Technical Advance

Reconstructed β -Catenin/TCF4 Signaling in Yeast Applicable to Functional Evaluation of APC Mutations

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In human genetics and molecular oncology, mutation research is necessary not only to identify mutations in nucleic acid sequences, but also to analyze the loss of function caused by mutant proteins. We reconstructed a protein-protein network system of human β -catenin and TCF4, in *Saccharomyces cerevisiae*. β -Catenin and TCF4 proteins form a complex and transactivate reporter genes. Co-expressed wild-type APC with β -catenin and TCF4 inhibit the transcriptional activity of the β -catenin/TCF4 complex in yeast, as well as in mammals. This unique method in which the β -catenin/TCF4 signaling pathway is reconstructed *in vivo* may prove useful for the functional evaluation of APC mutants, including a type of APC truncated and missense mutants influenced to the ability of binding to β -catenin. (*Am J Pathol* 2003, 163:2201–2209)

APC, a tumor suppressor gene, was first identified as a causal gene in cases of familial adenomatous polyposis. Later, mutation of this gene was detected at a high rate not only in non-hereditary colorectal cancer, but also in other cancers originating from various tissues of the digestive tract, such as the esophagus, stomach, and pancreas. The mutation of the APC gene is now considered to be involved in the generation and progression of many cancers.^{1–8} Recently, it became clear that APC protein

might play a critical role in the decomposition of β -catenin through the formation of a complex of β -catenin with GSK3- β and AXIN1.^{9,10} Accordingly, impaired decomposition of β -catenin due to dysfunction of the APC gene results in accumulation of β -catenin in the cytoplasm, thus permitting its binding to TCF4, a transcription factor and its transport to the nucleus. This mechanism is considered to promote carcinogenesis, since the complex stimulates the transcription of various growth factors such as c-myc and cyclin D1.^{11,12} Therefore, identification of mutated APC gene seems to be important not only for the diagnosis of FAP, but also for the elucidation of the biological characteristics of cancer.

Protein-protein interactions *in vivo* should be further analyzed in the context of postgenomic research projects, since many proteins constitute a complicated network inside these cells, and such interactions are involved in the regulation of protein function. From this standpoint, the target of gene mutation research should not be limited to analysis of abnormal base sequences of DNA, but instead broadened to include those phenomena brought about by mutated proteins. Several reports have thus far been published on the technology relevant to such research; this technology enables the expression in yeast of mutated human proteins, makes possible the identification of their sites of mutation, and evaluates the functions of various proteins.^{13–24} These assays, analyses, and observations have all provided evidence that human proteins expressed in yeast are functionally reproduced as they exist in human cells.

We previously reported the development of an “APC Yeast Color Assay” using yeast cells, by which nonsense and frameshift mutations in full length cDNA of the APC could efficiently be detected. The usefulness and practicability of this assay was previously determined.²¹ However, under the present conditions, this assay was not able to analyze the function of missense and truncated

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mutant APC, which are considered to be associated with carcinogenesis.^{2,5,24–27} Here, we attempted to overcome the shortcomings of our previous technique and consequently succeeded in development of a functional diagnosis of APC. Our newly developed technique includes reconstruction of the transcriptional mechanism of the human β -catenin/TCF4 complex in yeast. Our present results are unique, as they reconstruct *in vivo* a human molecular system in yeast for the purpose of intracellular gene network research.

Materials and Methods

Plasmid Construction

pTOP-ADE reporter plasmid was constructed by substituting the TCF binding sequence (CCTTTGAAC) for the p53 binding sites in pLS210,²⁰ which was a gift of Dr. R. Iggo (Swiss Institute for Experimental Cancer Research (ISREC), Epalinges, Switzerland). The pFOP-ADE reporter vector was constructed by substituting the mutant TCF binding sequence (CCTTTGGCC) in pLS210. pTOP-LUC reporter plasmid was constructed as follows: the firefly luciferase open reading frame, cloned from pGL3 (Promega, Tokyo, Japan) was inserted into *Bam*HI site under the CYC1 minimal promoter in pTOP-ADE. The *Saccharomyces cerevisiae*/*Escherichia coli* shuttle expression vectors pSY3, pSY4, pSY5, pSY3dual, and pSY4dualHA were constructed as follows: pRS313, 314, and 315 were kindly supplied from Y. Takai (Osaka University, Suita, Japan). The ADH1 promoter was amplified by PCR from pLS76¹⁴ using a set of primers containing an *Eco*RI site or a *Bam*HI site at the 5' end. The *Eco*RI/*Bam*HI fragment was inserted into pRS314³⁴ to yield pRS314Ap. The CYC1 terminator with a *Bam*HI/*Sac*I linker was amplified by PCR and inserted into the *Bam*HI/*Sac*I site of pRS314Ap to construct pSY4. CYC1 promoter was amplified by PCR from pLS381¹⁶ using a set of primers containing an *Eco*RI site or *Bgl*II-*Eco*RI sites at the 5' end. The *Eco*RI fragment was inserted into pSY4 to construct pSY4Cp. CYC1 terminator with a *Bgl*II/*Apal* linker was amplified by PCR and cloned into the *Bgl*II/*Apal* site of pSY4Cp to construct pSY4dual. To construct pSY4dualHA, the fragment encoding the HA epitope synthesized by PCR was inserted into pSY4dual, which was digested with *Bam*HI. The fragment with *Sac*I and *Apal* sites containing ADH1 promoter and CYC1 terminator derived from pSY4 was inserted into the *Sac*I- and *Apal*-cut pRS313 and pRS315 to construct pSY3 and pSY5. The fragment with *Sac*I and *Apal* sites containing CYC1 promoter, CYC1 terminator, ADH1 promoter, and the CYC1 terminator derived from pSY4dual, was inserted into the *Sac*I/*Apal* site of pRS313 to produce pSY3dual. The human β -catenin cDNA with *Bam*HI linker was prepared by PCR and cloned into the *Bam*HI site of pBSSK to construct pBSSK-BC. pBSSK-BC was digested with *Bam*HI and the fragment encoding β -catenin (1–783) was inserted into the *Bam*HI-cut pSY3 to construct pSY3(BC). β -catenin (1–676) with the *Bam*HI site at the 5' end was synthesized by PCR. This fragment was digested with the

*Bam*HI site and inserted into the *Bam*HI-cut pSY3 to construct pSY3(BC Δ AD). To construct pSY3(BC Δ arm), the central portions of the inserted β -catenin cDNA fragments in pSY3(BC) between nt 343–423 were replaced by a *Eco*RI site by using a PCR mutagenesis method. To construct pBSSK-BCala, a PCR mutagenesis strategy was used to generate mutations. BCala contained substitutions of alanine for serine and threonine residues (Ser34, Ser37, Thr41, and Ser45) at the phosphorylation sites by GSK3- β . To construct pSY3(BCala), pBSSK-BCala was digested with *Bam*HI and the fragment encoding β -catenin (34/37/41/45A) was inserted into *Bam*HI-cut pSY3. The human TCF4 cDNA with *Bam*HI linker was prepared by PCR and cloned into the *Bam*HI site of pBSSK to construct pBSSK-T4. To construct pSY4(T4), pBSSK-T4 was digested with *Bam*HI and the fragment encoding TCF4 (1–597) was inserted into *Bam*HI-cut pSY4. To construct pSY4(T4 Δ HMG), TCF4 (1–311) with the *Bam*HI site at the 5' end, synthesized by PCR, was digested with *Bam*HI and was inserted into *Bam*HI-cut pSY4. To construct pSY4(T4 Δ β BD), TCF4 (73–597) with the *Bam*HI site at the 5' end, synthesized by PCR, was digested with *Bam*HI and was inserted into *Bam*HI-cut pSY4. To construct pSY3dual(T4), TCF4 (1–597) digested with *Bam*HI was inserted into *Bgl*II-cut pSY3dual. To construct pSY3dual(BC+T4), which co-expressed β -catenin and TCF4, β -catenin (1–783) digested with *Bam*HI was inserted into *Bam*HI-cut pSY3dual(T4). The human AXIN1 cDNA with *Bgl*II was amplified and inserted into *Bam*HI-cut pSY4dualHA, to construct pSY4dualHA(AX). To construct pSYdualHA(AX+G3), which co-expressed AXIN1 and GSK3- β , the human GSK3- β cDNA with the *Bgl*II site at the 5' end, synthesized by PCR, was digested with *Bgl*II and inserted into the *Bgl*II-cut pSY4dualHA(AX). Renilla reniformis-luciferase (RLuc) cDNA with *Hind*III and *Eag*I linker was prepared by PCR and cloned into the *Hind*III/*Eag*I site of pLS76 to construct pMT31. A *Bgl*II site was engineered between LEU2 and CEN/ARS of pLS76 by using a PCR mutagenesis strategy. RLuc+CYC1 terminator with the *Bgl*II site at the 5' end, synthesized by PCR, was digested with *Bgl*II and was inserted into the *Bgl*II-cut pMT31 to construct pMT33. The fragment with *Sac*I and *Apal* sites containing ADH1 promoter and CYC1 terminator derived from pSY5 was inserted into the *Sac*I- and *Apal*-cut pMT33 to construct pSY5R. pBSKS-APC was kindly supplied from Dr. Y. Nakamura (Tokyo University, Tokyo, Japan). To construct pSY5(AP) and pSY5R(AP), the fragment encoding APC cDNA (1–2348) with *Bam*HI linker, synthesized by PCR, was ligated into the *Bam*HI-cut pSY5 and pSY5R. To construct pBSKS-AP Δ BD, pBSKS-APC was digested with *Bgl*II and was self-ligated. The fragment encoding APC (1–1011, 2144–2348) with the *Bam*HI site at the 5' end, synthesized by PCR, was digested with *Bam*HI and was inserted into the *Bam*HI-cut pSY5R to construct pSY5R(AP Δ BD). The gap repair vectors, pSY5R(AP Δ NES), pSY5R(gAPII), pSY5R(gAPIII), and pSY5R(gAPIV), were identical to pSY5R(AP), except that the central portions of the inserted APC cDNA fragments between amino acids 21–448, 449–1011, 1012–1516, and 1517–2143 were replaced by a *Sac*II site by

using a PCR mutagenesis method. Using a PCR mutagenesis strategy to construct missense mutant APC (R414C, G1120E, I1307K, E1317Q, S1395C) expression vector pSY5 (APR414C), pSY5 (APG1120E), pSY5(I1307K), pSY5(E1317Q), and pSY5(S1395C) generated mutations.

RT-PCR

Extraction of mRNA from cell lines or tumor tissues and first-strand cDNA synthesis has previously been described.²¹ All mutant APC fragments were amplified from cDNA by using Pfu polymerase (Stratagene, La Jolla, CA). The primers of amplification of three APC cDNA fragments and the PCR parameters were previously reported.

Yeast Strain

Yeast strain YPH499 was used in this study as the parent strain, and was kindly supplied by Dr. Y. Takai and K. Tanaka (genotype: MATa, lys2-801amber, ade2-101orcher, leu2 Δ 1, trp1 Δ 63, his3 Δ 200, ura3-52). The TBE (TCF-binding element)-ADE2, mutant TBE-ADE2, TBE-LUCIFERASE constructs pTOP-ADE, pFOP-ADE, and pTOP-LUC were linearized with *Apal* within the URA3 gene and integrated into the ura3 locus of YPH499 to yield strains yTOP-ADE, yFOP-ADE, and yTOP-LUC.

Yeast Transformation

Yeast was cultured in YPD medium supplemented with an excessive amount of adenine (200 μ g/ml) until OD600 nm reached 0.8. Then the yeast was washed in LiOAc solution containing 0.1 mol/L lithium acetate, 10 mmol/L Tris-HCl (pH 8.0), and 1 mmol/L Na₂-EDTA; samples were pelleted again, and 50 μ l yeast suspensions were mixed with 100 ng of APC, β -catenin, TCF4 expression vector or linearized gap vector and PCR product, 50 ng of sonicated single-stranded salmon sperm DNA, and 300 μ l of LiOAc containing 40% polyethylene glycol 4000. The mixture was incubated at 30°C for 30 minutes and heat-shocked at 42°C for 15 minutes. Yeast samples were then plated on synthetic dropout medium minus histidine, triptophane, leucine, and uracil, but containing a limited amount of adenine (5 μ g/ml) and incubated for 48 hours in a 30°C humidified atmosphere. The yeast cells formed red colonies because of the accumulation of an intermediate in adenine metabolism under a limited amount of adenine.³⁵

Immunoblotting

Yeast colonies were inoculated to YPD medium supplemented with a sufficient amount of adenine (200 μ g/ml) and were cultured for 18 hours. The yeast samples were pelleted by centrifugation and resuspended in yeast lysis buffer (Tris-HCl, pH7.5, 1 mmol/L KOH, 0.1% Triton-X) supplemented with protease inhibitors (10 mg/ml leupep-

tin, 1 mg/ml pepstatin A, 10 mg/ml aprotinin, and 5 mg/ml phenylmethylsulfonyl fluoride). The yeast whole-cell lysate was extracted by the glass beads method according to the standard protocol. Yeast lysates were electrophoresed by 4% to 7.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane, which was then incubated with anti- β -catenin (Transduction Laboratories, Lexington, KY), anti-TCF4 (Upstate Biotechnology, Lake Placid, NY), anti-APC (Calbiochem, San Diego, CA), anti-HA (Invitrogen, Carlsbad, CA), anti-GSK3- β (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with horseradish peroxidase-conjugated antibody to mouse IgG (Amersham Biosciences, Buckinghamshire, UK). The blots were developed with an enhanced chemiluminescence kit (Amersham Biosciences).

Detection of Luciferase Activity in Yeast

yTOP-LUC and yFOP-LUC, transiently transfected with expression vectors, were lysed in yeast lysis buffer (25 mmol/L Tris-HCl, pH7.4, 2.5 mmol/L Na₂-EDTA, pH7.4, 0.5% Triton-X, 36 mmol/L sodium deoxycholate, and 1X PBS pH 7.4) supplemented with protease inhibitors (10 mg/ml leupeptin, 1 mg/ml pepstatin A, 10 mg/ml aprotinin, and 5 mg/ml phenylmethylsulfonyl fluoride). The yeast whole cell lysate was extracted by the glass beads method. Yeast cell lysate (20 μ g) was measured for firefly luciferase activity/renilla luciferase activity (RLU) using Mini Lumimat LB950L (Berthold Technologies, Wildbad, Germany), according to a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) protocol.

DNA Sequencing

DNA sequencing of each construct was performed using a dye-terminator sequencing kit on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA).

Results

Expression and Reconstruction of Transcription Mediated by the β -Catenin and TCF4 Systems in Yeast

We examined the expression of normal human β -catenin and TCF4 proteins in yeast and confirmed the generation of these proteins by SDS-PAGE (Figure 1, A and B). Then, we examined the expression of β -catenin or TCF4 protein alone in cells of a yeast strain, yTOP-ADE, to which ADE2, a reporter gene (with CTTTGAAC in the upstream activation sequence, a DNA-binding site needed for transcription mediated by the β -catenin/TCF4 complex), was introduced. Consequently, the yeast cells formed red colonies when included with the ADE2 reporter gene in combination with either the β -catenin or TCF4 gene; under these conditions, the protein complex needed for transcription could not be constituted (the appearance rate of red colonies was 99.333 ± 0.577 SD %). On the

Figure 1. Determination of the direct interaction of β -catenin and TCF4 in yeast. **A:** Schematic presentation of β -catenin and TCF4 mutants. SSTS in β -catenin indicates phosphorylation sites (34S, 37S, 41T, 45S) and AD indicates activation domain. AAAA indicates the point mutation of S34A, S37A, T41A, and S45A. β BD is the β -catenin binding domain, and HMG (high-mobility group box) is the DNA binding domain. **B:** Detection of β -catenin and TCF4 proteins in yeast by immunoblot analysis. Lane 1, wild-type β -catenin; lanes 2 to 4, β -catenin mutants; lane 5, wild-type TCF4; lanes 6 and 7, TCF4 mutants. **C:** β -catenin and TCF4 transactivation for specific DNA sequence. yTOP-ADE with the true binding-site formed white colonies by β -catenin and TCF4 co-expression. In contrast, yFOP-ADE with the false binding-site formed red colonies, which indicated no transactivation by the β -catenin/TCF4 complex. **D:** Yeast transformed with dual expression vector encoding β -catenin and TCF4, and the different mutants represented, were seeded in minimal adenine plates.

other hand, the yeast cells formed white colonies when included with the reporter gene in combination with both the β -catenin and TCF4 genes, a condition under which the complex formation of the β -catenin and TCF4 proteins needed for the transcription could be expected to be constituted (the appearance rate of red colonies was $3.218 \pm 1.088\%$).

Next, we examined the effects of mutation of the DNA-binding sequence on transcription to verify that the DNA-binding sequence was specific for β -catenin/TCF4 complex-mediated transcription. It was shown that yeast cells of yTOP-ADE, with the co-expression of β -catenin and TCF4 proteins, formed white colonies, indicating that ADE2 gene with CTTTGAAC, a normal DNA-binding sequence, was reactive to the β -catenin/TCF4 protein complex-mediated transcription. In contrast, the cells with

CTTTGGCC, a mutated DNA-binding sequence, formed red colonies, indicating that it was not reactive in response to the complex-mediated transcription. These findings revealed that the β -catenin and TCF4 protein complex was specifically bound to the CTTTGAAC sequence of the reporter gene to mediate transcription (Figure 1C).

To confirm further that β -catenin directly interacted with TCF4 in yeast, each of the β -catenin or TCF4 gene mutants was co-expressed with the wild-type of the counterpart gene. The combinations were as follows: 1) BC Δ AD (the mutant in which the carboxyl terminus of β -catenin, including its transcription-activation ability, was removed) and wild-type TCF4; 2) BC Δ arm (the mutant in which arm repeats number 5 and number 6 of β -catenin were removed) and wild-type TCF4; 3) wild-

type β -catenin and T4 Δ HMG (the mutant in which the DNA-binding site of TCF4 was removed); and 4) wild-type β -catenin and T4 Δ β BD (the mutant in which the β -catenin binding domain of TCF4 was removed).

Consequently, all of the yeast cells formed red or pink colonies, showing decreased ADE2 reporter activity. In contrast, the yeast with the two combinations of wild-type β -catenin and wild-type TCF4, and BCal α (the mutant in which the serine and threonine residues of β -catenin, corresponding to codons 34, 37, 41, and 45 of the β -catenin gene were replaced by the alanine residue) and wild-type TCF4 formed white colonies. These findings provide evidence that in yeast cells, β -catenin and TCF4 interacted directly to form a complex; it was also suggested that the resulting interactions induced the transcription of the reporter (Figure 1D).

Introduction of Negative Regulators for β -Catenin and TCF4 Complex

We examined whether APC inhibited the transcriptional activity of the β -catenin/TCF4 complex. For this purpose, negative regulators, APC, AXIN1, and GSK3- β , were expressed in combination of both β -catenin and TCF4 (Figure 2A). The expressions of several type of APC, wild-type AXIN1, and wild-type GSK3- β in yeast were confirmed by SDS-PAGE (Figure 2B). The expressions of wild-type APC, AP Δ β BD, and AP Δ NES in yeast were confirmed by SDS-PAGE (Figure 2, A and B). When wild-type APC was co-expressed, wild-type APC permitted the color of the yeast colonies to become red (Figure 2C). Next, the co-expression of AP Δ β BD was examined (a mutant in which 15 and 20 amino acid repeats located in the center third of APC were removed) with a combination of β -catenin and TCF4. In this case, the yeast cells formed white colonies, indicating transcription mediation by the β -catenin/TCF4 complex. However, the coexpression of AP Δ NES (the mutant in which LxxLxL, which is located in the amino terminal of APC and acts as a transfer signal to the extranuclear environment,²⁹ was removed) in combination with β -catenin and TCF4, allowed the yeast cells to form red colonies like those observed with the wild-type APC. When AXIN1 and GSK3- β were co-expressed, yeast cells formed white-colored colonies. These two negative regulators did not work for β -catenin/TCF4 complex. These findings demonstrate that the wild-type APC inhibited the transcriptional activity of the β -catenin/TCF4 complex in yeast as well as in mammalian cells.

Moreover, a dual luciferase assay was carried out to confirm the results of the color change assay using yeast colonies, generated by ADE2 reporter activity, which was the result of binding to the β -catenin/TCF4 complex (Figure 2C). Quantitative determination of the transcriptional activity of the β -catenin/TCF4 complex was carried out. In this study, we introduced firefly luciferase to a yeast strain, yTOP-LUC, including a reporter plasmid with reactivity to the β -catenin/TCF4 complex. Renilla luciferase was used as an inner control. When an empty vector including renilla luciferase alone was introduced, the

mean relative luciferase activity was 0.335. When wild-type APC was co-expressed in combination with β -catenin and TCF4, the mean activity was 4.763. This value was lower than that when a combination of β -catenin and TCF4 was expressed without wild-type APC (10.897). These findings indicate that wild-type APC strongly inhibited the transcription mediated by the β -catenin/TCF4 complex. The co-expression of AP Δ β BD in combination with β -catenin and TCF4 showed transcriptional activity as high (9.867) as that observed with combined expression of β -catenin and TCF4, but in the absence of APC. This indicates that AP Δ β BD, lacking the central β -catenin-binding site, entirely lost its transcription-inhibitory activity. The co-expression of AP Δ NES showed low transcriptional activity (2.433) as that observed with the co-expression of wild-type APC. This result indicates that AP Δ NES has the ability to regulate the β -catenin and TCF4 transactivation. The co-expression of AXIN1 and GSK3- β in combination with β -catenin and TCF4 showed transcriptional activity as high (13.900), but in the existence of APC the relative transcriptional activity was regulated (4.520). These findings suggest that the luciferase activity coincides well with the color change from red to white in the yeast colonies, and the transcriptional activity can be evaluated simply based on the observation of the color change among the yeast colonies.

Co-Expression of β -Catenin, TCF4, and APC and Their Applications to Functional Diagnosis

To verify that our colony color assay can be applied to evaluate the transcriptional activation, we carried out further investigations of the effects on colony colorization after introduction of the known APC mutants, in combination with β -catenin and TCF4, to yeast cells. When each of the deficient-type mutants of APC (811:TCA to TGA, 853:GAG to TAG, 1338:CAG to TAG, and 1414:GGA to GG; derived from the cell lines Colo320DM, HT29, SW480, and DLD1, respectively), was expressed in place of wild-type APC, the yeast cells formed white colonies. When each of the deficient-type mutants of APC (1114:CGA to TGA, 1545:TCA to TAA, and 1554:GAA to GAAA; derived from colorectal cancer tissue in previous analysis⁷) was introduced, all yeast cells formed white colonies, indicating that transcriptional activation of the ADE2 reporter gene mediated by the β -catenin/TCF4 complex was induced.

When missense mutants of APC R414C, G1120E, I1307K, E1317Q, and S1395C registered in Online Mendelian Inheritance in Man (OMIM: <http://www.ncbi.nlm.nih.gov/Omim/>) were introduced into the yeast cells, all of the yeast cells formed red colonies. This result indicated that the transcriptional activation of the ADE2 reporter, under normal conditions mediated by the β -catenin/TCF4 complex, was inhibited. The expression of mutant APC in yeast cells was detected by SDS-PAGE (Figure 3, A to C). These findings revealed that mutant APC of the deficient type, which accounts for 90% of the known APC mutations, lost its inhibitory activity on the transcription mediated by the β -catenin/TCF4 complex.

Figure 2. Negative regulators, APC, GSK3- β , and AXIN1 cotransformed in yeast with β -catenin and TCF4. **A:** Structure of APC mutants, wild-type GSK3- β , and wild-type AXIN1. In APC, **gray bars** indicate nuclear export signals,²⁹ and **black bars** indicate 15 and 20 amino acid repeats which bind β -catenin. In AXIN1 RGS, G3BD, and β BD indicate APC, GSK3- β , and β -catenin binding domain. **B:** Detection of APC, GSK3- β , and AXIN1 protein in yeast by immunoblot analysis. Lane 1, wild-type APC; lanes 2 and 3, APC mutants; lane 4, wild-type GSK3- β ; lane 5, wild-type AXIN1. **C:** Yeast transformed with dual expression vector encoding β -catenin and TCF4 and expression vectors encoding the several negative regulators of APC, AXIN1, and GSK3- β represented, were seeded in minimal adenine plates. Transcriptional activities of luciferase reporter were measured by co-expression of β -catenin, TCF4, APC, AXIN1, and GSK3- β in γ TOP-LUC yeast strains. The results are the averages of triplicate determinations.

However, it is of note that the mutant missense-type APC registered in OMIM maintained inhibitory activity on the transcription mediated by the β -catenin/TCF4 complex, as did wild-type APC.

Discussion

In the present study, we showed in yeast cultures that β -catenin interacted with TCF4 and that binding of this complex to the specific DNA sequence initiated the transcription. We also reproduced *in vivo* APC inhibition of this transcription. Based on these findings, we demon-

strated that a complicated human protein network could be reconstructed in yeast cells (Figure 4).

The major difference between this method and the previous method²⁸ reside in the use of a different reporter gene (color change *versus* ability to grow on medium lacking histidine). The *ADE2* reporter gene is used for several yeast assays because of sensitivity and convenience for the genetic assay.^{19-21,23} In this study, when BC Δ AD and wild-type TCF4 were co-expressed, the color of yeast colony appeared pink. This result supports that β -catenin has two transactivation domains in the N terminus and C terminus, and BC Δ AD have a weak tran-

Figure 3. Analysis of the transactivation of β -catenin and TCF4 influenced by APC mutations. **A:** Yeast cotransformed with dual expression vector encoding β -catenin and TCF4, and expression vector encoding the several APC mutations represented were seeded in minimal adenine plates. **B:** Detection of APC mutant protein in yeast by immunoblot analysis. Lane 1: wild-type APC; lanes 2–6: missense mutation APC. **C:** Lanes 7 to 13: truncated mutation APC.

script activity because BC Δ AD have N-terminal transactivation domain. It seems to be difficult that the yeast colony growth assay detect the moderate changes of reporter gene activity. Furthermore, our assay does not require a replica-plating step and can easily distinguish yeast containing mutant APC as red colonies. Moreover, mutant APC plasmid can be easily recovered from red colonies and subjected to DNA sequence analysis. ADE2 reporter assay is able to screen the inhibitor of transac-

tivation by yeast colony color change. Medium lacking assay is unable to screen because yeast colony is not grown by co-expression of inhibitor clone and expression plasmids of the inhibitors cannot be recovered.

We showed that even in yeast cells, human β -catenin, TCF4, and APC proteins functioned just as they do in human cells, as previously reported in the case of p53, MLH1, and ER proteins. Furthermore, the yeast system proved most suitable for the functional diagnosis of APC when β -catenin and TCF4 were co-expressed. This assay system is unique in that the β -catenin and TCF4 proteins are reproduced, intact with their original functional forms, as in human cells: β -catenin with the transcription-activating domain and TCF4 with the DNA-binding domain interact with each other to form a complex and mediate the transcription of the reporter as its native form, unlike as GAL4 protein in its hybrid form.

It has been assumed that the central third of the amino acid repeats located in the center of APC bound to β -catenin is the most important site for inhibiting transcription mediated by the β -catenin/TCF4 complex.³⁰ In support of this assumption, we demonstrated with our yeast assay system that AP Δ BBDD, a mutant lacking this portion of APC, did not inhibit transcription mediated by the β -catenin/TCF4 complex. This finding indicates the fidelity of the function of the three human proteins included in this novel yeast assay system.

Figure 4. The schematic diagram of β -catenin, TCF4, and APC-mediated transcription in yeast. RT-PCR amplified fragments derived from cells or tissues were introduced with the linearized gap vector and a dual expression vector encoding β -catenin and TCF4 into yeast strain YTOP-ADE. The yeast containing cDNA fragments of wild-type APC or mutant-type APC formed white or red colonies.

We also showed that the expression of AP Δ NES, a mutant lacking the nuclear export signal of APC, did not inhibit transcription mediated by this complex. This result, inconsistent with that of a previous report, supports the reproduction of the *in vivo* function in our assay system.³¹ However, as regards the nuclear export signal, as well as the nuclear localization signal, further analyses will be needed that include investigation of the localization of the signal and the effects of introduction of mutants.

Many yeast assay systems have thus far been developed as a means of genetic diagnosis. The characteristic high ability of homologous recombination of yeast is used in all of these assay systems. In these systems, the PCR fragments are used as a template of the target gene, and gap vectors are introduced into yeast to initiate the homologous recombination. The mutated sequence included in the fragment induces the expression of the mutated protein, and thus permits the development of simple and quick diagnosis of gene mutation.^{32,33} However, it is still not able to detect APC missense mutations or mutations affecting nuclear export sequences by these yeast assay systems. The missense mutations were detected from cancer tissues. It is not reported that these missense APC mutants lose the function of inhibiting the β -catenin/TCF4 transactivation or other functions. We cleared by using our new assay that these APC mutations have direct β -catenin/TCF4 transcript activity, which is the same as wild-type APC. Although no natural pathogenic missense mutation is available, this assay can test an artificially constructed missense mutation that disrupts APC and β -catenin interaction. This yeast system potentially has the ability to detect APC pathogenic missense mutations.

Our new yeast assay has only the ability to evaluate the interaction of the APC to β -catenin. The effect of APC missense mutations may not relate directly or solely to β -catenin binding and may require the full components of the Wnt pathway to exhibit their effects. We showed that AXIN1 or GSK3- β expressed alone did not inhibit transcription mediated by the β -catenin/TCF4 complex. These findings suggest that the pathways of ubiquitination, phosphorylation, and decomposition of β -catenin are regulated in a more complicated manner. Combined with the fact that GSK3- β and AXIN1 do not work in the yeast, this yeast assay was not reconstructed completely as in mammalian cells. Therefore, we mentioned that this APC functional assay has the limits to detect all types of APC mutations.

In the present assay, the regulation of proteins (eg, phosphorylation, ubiquitination, and decomposition), and the regulation of genes (eg, loss of heterozygosity and methylation of the promoter) were not completely reconstructed. However, this assay did enable the simple evaluation of the function of the β -catenin/TCF4 complex. This system is expected to pave the way for the following developments:¹ Diagnosis of more functional changes in APC than the presently recognized changes,² screening for target genes by preparing the reporter gene, including DNA fragments,³ screening for new proteins related to the transcription mediated by the β -catenin/TCF4 com-

plex,⁴ and comparison of the functions of individual protein homologs (eg, APCL, γ -catenin, LEF1, TCF3, etc).

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