gBR1EX11F, gctgctgtgaatttctgagacg, nt. 33871–33894 from codon 224	gBREX11R2	2232 bp 58°C
BRCA1 exon 11 lower 2/3	L78833	External	BR1EX11F2	BR1EX11Rn, gggcaaacacaaaaacctggttc, nt. 37303–37326	2306 bp 60°C
		Internal	gBREX11F2	gBR1EX11R, tccaatacctaagttgaatccatgc, nt. 37280–37305 to codon 1366 + 6 bp	2220 bp 58°C
APC exon 15	M74088	External	APCEX15F, aaatgaaacctcgattgaatcc, nt. 2951–2973	APCEX15R, gctgctctgattctgtttcattc, nt. 4620–4642	1692 bp 55°C
		Internal	gAPCMCRF, (vs)+aatcgagtggttctaatac, nt. 3355–3373 from codon 1113	gAPCMCRR, (vs)+cgtggcaaaagttaataa, nt. 4480–4497 to codon 1493	1143 bp 51°C
hMSH6 cDNA	U54777	External	MSH6F1, agggaggtcattttacagtcg, nt. 552–573	MSH6R1, ctacatcgtgcctccatcattt, nt. 1539–1560	1009 bp 55°C
		Internal	gMSH6F1, (vs)+cctgaataactgagagcaa, nt. 578–596 from codon 170	gMSH6R1, (vs)+gaaatcctcaggcacatag, nt. 1258–1276 to codon 402	699 bp 52°C
E-cadherin 1/3 part	Z13009	External	EcadF1, ccatgggccccttgagccgc, nt. 93–112	EcadR1, ctggaagagcaccttccatgac, nt. 905–926	834 bp 58°C
			gEcad1Fc, (vs)+ctctcggcgtgctgctgct, nt. 116–135 from codon 8	gEcad1Rc, (vs)+agcaccttccatgacagaccctt, nt. 896–919 to codon 275	804 bp 60°C
E-cadherin 2/3 part	Z13009	External	EcadF3, aacgcattgccacatacactc, nt. 762–782	EcadR3B, ggggcttcattcacatccag, nt. 1526–1545	784 bp 57°C
		Internal	gEcadF2, (vs)+ttctctcacgctgtgcattcaac, nt. 785–808 from codon 231	gEcadR2, (vs)+ggtgacggtggtgtggagg, nt. 1497–1516 to codon 474	732 bp 58°C
E-cadherin 3/3 part	Z13009	External	EcadF4, cgtagcagtgacgaatgtggt, nt. 1453–1473	EcadR4, ggggaaggagctgaaaaaac, nt. 2807–2826	1374 bp 57°C
		Internal	gEcad3F2, (vs)+gtctctctcaccacctccaca, nt. 1484–1504 from codon 464	gEcad3R2, (vs)+gccccattcgttcaagtagtc, nt. 2672–2692 to codon 866	1209 bp 57°C

*Gene, accession no.: gene name and accession number denoted in GenBank.

†Use: external or internal primer set. To an internal primer (name starting with "g"), vector end sequences ("(vs)+") for gap repair was added at the 5'-terminus, as described in Materials and Methods.

‡Position; positions in nucleotide number were of GenBank data.

§Size; size without gap repair sequences; Tm; annealing temperature used for PCR.

matching the codon frame of a target sequence (Table 1). Length and position of the primers were selected not to form a dimer of more than 4 bases in the 3'-termini. To the 5' end of the gene-specific part of an internal primer, a sequence for gap repair (homologous recombination) matching the corresponding cut-end of the vector was added as follows. For gap repair into pMT18, 5'-CAC ACT AAA TTA ATA ATG ACC GGC ATG-3' (last ATG for translational start) and 5'-ACC AAC TGT TCT AGA ATC CAT GGC-3' were added to the 5'-termini of forward and reverse primers respectively. For gap repair into pMT18flag, 5'-GTC GTC ATC CTT GTA ATC CAT GGC-3' was added to the 5' end of reverse primers.

To examine the effect of length of sequences for gap repair on fidelity of the recombination, we added three sized gap-repair parts (18 bases, forward 5'-AAA TTA ATA ATG ACC GGC-3', reverse 5'-TGT TCT AGA ATC CAT GGC-3'; 24 bases, forward 5'-CAC ACT AAA TTA ATA ATG ACC GGC-3', reverse 5'-ACC AAC TGT TCT AGA ATC CAT GGC-3'; and 30 bases, forward 5'-AAT ACA CAC ACT AAA TTA ATA ATG ACC GGC-3', reverse 5'-TAA TAT ACC AAC TGT TCT AGA ATC CAT GGC-3') to the 5' end of respective forward and reverse *BRCA1*-specific parts (5'-(ATG) AGA AAT CTA AGC CCA CCT AAT-3' and 5'-AGT AAT GAG TCC AGT TTC GTT-3'),

and named g18BR1EX11F2 (total length, 42 bases) and g18BR1EX11R2 (39 bases), g(24)BR1EX11F2 (48 bases), and g(24)BR1EX11R2 (45 bases), and g30BR1EX11F2 (54 bases) and g30BR1EX11R2 (51 bases), respectively.

Nucleic Acid Extraction and cDNA Synthesis

Seven breast cancer cell lines (MCF-7, T-47-D, MDA-MB-435s, MDA-MB-231, ZR-75-1, MDA-MB-436, BT549, SKBr-3) and seven colon cancer cell lines (HCT116, HT29, HCT15/DLD1, HCC2998, KM12, SW480, COLO201) were used in this study. Cells were cultured in 10-cm dishes, and genomic DNA and total RNA were extracted with the use of DNAzol and Trizol reagents (Gibco-BRL) according to the manufacturer's instructions. Three colon cancer tissues and 10 cervical cancer tissues resected by the standard surgical procedure at the First Department of Surgery, Hokkaido University Hospital and the Gynecology Department, University of Indonesia, were snap-frozen in liquid nitrogen and served for nucleic acid extraction. For RNA, cDNA synthesis was done at 37°C for 60 minutes in a 20- μ l reaction mixture containing 1 μ g RNA, 1 $\times$ reverse transcriptase

(RT) buffer, 7.5 mmol/L dithiothreitol, 2.0 mmol/L MgCl₂, 0.5 mmol/L each dNTP, 10 ng/ μ l random pdN6 primer, and 10 U/ μ l Moloney murine leukemia virus reverse transcriptase (Gibco-BRL).

Nested PCR

Amplification of the target gene with addition of sequences for gap repair was done in a nested PCR procedure using *Pfu TURBO* polymerase (Stratagene, La Jolla, CA) on a Thermal Cycler 2400 (Perkin Elmer, Chiba, Japan). The first PCR was done on 100 ng of genomic DNA or 2 μ l of RT product (cDNA) in a 25- μ l reaction mixture containing 1 $\times$ cloned *Pfu* buffer, 0.05 U/ μ l *Pfu TURBO* polymerase, 0.2 mmol/L each dNTP, and 0.4 μ mol/L each external primer. PCR cycles consisted of a 40-second initial denaturation at 95°C (hot start), and then 10 cycles of denaturation at 95°C for 40 seconds, annealing at an indicated temperature (Table 1) for 40 seconds, and extension at 78°C for an extension time (2.0 minutes/kb target size); and after-extension at 78°C for 7 minutes. The second PCR was done with 2 μ l of the 100-fold diluted first PCR product and an internal primer pair, in 35 cycles at the annealing temperature indicated in Table 1. Satisfactory amplification was verified by electrophoresis in a 1% agarose gel and visualization by ethidium bromide staining under UV light.

Transformation of Yeast

The yeast strain yPH857 [MATa *ura3-53 lys2-801 ade2-101 his3- Δ 200 trp1- Δ 63 leu2- Δ 1 cyh2R*] was transformed with crude PCR product (1 to 5 μ l) and the linearized vector (50 ng) by a lithium acetate/heat-shock method as described previously.⁴ Yeast were then plated and grown at 30°C for 2 to 3 days on a synthetic medium CAu10 (SD; *ura*–; 0.67% yeast nitrogen base, 2% glucose, 1% casamino acids, 20 mg/L L-tryptophane, 10 mg/L adenine, and 2.5% agar). After color intensification by incubating the culture plate at 4°C, formed white and red colonies were counted. Assay results were expressed as percentages of red colonies in more than 200 colonies per plate.

Recovery of Plasmids and Sequencing

Yeast were digested for 1 hour with zymolyase 100T (Seikagaku-Kogyo, Tokyo, Japan) and plasmids were then recovered by an alkaline lysis procedure (QIAprep plasmid kit; Qiagen GmbH, Hilden, Germany) and introduced into XL-1blue *E. coli* cells by electroporation. The presence of a gap-repaired insert of the expected size was confirmed by enzyme digestion with *Sph*I and *Eco*RV. Plasmids were sequenced with a DyeDeoxy terminator kit (Perkin-Elmer) on an ABI 377 automated sequencer (Perkin Elmer/Applied Biosystems). Primers used for sequencing were VF-1, 5'-CTTCTATAGACACGCAAACAC-3' and VR-1, 5'-CAATCATACGTCCTCAATTGTC-3, which anneal the vector outside the gap, and primers specific to each gene.

Comparison with the Conventional Stop Codon Assay

The assay was performed for *APC* gene (codons 1113 to 1493) on genomic DNA extracted from three colon cancer cell lines (COLO201, DLD1, SW480) and three colon cancer tissues (I, H, M). The assay results were compared to those of the yeast color assay specific to *APC* gene (Furuuchi and colleagues⁵).

Assay for *hMSH6*

A part of *hMSH6* cDNA (codons 170 to 402) was amplified with nested PCR from RT products of five colon cancer cell lines (HCT15/DLD1, HCT116, HT29, HCC2998, and KM12) using primers shown in Table 1. yPH857 was transformed with each crude PCR product and linearized assay vector and cultured as described above.

Assay for *E-Cadherin*

The entire coding region of E-cadherin cDNA consists of 2649 bp (882 amino acids). We divided the region into three parts to amplify 804-, 732-, and 1209-bp fragments for respective codons 8 to 275, 231 to 474, and 464 to 866 in nested PCR procedures. The assay of each part was performed in 10 cases of cervical cancer. For the first part assay, adenine concentration was increased from 10 to 12.5 or 15 μ g/ml enough to supplement the partial ADE2 activity observed as pink colonies.

Results

Preventive Effect of *Sfo*I Digestion on Self-Circularization of the Vector

Yeast strain yPH857 was transformed with 50 ng of *Bam*HI-linearized pLS381 (original vector) or *Sfo*I-linearized pMT18 (new vector) in duplicate independent procedures, and resultant yeast colonies were counted for assessment of self-circularization of the vectors with cohesive versus smooth ends in yeast. *Bam*HI-linearized pLS381 yielded 77 and 72 colonies, whereas *Sfo*I-linearized pMT18 gave 1 and 0 colony only.

Fidelity of Gap-Repair

Nested PCR

In the initial stage of the present study, we observed incorporation of a primer dimer or PCR products with artificial mutations into the vector. To solve the problem, we introduced the following modifications into PCR amplification of gene fragments: 1) to avoid primer duplexing, which may result in formation of a short fragment flanked by recombinable sequences at the both ends, we designed primers to anneal each other with no more than 3 bases; and 2) to minimize base-misincorporation, we

changed polymerase from *Taq* to *Pfu TURBO*, which has a proofreading function. We also reduced the amplicon size to below 2.5 kb.

In some cases of PCR amplification, we had difficulty in obtaining a good amplification of GOI fragment, probably because of the addition of a long gap repair part to the primers. And in such cases, we often observed incorporation of nonspecific PCR product into the vector. We therefore used nested PCR, to maximize the efficiency and specificity of PCR amplification.

Applying these strategies, we amplified middle 1/3 (1.0 kb), upper 2/3 (2.2 kb), and lower 2/3 (2.2 kb) parts of *BRCA1* exon 11 with the primer sets shown in Table 1, and tested the assay. Consequently, we observed reduction in percentages of red colonies respectively to 4.0 to 10.7% ($n = 10$; mean, 8.1%), 3.8 to 7.3% ($n = 6$; mean, 5.5%), and 10.3 to 16.0% ($n = 6$; mean, 13.3%). Sequence analysis of the plasmids recovered from white colonies demonstrated that all of the plasmids were incorporated with right gene fragments.

Effects of Primer Length on Assay Background

Further to see whether length of sequences used in homologous recombination could affect the fidelity of recombination, we amplified middle 1/3 of *BRCA1* exon 11 using a primer set (gBEX11F2 and gBEX11R2) with additional 18-, 24-, 30-, or 39-base recombination sequences at the 5'-termini, from DNA of cell lines MCF-7 and ZR-75-1, and tested the products in the assay. For each cell line, percentages of the background red colonies were respectively 4.8%, 7.9% (18 bases); 9.8%, 7.2% (24 bases); 11.1%, 12.7% (30 bases); and 7.7%, 8.5% (39 bases).

Effects of Primer Length on Fidelity of Homologous Recombination

We then examined the presence of the insert in the recovered plasmids by *SphI* and *EcoRV* digestion. Expected sized inserts were confirmed in 20 of 20 white colonies for the 18-base recombination sequences (95% confidence interval 0.832 to 1.000), 40/40 for 24 bases (0.911 to 1.000), 20/20 for 30 bases (0.832 to 1.000), and 18/20 for 39 bases (0.683 to 0.988). Sequence of the plasmids with inserts verified precise recombination of the insert to the expected place without frameshift or mutation. From these data and the consistent data given by Hua and colleagues⁹ on the efficiency of homologous recombination in yeast, we determined to add 24-base sequences to the gene-specific part of internal primers for homologous recombination in our system thereafter.

Detection of Mutations

Assay for BRCA1 Exon 11 in Breast Cancer Cell Lines

Assays for *BRCA1* exon 11 in two parts were performed in seven breast cancer cell lines. Obtained percentages of red colonies for upper 2/3 (codons 224 to

967) and lower 2/3 parts (codons 629 to 1366) were, respectively, as follows: 5.5 and 5.9% (T-47-D); 5.4 and 10.4% (MDA-MB435s); 4.3 and 12.4% (MDA-MB-231); 5.0 and 15.4% (ZR-75-1); 6.2 and 11.6% (MDA-MB-436); 8.2 and 11.1% (BT549); and 8.2 and 18.1% (SKBr-3). Sequence analysis of the plasmids recovered from red colonies denied the presence of clonal mutation. These results showed that mutation was absent in this region of *BRCA1* gene in all of the samples tested, and that the false-positive levels (background red colonies) were <20% for 2.2-kb-long gene fragments (Figure 2a).

Detection of APC Mutation

Assay for *APC* exon 15 (codons 1113 to 1493) was performed on three colon cancer cell lines and three colon cancer tissues, and results were compared with the corresponding results previously determined by *APC* color assay.⁵ Results of the universal stop codon assay expressed in percentage of red colonies were: 8.9% for COLO201 (wild-type), 99.1% for HCT15/DLD1 (codon 1414 GGC>GG), 99.5% for SW480 (codon 1338 CAG>TAG), 44.6% for tumor sample I (codon 1450 CGA>TGA), 42.3% for tumor sample H (codon 1465 AGT>T, Figure 2b), and 44.6% for tumor sample M (codons 1309 to 1311 GAAAAGA>GA). These results were consistent with the results by Furuuchi and colleagues⁵ (respectively, 4.9%, 97.7%, 99.3%, 56.3%, 33.7%, and 32.7%).

Detection of hMSH6 Mutation

Assay for *hMSH6* (codons 170 to 402) was performed on five colon cancer cell lines. RNA was extracted from these cells, and subjected to RT-PCR amplification of cDNA using the external and internal primer sets shown in Table 1. The assay gave red colonies of 55.9% for HCT15, 8.9% for HCT116, 10.1% for HT29, 7.6% for HCC2998, and 9.8% for KM12. Sequence analysis of the recovered plasmids from red colonies for HCT15 revealed one base deletion of codon 289/299 (GGCCTG→GGCTG; Figure 2c).

Detection of E-Cadherin Mutation

Assay for E-cadherin gene was done by dividing the entire coding region of 2649 bp into overlapping three parts (first part, codons 8 to 275; second part, codons 231 to 474; third part, codons 464 to 866) as shown in Table 1. A total of 10 tumors of uterine cervix were subjected to the study. On testing the first part, wild-type cDNA gave pink colonies, making discrimination from red colonies difficult. Computer analysis of the protein secondary structure revealed a massive cluster of β -sheet structures in this region (data not shown), suggesting a stiff structure of the protein that might have interfered with ADE2 enzymatic activity by concealing the catalytic site. This problem was overcome by using a medium with an increased amount of adenine (12.5 or 15 μ g/ml) and a molecular spacer (FLAG sequence) between E-cadherin

Figure 2. Assay results. **a:** The background level of the assay. Samples without mutation gave red colonies of <20%. **b:** The result of an assay for *APC* gene exon 15 in colon cancer case H, showing 42% red colonies. Genomic DNA was used as the starting material. **c:** Detection of *hMSH6* mutation in HCT15/DLD1 colon cancer cell line, as 56% red colonies. mRNA was used as the starting material. **d:** Detection of mutation in the middle part (codon 231 to 474) of E-cadherin mRNA in a cervical cancer, as 52% red colonies.

cDNA and *ADE2* cDNA (pMT18flag). Of 10 cases of cervical cancer, two cases showed red colonies of >20% in the third part: 52.0% in case 2 and 65.8% in case 6 (Figure 2d). Sequence analysis of the plasmids recovered from red colonies demonstrated one base deletion (GATTTT→GATTT) at codon 552/553 in case 2 and 146-bp deletion (whole exon 11 skipping) in case 6.

Discussion

Homologous recombination is a useful property of yeast. It permits highly efficient cloning of DNA fragments into plasmid vectors^{10–13} as well as integration/targeting of a gene in yeast genome. Furthermore, it has been successfully used for mutation detection in combination with an appropriate reporter system.^{3–6,14–16} These studies used 100- to 200-bp-long homologous sequences for recombination. The present study demonstrated, however, that 18-bp sequences were sufficient for precise recombination. This enabled us to use short sequences added by PCR to insert any target gene fragments into a common yeast-expression vector *in vivo* in yeast. It dramatically increased applicability and efficiency of the yeast-based stop codon assay.

Because the present assay, namely “universal stop codon assay,” reports prevalence of mutant allele in a given sample as percentage of red colonies, false posi-

tivity (background red colonies) must be suppressed to a minimum. Considering the clonality of mutant-harboring tumor cells and contamination of normal cells in case of somatic mutation, and unbalanced expression of mutant allele because of nonsense-mediated RNA decay^{4,17} for both somatic and germline mutation, the background is required to be <20%. As homologous recombination into vector utilizes common sequences, the flanked part of amplified PCR product must be precise and specific to the tested gene, devoid of nonspecific amplification of untoward gene fragments with sequences homologous to the primer sequences. To achieve this, we used a nested PCR procedure. In addition, we designed primers free of duplex formation to avoid a primer dimer, which may be incorporated into the assay vector, as seen in the beginning of the study. We also used a proofreading enzyme, *Pfu* polymerase, to minimize base misincorporation.¹⁸ With these means, we successfully achieved low assay background levels in all of the instances of application.

The universal stop codon assay successfully detected mutations in *APC*, *hMSH6*, and E-cadherin genes. Quantitativeness of the assay was also satisfactory. As the assay takes only several days beginning with primer design and can process a large number of samples at once, it can serve as a high-throughput screening method of mutations in human genetic diseases. Although the spectrum of mutations ranges from cyto-

netically visible chromosomal arrangements to single-base alterations, the most prevalent mutation type in human genetic diseases is short-base deletion/insertion or base substitution leading to protein-truncation, accounting for >50%.⁸ As this assay itself provides an efficient cloning system of gene fragments spanning up to 2 kb, it facilitates sequence determination of missense mutations from yeast white colonies as well as nonsense mutations from red colonies.

In conclusion, the universal stop codon assay provides a powerful means of general utility for diagnosis of genetic diseases caused by germline mutation or somatic mutation of genes. This might bring to a solution, at least in part, for increasing demand of genetic diagnosis in the postgenomic era.

Acknowledgments

We thank Ms. M. Yanome for help in manuscript preparation and Ms. N. Furuuchi for technical help.

References

1. Grompe M: The rapid detection of unknown mutations in nucleic acids. *Nat Genet* 1993, 5:111–117
2. Kashiwazaki H, Tonoki H, Tada M, Chiba I, Shindoh M, Totsuka Y, Iggo R, Moriuchi T: High frequency of p53 mutations in human oral epithelial dysplasia and primary squamous cell carcinoma detected by yeast functional assay. *Oncogene* 1997, 15:2667–2674
3. Ishioka C, Suzuki T, FitzGerald M, Krainer M, Shimodaira H, Shimada A, Nomizu T, Isselbacher KJ, Haber D, Kanamaru R: Detection of heterozygous truncating mutations in the BRCA1 and APC genes by using a rapid screening assay in yeast. *Proc Natl Acad Sci USA* 1997, 94:2449–2453
4. Andreutti-Zaugg C, Scott RJ, Iggo R: Inhibition of nonsense-mediated messenger RNA decay in clinical samples facilitates detection of human MSH2 mutations with an in vivo fusion protein assay and conventional techniques. *Cancer Res* 1997, 57:3288–3293
5. Furuuchi K, Tada M, Yamada H, Kataoka A, Furuuchi N, Hamada J, Takahashi M, Todo S, Moriuchi T: Somatic mutations of the APC gene in primary breast cancers. *Am J Pathol* 2000, 156:1997–2005
6. Zhang C-L, Tada M, Kobayashi H, Nozaki M, Moriuchi T, Abe H: Detection of PTEN nonsense mutation and psiPTEN expression in central nervous system high-grade astrocytic tumors by a yeast-based stop codon assay. *Oncogene* 2000, 19:4346–4353
7. Weisman LS, Bacallao R, Wickner W: Multiple methods of visualizing the yeast vacuole permit evaluation of its morphology and inheritance during the cell cycle. *J Cell Biol* 1987, 105:1539–1547
8. McKusick VA: Mendelian inheritance in man. A Catalog of Human Genes and Genetic Disorders, ed 12, vols 1–3. Baltimore, The Johns Hopkins University Press, 1998
9. Hua SB, Qiu M, Chan E, Zhu L, Luo Y: Minimum length of sequence homology required for in vivo cloning by homologous recombination in yeast. *Plasmid* 1997, 38:91–96
10. Ma H, Kunes S, Schatz PJ, Botstein D: Plasmid construction by homologous recombination in yeast. *Gene* 1987, 58:201–216
11. Degryse E, Dumas B, Dietrich M, Laruelle L, Achstetter T: In vivo cloning by homologous recombination in yeast using a two-plasmid-based system. *Yeast* 1995, 11:629–640
12. Prado F, Aguilera A: New in-vivo cloning methods by homologous recombination in yeast. *Curr Genet* 1994, 25:180–183
13. Hudson Jr JR, Dawson EP, Rushing KL, Jackson CH, Lockshon D, Conover D, Lanciault C, Harris JR, Simmons SJ, Rothstein R, Fields S: The complete set of predicted genes from *Saccharomyces cerevisiae* in a readily usable form. *Genome Res* 1997, 7:1169–1173
14. Ishioka C, Frebourg T, Yan YX, Vidal M, Friend SH, Schmidt S, Iggo R: Screening patients for heterozygous p53 mutations using a functional assay in yeast. *Nat Genet* 1993, 5:124–129
15. Flaman J-M, Frebourg T, Moreau V, Charbonnier F, Martin C, Chapuis P, Sappino A-P, Limacher J-M, Brons L, Benhattar J, Tada M, Van Meir EG, Estreicher A, Iggo RD: A simple p53 functional assay for screening cell lines, blood, and tumors. *Proc Natl Acad Sci USA* 1995, 92:3963–3967
16. Balmelli-Gallacchi P, Schoumacher F, Liu JW, Eppenberger U, Mueller H, Picard D: A yeast-based bioassay for the determination of functional and non-functional estrogen receptors. *Nucleic Acids Res* 1999, 27:1875–1881
17. Maquat LE: When cells stop making sense: effects of nonsense codons on RNA metabolism in vertebrate cells. *RNA* 1995, 1:453–465
18. Flaman J-M, Frebourg T, Moreau V, Charbonnier F, Martin C, Ishioka C, Friend SH, Iggo R: A rapid PCR fidelity assay. *Nucleic Acid Res* 1994, 22:3259–3260