RESEARCH COMMUNICATION

Fibroblast growth factor-1 stimulation of quiescent NIH 3T3 cells increases G/T mismatch-binding protein expression

Patrick J. DONOHUE*‡, Sheau-Line Y. FENG*, Gregory F. ALBERTS*, Yan GUO*, Kimberly A. PEIFLEY*, Debbie K. W. HSU* and Jeffrey A. WINKLES*†§

*Department of Molecular Biology, Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855, and †Department of Biochemistry and Molecular Biology, George Washington University Medical Center, Washington, DC 20037, U.S.A.

Polypeptide growth factors promote cell-cycle progression in part by the transcriptional activation of a diverse group of specific genes. We have used an mRNA differential-display approach to identify several fibroblast growth factor (FGF)-1 (acidic FGF)-inducible genes in NIH 3T3 cells. Here we report that one of these genes, called FGF-regulated (FR)-3, is predicted to encode G/T mismatch-binding protein (GTBP), a component of the mammalian DNA mismatch correction system. The murine GTBP gene is transiently expressed after FGF-1 or calf serum treatment, with maximal mRNA levels detected at 12 and 18 h post-stimulation. FGF-1-stimulated NIH 3T3 cells also express an increased amount of GTBP as determined by immunoblot analysis. These results indicate that elevated levels of GTBP may be required during the DNA synthesis phase of the cell cycle for efficient G/T mismatch recognition and repair.

INTRODUCTION

Polypeptide growth factors exert their biological effects by binding to specific high-affinity cell-surface receptors. The binding of ligand to receptor initiates a complex series of cytoplasmic events that ultimately promote the transcriptional activation of numerous cellular genes [1,2]. The genes activated during mammalian cell-cycle progression are generally classified as either immediate-early-response (e.g. c-*fos*), delayed-early-response (e.g. proliferin) or late-response (e.g. histone H3) genes. Several of these growth-factor-regulated genes have been shown to encode proteins necessary for cellular proliferation [3–5].

Fibroblast growth factor (FGF)-1 (acidic FGF), a member of a family of structurally related polypeptides, is a multifunctional protein that can stimulate cellular proliferation, migration and differentiation *in vitro* and promote blood-vessel formation and wound repair *in vivo* [6–8]. These biological activities are mediated via binding to a family of protein tyrosine kinase cell-surface receptors [6,9]. FGF-receptor autophosphorylation initiates a signal-transduction cascade characterized by protein kinase activation and gene induction. We have used an mRNA differential-display approach to identify genes that are activated after FGF-1 stimulation of quiescent NIH 3T3 fibroblasts [10]. Genes identified to date using this strategy include those coding for enzymes in metabolic pathways [11,12], a serine/threonine protein kinase [13], a cytoskeletal protein [14] and a DNA-binding transcription factor [15]. Here, we report that the FGF-regulated (FR)-3 gene is the murine homologue of the human G/T mismatch-binding protein (GTBP) gene recently described by Palombo et al. [16] and Nicolaides et al. [17]. Indeed, the deduced FR-3 amino acid sequence has 100% identity with a murine GTBP sequence submitted to the GenBank database by Corradi et al. (accession number U42190). GTBP is an ~ 160 kDa polypeptide that is structurally related to the Mut S homologue (MSH) family of DNA mismatch repair proteins (reviewed in [18,19]). GTBP exists as a heterodimer with MSH2, and together this complex can bind to G/T mismatches [16,20].

MATERIALS AND METHODS

Cell culture

Murine NIH 3T3 cells (American Type Culture Collection) were grown at 37 °C in Dulbecco's modified Eagle's medium (Mediatech) supplemented with 10% (v/v) heat-inactivated bovine calf serum (Hyclone Laboratories) and a 1:100 dilution of a 10^4 units/ml penicillin/10 mg/ml streptomycin/25 μg/ml amphotericin solution (JRH Biosciences). The cells were expanded by trypsin/EDTA (JRH Biosciences) treatment and subculturing at a split ratio of 1:7 every 2–3 days. To induce a relatively quiescent cell population, subconfluent cells were incubated for ~ 72 h in the above medium containing a reduced serum concentration (0.5%). Cells were then left either untreated or treated for various times with either 10 ng/ml recombinant human FGF-1 (kind gift of W. Burgess, American Red Cross)/5 units/ml heparin (Upjohn) or 10% calf serum.

RNA isolation

Cells were harvested by trypsin/EDTA treatment, and total RNA was isolated using RNA Stat-60 (Tel-Test, Inc.) according

Abbreviations used: FGF, fibroblast growth factor; FR, FGF-1-regulated; GTBP, G/T mismatch-binding protein; MSH, Mut S homologue.

§ To whom correspondence should be addressed.

‡ Present address: Laboratory of Neurochemistry, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Rockville, MD 20850, U.S.A.

The nucleotide sequences reported in this paper have been deposited in GenBank (accession numbers U61388 and U61389).

to the manufacturer's instructions. RNA concentrations were calculated by measuring UV light absorbance at 260 nm.

Differential display

RNA was isolated from quiescent or FGF-1-stimulated cells, and equivalent amounts (1 μg) were converted into cDNA using random hexamer primers (Boehringer Mannheim) as described previously [13]. PCR assays were then performed using the same conditions we described previously [13]. The degenerate sense protein-kinase-domain primer and the degenerate antisense zinc-finger-domain primer have been described elsewhere [11,13]. An equivalent aliquot of each amplification mixture was subjected to electrophoresis in a 1.8 % agarose gel. ΦX174/*Hae*III restriction fragments (Clontech Laboratories) were used as size standards. DNA was detected by ethidium bromide staining. In this differential-display experiment, 2 cDNA fragments ($\sim$ 200 and $\sim$ 700 bp in size) were isolated and cloned as described previously [13]. The shorter cDNA, encoding a putative protein kinase called Fnk, was described previously [13]. The longer cDNA, termed FR-3, is the subject of this report.

cDNA library screening

A mouse brain λgt10 cDNA library (Clontech Laboratories) was screened with the subcloned PCR-derived FR-3 cDNA fragment to obtain a longer cDNA clone as described previously [13]. One positive phage was plaque purified, and the cDNA insert was released from the λgt10 vector as three fragments after *Eco*RI digestion. Each restriction fragment was subcloned into the plasmid vector pGEM3Zf+ (Promega Corp.).

cDNA sequence analysis

Plasmid DNA was purified using a Magic Miniprep Kit (Promega Corp.), and both strands of the 5′ and 3′ ends of each cDNA restriction fragment were sequenced by the dideoxynucleotide chain termination method either automatically using an Applied Biosystems Model 373A DNA sequencer or manually using a Sequenase 2.0 kit (U.S. Biochemical Corp.). The nucleic acid and deduced protein sequences were compared with sequences in the database server at the National Center for Biotechnology, using the Blast network service [21]. Protein sequences were aligned using the University of Wisconsin GCG package and were displayed using Microsoft Excel 5.0a.

RNA gel-blot hybridization

Each RNA sample (10 μg) was denatured and subjected to electrophoresis in 1.2 % agarose gels containing 2.2 M formaldehyde. The gels were stained with ethidium bromide to verify that each lane contained similar amounts of undegraded rRNA. RNA was electroblotted on to Zetabind nylon membranes (Cuno Inc.) and was cross-linked by UV irradiation using a Stratalinker (Stratagene). The $\sim$ 2.3 kb cDNA restriction fragment was radiolabelled as described above for cDNA library screening. Membrane hybridization and washing conditions were as described previously [15].

Immunoblot analysis

Cellular lysates were prepared as described previously [14], and the amount of protein in each clarified lysate was determined using the bicinchoninic acid protein-assay reagent (Pierce Chemical Co.). Equivalent amounts of each protein sample (50 μg) were mixed with 2 × sample buffer [125 mM Tris/HCl, pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol and 10 % (v/v) 2-mercaptoethanol], heated at 95 °C for 4 min, and subjected to electrophoresis in a 7.5 % polyacrylamide/SDS slab gel. Prestained high-molecular-mass protein standards (Bio-Rad Laboratories) and 10 μg of HeLa cell nuclear extract (Santa Cruz Biotechnology, Inc.) were also included on the gel. Proteins were transferred on to a nitrocellulose membrane (Schleicher & Schuell) by electroblotting. The membrane was stained with Ponceau S (Sigma) to verify that equivalent amounts of cellular protein were present in each gel lane and then incubated at 4 °C for 18 h in TBST/milk [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5 % (v/v) Tween-20 and 5 % (w/v) non-fat dry milk]. The membrane was then incubated at room temperature for 1 h in TBST/milk containing a 1:1000 dilution of anti-GTBP serum (kind gift of J. Jiricny, Istituto di Ricerche di Biologia Molecolare P. Angeletti, Pomezia, Italy), washed several times with TBST/milk, and then incubated at room temperature for 1 h in TBST/milk containing a 1:1500 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (U.S. Biochemical Corp.). The membrane was washed several times with TBST/milk and then with AP buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$). Immunoreactive proteins were detected after the addition of fresh AP buffer containing 0.33 mg/ml Nitro Blue Tetrazolium (Promega Corp.) and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Promega Corp.).

RESULTS AND DISCUSSION

Identification of an FGF-1-inducible gene by mRNA differential display

RNA isolated from quiescent or FGF-1-stimulated NIH 3T3 cells was converted into cDNA using reverse transcriptase and random-hexamer primers. PCR assays were then performed using degenerate zinc-finger- and protein-kinase-domain oligonucleotide primers. Amplification products were displayed using agarose-gel electrophoresis and ethidium bromide staining. The pattern of amplified cDNAs obtained from quiescent or FGF-1-stimulated cellular RNA were, for the most part, similar (Figure 1). However, an $\sim$ 700 bp DNA fragment was amplified to a greater degree when cDNA representing the RNA isolated from cells treated with FGF-1 for 12 h was used as template. Initial

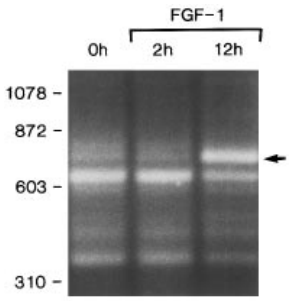

Figure 1 Identification of an FGF-1-inducible mRNA in NIH 3T3 fibroblasts by differential display

Serum-starved cells were left either untreated or treated with FGF-1 for 2 or 12 h. RNA was isolated, cDNA was synthesized, and PCR was performed using zinc-finger- and protein-kinase-domain oligonucleotide primers. Amplification products were separated by agarose-gel electrophoresis and detected by ethidium bromide staining. DNA size markers (in bp) are shown on the left. The arrow on the right denotes a cDNA fragment representing a differentially expressed transcript.

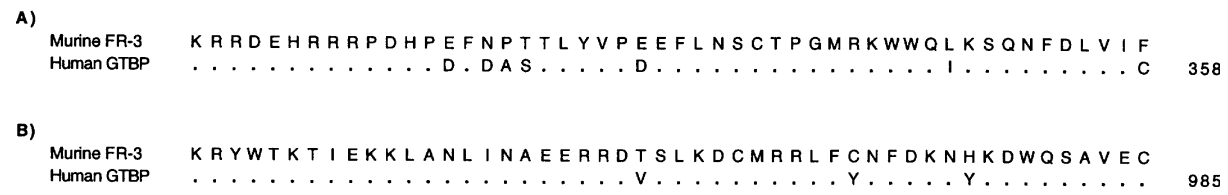

Figure 2 Sequence identity between the predicted murine FR-3 amino acid sequence and the human GTBP sequence

The amino acid sequence deduced from the nucleotide sequence of the 5′ end (**A**) or 3′ end (**B**) of the 2.3 kb restriction fragment of FR-3 cDNA is compared with the corresponding regions of human GTBP. Numbers at the right side refer to the last human GTBP amino acid residue [16] on the line. Dots represent amino acid sequence identity.

RNA gel-blot hybridization experiments indicated that this DNA fragment hybridized to an ~4.5 kb transcript. Therefore this fragment, termed FR-3, was radiolabelled and used to screen a mouse brain cDNA library in order to isolate longer cDNA clones. One positive phage was isolated that contained an ~3.2 kb cDNA insert. The insert contained two internal *Eco*RI sites; therefore three cDNA fragments (~2.3, 0.8 and 0.15 kb) were released after *Eco*RI digestion of purified phage DNA. All three *Eco*RI restriction fragments were subcloned.

FR-3 cDNA sequence analysis

The 5′ and 3′ ends of the FR-3 cDNA 2.3, 0.8 and 0.15 kb *Eco*RI restriction fragments were sequenced on both strands. A search of the available sequence databases indicated that both the 2.3 and 0.15 kb cDNA fragments had 100% nucleotide sequence identity with an unpublished murine GTBP cDNA sequence (accession number GB U42190). Also, the 2.3 and 0.15 kb cDNA fragments had 84% and 75% nucleotide sequence identity, respectively, with the corresponding regions of a human GTBP cDNA clone recently described by Palombo et al. [16]. An alignment of the deduced FR-3 amino acid sequence obtained from both ends of the 2.3 kb cDNA fragment and the corresponding regions of human GTBP is shown in Figure 2. The N-terminal FR-3 sequence has 86% identity with human GTBP, whereas the C-terminal sequence has 94% identity. In contrast to the FR-3 cDNA 2.3 and 0.15 kb *Eco*RI restriction fragments, the 5′ and 3′ ends of the 0.8 kb *Eco*RI fragment had no significant nucleotide sequence identity with database sequences. This result indicates that the 3.2 kb FR-3 cDNA was a chimaeric molecule containing two different cDNAs, only one of which encoded murine GTBP, that were presumably ligated to one another during library construction.

The original FR-3 cDNA was amplified in a PCR assay containing degenerate oligonucleotide primers designed to recognize DNA sequences encoding proteins with both a zinc finger and a protein kinase structural domain. Comparison of the PCR-primer sequences with the murine GTBP sequence (GB U42190) identified the two regions that flanked the 700 bp cDNA clone originally identified by differential display. This analysis indicated that the sense protein kinase oligonucleotide functioned as both a sense and antisense primer, and, under the PCR conditions used, the primer annealed to two regions of low nucleotide sequence identity (~56%). Thus, in this case, and in several others [11,12,14,15], our differential-display approach using motif primers did not identify a cDNA clone encoding a protein with the targeted structural domains. However, we have successfully used this targeted differential-display method to identify an FGF-1-inducible gene encoding a novel serine/theonine kinase [13].

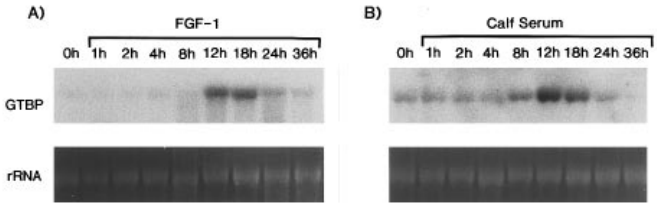

Figure 3 Effect of FGF-1 or calf serum treatment on GTBP mRNA levels in NIH 3T3 fibroblasts

Serum-starved cells were left either untreated or treated with FGF-1 (**A**) or calf serum (**B**) for the indicated time periods. RNA was isolated, and equivalent amounts of each sample were analysed by RNA gel-blot hybridization. Only the region of the autoradiogram that contained a hybridization signal is shown. The bottom part of each panel is a photograph of the 28 S rRNA band demonstrating that equivalent amounts of RNA were present in each gel lane.

Regulation of GTBP expression in NIH 3T3 cells

RNA gel-blot hybridization analysis was performed to confirm the differential-display results indicating that GTBP mRNA levels were elevated in FGF-1-stimulated NIH 3T3 cells. Quiescent NIH 3T3 cells were left either untreated or stimulated with FGF-1 for various times. Total RNA was isolated, fractionated in formaldehyde-containing agarose gels, transferred to a nylon membrane and probed with the 2.3 kb GTBP cDNA fragment. A single GTBP transcript of ~4.5 kb was detected in both quiescent and FGF-1-stimulated cells; however, elevated GTBP mRNA levels were apparent at 12 and 18 h after FGF-1 addition (Figure 3A).

We also investigated whether calf serum stimulation of NIH 3T3 cells would also increase GTBP mRNA expression. Serum contains numerous polypeptide mitogens but does not contain significant amounts of FGF-1 or the related mitogen FGF-2 (basic FGF) [22,23]. RNA was isolated from either quiescent cells or cells treated with calf serum for various time periods, and equivalent amounts were analysed by RNA gel-blot hybridization. Serum treatment increased GTBP mRNA expression with similar kinetics as observed following FGF-1 treatment (Figure 3B).

Immunoblot analysis was then performed to investigate whether FGF-1 treatment of NIH 3T3 cells resulted in elevated GTBP expression. Quiescent cells were left either untreated or treated with FGF-1 for different lengths of time. Cells were harvested, and equivalent amounts of protein were analysed by SDS/PAGE and immunoblotting with antiserum obtained from rabbits immunized with a recombinant glutathione S-transfer-

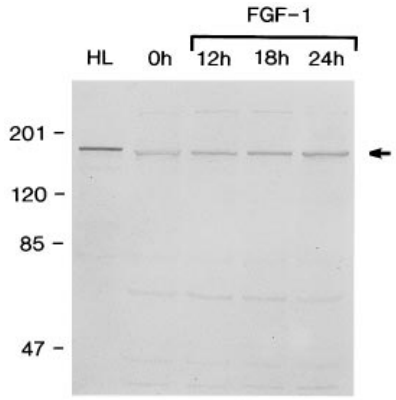

Figure 4 Effect of FGF-1 treatment on GTBP expression levels in NIH 3T3 fibroblasts

Serum-starved cells were left either untreated or treated with FGF-1 for the indicated time periods. Cell lysates were prepared, and equivalent amounts of protein, as well as 10 μg of HeLa cell nuclear extract (HL), were subjected to SDS/PAGE and immunoblot analysis using anti-GTBP polyclonal serum. Molecular masses of protein size standards (in kDa) are shown on the left. The arrow on the right denotes the major immunoreactive protein detected in fibroblasts.

ase–human GTBP (amino acids 750–928) fusion protein [16]. This antiserum has been shown previously to recognize specifically the $\sim$ 160 kDa GTBP expressed in HeLa cells [16]. We also detected an immunoreactive protein of $\sim$ 160 kDa in HeLa nuclear extracts; in addition, a protein with a similar molecular mass was expressed in quiescent and FGF-1-treated NIH 3T3 fibroblasts (Figure 4). Furthermore, FGF-1 stimulation increased the relative abundance of this immunoreactive protein.

In summary, the results described above indicate that the murine FR-3 gene encodes GTBP. GTBP is a member of the MSH family of DNA mismatch-binding proteins (reviewed in [18,19]). It was originally identified in HeLa cells as an $\sim$ 160 kDa protein that preferentially recognized G/T mismatches and bound to heteroduplexes containing small nucleotide loops [24,25]. More recently, it has been reported that GTBP can form a heterodimer with MSH2 and that efficient mismatch binding requires both polypeptides [16,20]. The *Saccharomyces cerevisiae* MSH6 protein appears to be the yeast homologue of mammalian GTBP [26]. Frameshift mutations of the human GTBP gene leading to truncated polypeptide products have been identified in several cancer cell lines and may be partially responsible for the mutator phenotype of these cells [27]. We have found that FGF-1 stimulation of quiescent NIH 3T3 cells results in GTBP mRNA accumulation during the late G_1/early S phases of the fibroblast cell cycle. These results indicate that enhanced levels of GTBP may be required for effective repair of DNA replication errors.

Received 1 July 1996/22 July 1996; accepted 6 August 1996

We thank Dr. W. Burgess (American Red Cross, Rockville, MD, U.S.A.) for the FGF-1, Dr. R. Friesel (American Red Cross, Rockville, MD, U.S.A.) for the protein kinase oligonucleotide and Dr. J. Jiricny for the anti-GTBP polyclonal antiserum. We are also grateful to Ms. K. Wawzinski for excellent secretarial assistance. This research was supported in part by NIH research grants HL-39727 and HL-54710. D.K.W.H. was supported in part by an NIH training grant (HL-07698).

REFERENCES

1 Williams, G. T., Abler, A. S. and Lau, L. F. (1992) in Molecular and Cellular Approaches to the Control of Proliferation and Differentiation (Stein, G. S. and Lian, J. B., eds.), pp. 115–161, Academic Press, Orlando
2 Muller, R., Mumberg, D. and Lucibello, F. C. (1993) Biochim. Biophys. Acta **1155**, 151–179
3 Kovary, K. and Bravo, R. (1991) Mol. Cell. Biol. **11**, 4466–4472
4 Brach, M. A., Gruss, H.-J., Sott, C. and Herrmann, F. (1993) Mol. Cell. Biol. **13**, 4284–4290
5 Baldin, V., Lukas, J., Marcote, M. J., Pagano, M. and Draetta, G. (1993) Genes Dev. **7**, 812–821
6 Burgess, W. H. and Winkles, J. A. (1996) in Cell Proliferation in Cancer: Regulatory Mechanisms of Neoplastic Cell Growth (Pusztai, L., Lewis, C. E. and Yap, E., eds.), pp. 154–217, Oxford University Press, Oxford
7 Miyamoto, M., Naruo, K.-I., Seko, C., Matsumoto, S., Kondo, T. and Kurokawa, T. (1993) Mol. Cell. Biol. **13**, 4251–4259
8 Yamasaki, M., Miyake, A., Tagashira, S. and Itoh, N. (1996) J. Biol. Chem. **271**, 15918–15921
9 Johnson, D. E. and Williams, L. T. (1993) Adv. Cancer Res. **60**, 1–40
10 Winkles, J. A., Donohue, P. J., Hsu, D. K. W., Guo, Y., Alberts, G. F. and Peifley, K. A. (1995) in Cardiovascular Disease 2 (Gallo, L. L., ed.), pp. 109–120, Plenum Press, New York
11 Hsu, D. K. W., Donohue, P. J., Alberts, G. F. and Winkles, J. A. (1993) Biochem. Biophys. Res. Commun. **197**, 1483–1491
12 Donohue, P. J., Alberts, G. F., Hampton, B. S. and Winkles, J. A. (1994) J. Biol. Chem. **269**, 8604–8609
13 Donohue, P. J., Alberts, G. F., Guo, Y. and Winkles, J. A. (1995) J. Biol. Chem. **270**, 10351–10357
14 Hsu, D. K. W., Guo, Y., Alberts, G. F., Peifley, K. A. and Winkles, J. A. (1996) J. Cell. Physiol. **167**, 261–268
15 Hsu, D. K. W., Guo, Y., Alberts, G. F., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Peifley, K. A. and Winkles, J. A. (1996) J. Biol. Chem. **271**, 13786–13795
16 Palombo, F., Gallinari, P., Iaccarino, I., Lettieri, T., Hughes, M., D'Arrigo, A., Truong, O., Hsuan, J. J. and Jiricny, J. (1995) Science **268**, 1912–1914
17 Nicolaides, N. C., Palombo, F., Kinzler, K. W., Vogelstein, B. and Jiricny, J. (1996) Genomics **31**, 395–397
18 Kolodner, R. (1996) Genes Dev. **10**, 1433–1442
19 Umar, A. and Kunkel, T. A. (1996) Eur. J. Biochem. **238**, 297–307
20 Drummond, J. T., Li, G.-M., Longley, M. J. and Modrich, P. (1995) Science **268**, 1909–1912
21 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) J. Mol. Biol. **215**, 403–410
22 Yoshitake, Y. and Nishikawa, K. (1992) In Vitro Cell. Dev. Biol. **28A**, 419–428
23 Ii, M., Yoshida, H., Aramaki, Y., Masuya, H., Hada, T., Terada, M., Hatanaka, M. and Ichimori, Y. (1993) Biochem. Biophys. Res. Commun. **193**, 540–545
24 Jiricny, J., Hughes, M., Corman, N. and Rudkin, B. B. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 8860–8864
25 Hughes, M. J. and Jiricny, J. (1992) J. Biol. Chem. **267**, 23876–23882
26 Marsischky, G. T., Filosi, N., Kane, M. F. and Kolodner, R. (1996) Genes Dev. **10**, 407–420
27 Papadopoulos, N., Nicolaides, N. C., Liu, B., Parsons, R., Lengauer, C., Palombo, F., D'Arrigo, A., Markowitz, S., Willson, J. K. V., Kinzler, K. W., Jiricny, J. and Vogelstein, B. (1995) Science **268**, 1915–1917