Biochemical and Biophysical Research Communications **263,** 1–5 (1999)
Article ID bbrc.1999.1294, available online at http://www.idealibrary.com on IDEAL®

Mutations at Position 277 Modify the DNA-Binding Specificity of Human p53 *in Vitro*

Patrick Chène
Oncology Department, Novartis, CH-4002 Basel, Switzerland

Received July 29, 1999

p53 regulates the expression of different genes that contain in their promoter a DNA sequence with two copies of the 10-base motif $Pu_1Pu_2Pu_3C_4(A/T)_5(T/A)_6G_7Py_8Py_9Py_{10}$. This sequence is degenerated, and thymine or cytidine is found equally at position 3 or 8. These two bases make contact with cysteine-277 of the human p53. An *in vitro* study was carried out to determine whether p53 could be mutated at position 277 so that it binds preferentially to a sequence containing thymine or cytidine. Various mutant proteins were created and their DNA-binding specificity was determined by gel shift assay. Two of them show an altered specificity. The Cys277Ser protein binds preferentially to cytidine-containing sequences while the Cys277Ala mutant has a preference for thymine-containing sequences. This specificity is presumably achieved because an alanine residue at position 277 interacts with the thymine via hydrophobic interactions and a serine makes a hydrogen bond with the cytidine but not with the thymine. © 1999 Academic Press

Key Words: **p53; DNA-binding specificity; mutant.**

The p53 protein plays a critical role in the cell. In cases of hypoxia, metabolite changes or DNA damage, it accumulates in the cell and becomes active. This induction of p53 activity can lead to cell-cycle arrest and/or apoptosis (1). If p53 exerts its activity through transcription independent mechanisms (2), a large part of its cellular activity is due to its ability to bind to DNA and to regulate the expression of different genes.

The consensus DNA-binding sequence for p53 is known (3, 4). It corresponds to two copies of the 10-base motif 5′-Pu.Pu.Pu.C.(A/T).(T/A).G.Py.Py.Py-3′ (where Pu is A or G and Py is C or T), which can be separated by up to 13 bases. Different promoters containing a p53-binding element have been identified (5). Some of them, such as the $p21^{Waf1/Cip1}$ promoter, have few mismatches with the consensus DNA-binding sequence of p53, whereas others, such as the bax promoter, are more degenerated. This suggests that p53 has a different affinity for the promoters which it regulates (6, 7, 8). Thus, in cells, the p53 regulation of these promoters might be dependent on at least the concentration of p53 in the cell. A promoter for which it has a high affinity would then be activated by a lower p53 concentration than a promoter for which it has a low affinity. This is supported by the fact that p53 mutants with an altered DNA-binding activity can still interact with the $p21^{Waf1/Cip1}$ DNA-binding element (for which there is a relatively high affinity) but not with the bax DNA-binding element (for which there is a relatively low affinity) (9).

The determination of the three-dimensional structure of a p53-DNA complex has revealed the molecular basis of the p53-DNA interaction (10). The p53 DNA-binding domain can be divided into three parts: major groove contacts, minor groove contacts in the AT-rich region, and phosphate contacts. Only three residues, lysine-120, arginine-280 and cysteine-277, make contacts in the major grooves. Arginine-280 contacts the invariant GC pair, lysine-120 gives a hydrogen either to a guanine or to an adenine, and cysteine-277 accepts a hydrogen from a cytosine or donates a hydrogen to a thymine (Fig. 1). Since lysine-120 and cysteine-277 can interact with two possible bases, it might be possible to mutate these residues to generate proteins that bind preferentially to DNA sequences containing one of these two bases. These proteins could be used as tools to analyse more selectively some of the downstream pathways regulated by p53. Indeed, the mutation of residues located around lysine-120 induces a change in DNA-binding specificity (11). Whereas the two mutant proteins Ser121Phe and Thr123Ala show the same affinity as wild-type (wt) protein for DNA sequences containing two copies of the consensus AGACATGCCT, their affinity for a single copy of this sequence is greater than that of wt protein.

Since the base pair contacted by lysine-120 differs from the base pair contacted by cysteine-277 (Fig. 1), a study was carried out to determine whether proteins mutated at position 277 would show an altered DNA-binding specificity.

0006-291X/99 $30.00
Copyright © 1999 by Academic Press
All rights of reproduction in any form reserved.

MATERIAL AND METHODS

Plasmid construction. The cloning of the human wt p53 is described in Chène *et al.* (12). The plasmids coding for the different mutants were obtained from wt p53 plasmid by polymerase chain reaction with the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The DNA sequence of all the different constructs was verified by double-strand sequencing.

Gel shift assay. The different plasmids (150 ng) were translated *in vitro* at 30°C in rabbit reticulocyte lysate (25 μl total volume) (TNT T7 Quick Coupled Transcription/Translation System, Promega). 7 μl of the translation mixture was incubated in a total volume of 20 μl for 1 h at 22°C in binding buffer (25 mM Hepes · NaOH, pH 7.6, 50 mM KCl, 10% glycerol, 0.1% Triton X-100, 5 mM DTT, 400 μg/ml poly[dI · dC] · poly[dI · dC]) in the presence of 1.5×10^{-14} moles [^{32}P] 5′-radiolabelled oligonucleotide corresponding to the WAF1 p53 DNA-binding element (13) and 300 ng of the activating antibody Ab421 (Calbiochem) (14). The reaction mixtures were loaded onto a 4% polyacrylamide gel containing 0.5 Tris-borate. After electrophoresis the gels were dried and exposed to X-ray films.

Immunoprecipitation. The plasmids (150 ng) were cotranslated at 30°C in a total volume of 25 μl rabbit reticulocyte lysate in the presence of [^{35}S]-methionine. 20 μl of the translation mixture was incubated for 1 h at 4°C in 500 μl IP buffer (50 mM Tris · HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40 and 0.5% sodium deoxycholate) in the presence of 2 μg of Ab1620 (Calbiochem). 50 μl of protein G-agarose (Boehringer Mannheim) was added and the mixtures incubated for another 1 hour at 4°C. After reaction, the beads were washed twice with IP buffer and once with low salt buffer (50 mM Tris · HCl, pH 7.5, 0.1% Nonidet P40 and 0.05% sodium deoxycholate) at 4°C. The bound proteins were eluted at 95°C with 50 μl SDS-buffer and 40 μl of the denatured solution was loaded onto a 10% SDS-PAGE. After drying, the gels were exposed to X-ray films.

Analysis of the protein synthesis by SDS-PAGE. The plasmids (150 ng) were cotranslated at 30°C in a total volume of 25 μl rabbit reticulocyte lysate in the presence of [^{35}S]-methionine. 5 μl of the translation mixture were incubated with 35 μl SDS buffer and incubated for 5 min at 95°C. The denatured solution (10 μl) was loaded onto a 10% SDS-PAGE. After drying, the gels were exposed to X-ray films.

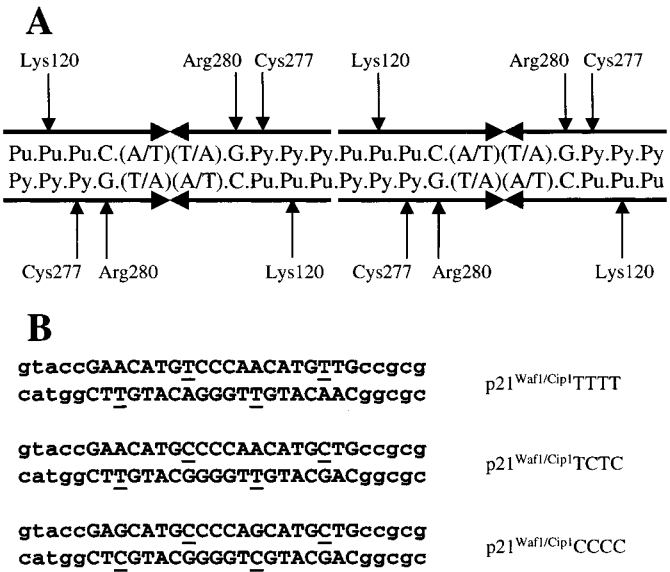

FIG. 1. p53 DNA-binding elements. (A) The consensus p53-binding site is represented and the contacts with arginine-280, cysteine-277, and lysine-120 are indicated. Arrows represent the half sites. (B) DNA sequences of the oligonucleotides used for the gel shift assay experiments. The lowercase letters indicate additional bases that have been added to the p53-binding element to favour the annealing of the oligonucleotides. The bases in contact with a residue at position 277 are underlined. The p21$^{Waf1/Cip1}$ DNA element (13) corresponds to p21$^{Waf1/Cip1}$TTTT.

RESULTS AND DISCUSSION

Cysteine-277 from human p53 was mutated to serine (Cys277Ser), alanine (Cys277Ala) and aspartic acid (Cys277Asp). The ability of these proteins to bind to an oligonucleotide containing the p53 binding site from the p21$^{Waf1/Cip1}$ promoter (13) (Fig. 1) was measured in a gel shift assay (Fig. 2A). The experimental results show that the Cys277Ala mutant has a DNA-binding activity similar to that of wt protein. The DNA-binding activity of the Cys277Ser protein is weak, while that of Cys277Asp is slightly greater. These different affinities for DNA could be due to the fact that the proteins are not synthesised in the same amounts *in vitro,* in which case the difference observed in the gel shift assay would not be related to their DNA-binding affinity. To exclude this possibility, the proteins were synthesised in the presence of [^{35}S]-methionine and analysed by SDS-PAGE (Fig. 2B). These results show that the different proteins are *in vitro* synthesised at similar levels. This indicates that the differences observed in the assay are not due to differences in the amount of protein synthetised. p53 mutations often lead to protein with an altered conformation (10, 15–16). This change in conformation can be monitored by the loss of the epitope for antibody Ab1620 (17–18). To verify if the mutations did not induce a loss of the wt conformation, the proteins were *in vitro* synthesised and immunoprecipitated with the antibody Ab1620 (Fig. 2C). The three mutant proteins Cys277Ser, Cys277Asp and Cys277Ala and the wt protein are immunoprecipitated in a similar fashion indicating that the mutations do not induce a loss of the wt conformation. This is supported by the analysis of p53 structure (10). The cysteine-277 is located in a loop at the surface of the protein. Therefore its mutation in alanine, serine or aspartate should not induce major changes in p53 conformation. Altogether these results show that the difference observed in gel shift assay are due to different DNA binding properties of the mutants.

Cho *et al.* (10) report that cysteine-277 could donate a hydrogen bond to the O4 of a thymine moiety. In this case, the Cys277Ser protein should bind to the p21$^{Waf1/Cip1}$ DNA-binding element more tightly than Cys277Asp, which cannot donate a hydrogen bond. Moreover, the wt and Cys277Ser proteins should show a similar DNA-binding activity because they are both capable of donating a hydrogen bond. These obser-

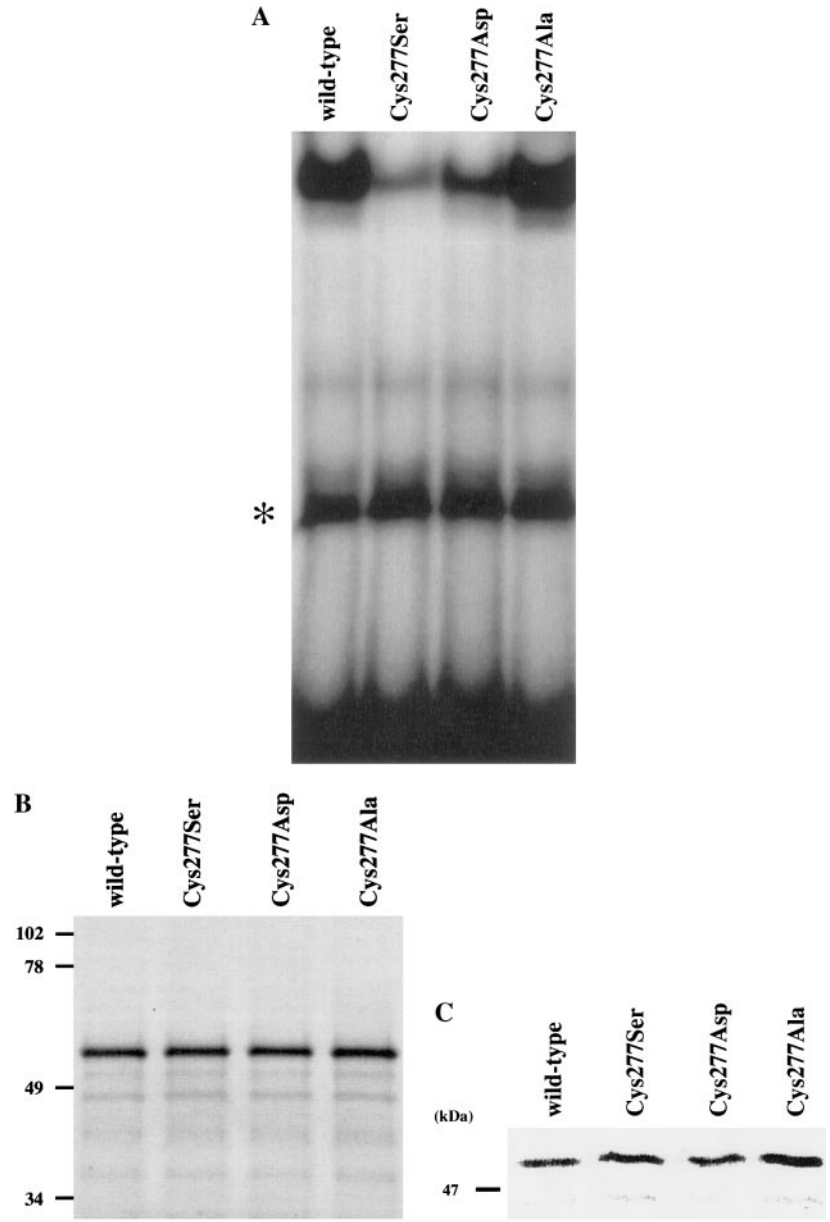

FIG. 2. DNA-binding properties of different proteins mutated at the position 277. (A) The wt, Cys277Ser, Cys277Asp, and Cys277Ala proteins were translated *in vitro* in rabbit reticulocyte lysate and their DNA-binding activity was determined in gel shift assay. An autoradiogram of the dried gel is shown. The star indicates a protein of the lysate that interacts with the oligonucleotide. (B) The same mutant proteins were translated *in vitro* in the presence of [^{35}S]-methionine. After translation, the lysates were heat denatured in the presence of SDS-buffer and loaded onto a 10% SDS-PAGE. An autoradiogram of the dried gel is shown. The molecular weights are indicated. (C) The different proteins were *in vitro* translated in the presence of [^{35}S]-methionine and immunoprecipitated with the antibody Ab1620. The proteins were denatured and loaded onto a 10% SDS-PAGE. An autoradiogram of the dried gel is shown.

vations suggest that the interaction between the cysteine-277 and the thymine moiety does not depend only on the creation of a hydrogen bond. One possibility is that a cysteine residue at position 277 interacts better with the thymine moiety than a serine residue because it is more hydrophobic. The role of hydrophobic interactions between thymine and the residue at position 277 is supported by the properties of the Cys277Ala mutant (Fig. 2A). The substitution of cysteine-277 by an amino acid unable to form a hydrogen bond with the thymine moiety leads to a protein which is as active as wt p53. Alanine residues show specific hydrophobic interactions with the methyl group of thymines (19), supporting the idea that the thymine moiety interacts with the p53 protein via hydrophobic interactions.

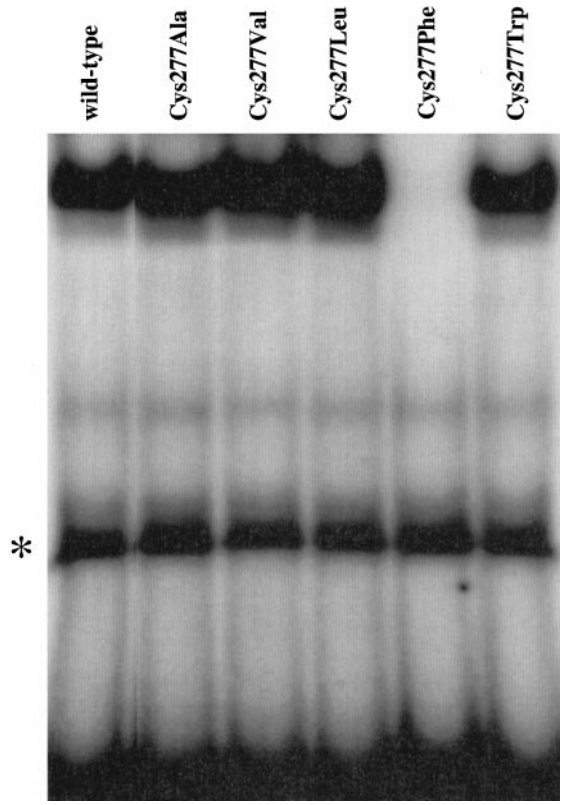

FIG. 3. DNA-binding properties of different proteins with hydrophobic side chains at position 277. The wt, Cys277Ala, Cys277Val, Cys277Phe, and Cys277Trp proteins were translated *in vitro* and their DNA-binding properties were analyzed in gel shift assay. An autoradiogram of the dried gel is shown. The star indicates a protein of the lysate that interacts nonspecifically with the oligonucleotide.

To further demonstrate this hydrophobic effect, the presence of other hydrophobic side chains at position 277 was investigated. The mutant proteins Cys277Val, Cys277Leu, Cys277Phe and Cys277Trp were evaluated for their DNA-binding activity (Fig. 3). The experimental results show that the introduction of a valine or a leucine residue does not influence DNA binding, which indicates that the p53 protein can accommodate different hydrophobic side chains at position 277. Even a leucine residue with a volume of 167 $Å^3$ (20) can replace the cysteine residue, which has a volume of 109 $Å^3$. However, the Cys277Phe (190 $Å^3$) mutant is completely inactive, indicating that larger hydrophobic side chains may not be tolerated at this position. The Cys277Trp mutant, however, which has an even larger side chain (228 $Å^3$), shows the same activity as wt protein. This could be explained by the fact that the Cβ of the tryptophan residue is linked to an indole ring and not directly to a phenyl ring, as is the case for the phenylalanine residue. Owing to the smaller size of the indole ring, the p53 protein could tolerate this mutation. In addition, the NH group of the indole could make a hydrogen bond with the O4 of the thymine. The different DNA-binding activities observed for these different mutants are not due to the presence of different amounts of these proteins in the assay, because they are all translated at similar levels (data not shown).

The fact that cysteine-277 can be substituted by alanine, valine or leucine without loss of activity shows that hydrophobic interactions are determinant for the interaction with the thymine moiety. The situation should be different when a cytidine is present at this position in the DNA element. Cytidines are present at this position in about 50% of the p53 DNA-binding sites (10). The cytidine moieties do not contain a methyl group and are therefore less hydrophobic than the thymine moieties. They can, however, donate a hydrogen to H-bond acceptors. The presence of a cytidine should therefore alter the relative DNA-binding activity of the wt, Cys277Ser and Cys277Ala proteins. To test this hypothesis, the $p21^{Waf1/Cip1}$ DNA-binding element was mutated (Fig. 1). While only 2 of the 4 thymines interacting with the residue at position 277 were replaced by cytidines in the $p21^{Waf1/Cip1}$TCTC DNA, all 4 thymines were replaced by cytidines in the $p21^{Waf1/Cip1}$CCCC DNA. The affinity of the wt, Cys277Ser and Cys277Ala proteins for these different DNA elements was evaluated in a gel shift assay (Fig. 4). When the $p21^{Waf1/Cip1}$TCTC DNA element is used in the assay, all three proteins bind to DNA. The binding of Cys277Ala, however, is slightly weaker than that of the other two proteins. Only the wt and Cys277Ser proteins bind to $p21^{Waf1/Cip1}$CCCC DNA, whereas no binding is observed with the Cys277Ala mutant. Alto-

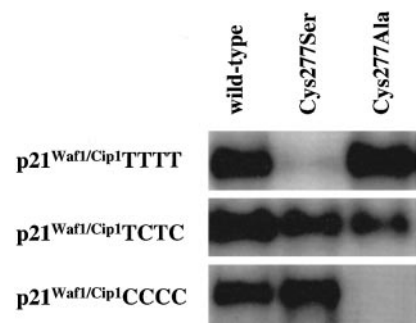

FIG. 4. DNA-binding specificity of wt, Cys277Ser, and Cys277Ala proteins. The wt, Cys277Ser, and Cys277Ala, Cys277Val proteins were translated *in vitro* and their DNA-binding properties were analyzed. Different DNA-binding elements were used: $p21^{Waf1/Cip1}$TTTT, $p21^{Waf1/Cip1}$TCTC, and $p21^{Waf1/Cip1}$CCCC. Their sequence is given in Fig. 1. An autoradiogram of the dried gel is shown. For a better presentation of the data, only the region of the gel corresponding to the protein-DNA complexes is shown. The $p21^{Waf1/Cip1}$CCCC oligonucleotide could not be 5′-phosphorylated by the T4 polynucleotide kinase to the same extent as the $p21^{Waf1/Cip1}$TTTT oligonucleotide. The specificity of this enzyme may differ for these two DNA sequences. Therefore the signals obtained with this oligonucleotide were always weaker.

gether these results show that the specificity of p53 DNA-binding can be modified by mutating the amino acid at position 277. The wt protein, which has a cysteine at position 277, interacts both with sequences containing thymine and with those containing cytidine, because it makes hydrophobic contacts with the methyl from the thymine or it accepts a hydrogen bond from the cytidine. The Cys277Ser protein cannot interact with thymine via hydrophobic interactions, and so binds very weakly to thymine-containing sequences. However, as the serine residue can accept hydrogen bonds, the Cys277Ser protein binds to cytidine-containing sequences. The opposite applies in the case of the Cys277Ala mutant. This protein can only interact via hydrophobic interactions with the methyl from thymine moieties. Since the alanine residues do not show strong hydrophobic interactions with the CH group from cytidine moieties (19) and are not H-bond acceptors, the Cys277Ala protein does not bind to cytidine-containing sequences. This also shows that p53 mutations can lead to complex phenotypes. The Cys277Ser mutant which is found in human cancers (21) would then be able to stimulate the activity of some of the promoters regulated by p53 (the cytidine-containing promoters) and not of the other one (the thymine-containing promoters.

In summary, it has been shown that mutations at position 277 in human p53 modify its DNA-binding specificity. Two mutants with opposite DNA-binding specificities have been identified: the Cys277Ser mutant, which binds only to DNA sequences that contain a cytidine at positions 3 and 8 of the consensus DNA element, and the Cys277Ala protein, which binds only to DNA sequences that contain a thymine at these positions. These two mutants could be very useful tools for analysing in more detail how p53 regulates its different gene targets.

REFERENCES

1. Levine, A. J. (1997) *Cell* **88,** 323–331.
2. Caelles, C., Helmberg, A., and Karin, M. (1994) *Nature* **370,** 220–223.
3. El-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992) *Nat. Genet.* **1,** 45–49.
4. Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., and Shay, J. W. (1992) *Mol. Cell. Biol.* **12,** 2866–2871.
5. Selivanova, G., and Wiman, K. G. (1995) *Adv. Cancer Res.* **66,** 143–180.
6. Martinez, J. D., Craven, M. T., and Pennington, M. E. (1998) *Oncogene* **16,** 453–458.
7. Friedlander, P., Haupt, Y., Prives, C., and Oren, M. (1996) *Mol. Cell. Biol.* **16,** 4961–4971.
8. Ludwig, R. L., Bates, S., and Vousden, K. H. (1996) *Mol. Cell. Biol.* **16,** 4952–4960.
9. Flaman, J. M., Robert, V., Lenglet, S., Moreau, V., Iggo, R., and Frebourg, T. (1998) *Oncogene* **16,** 1369–1372.
10. Cho, Y., Gorina, S., Jeffrey, P. D., and Pavletich, N. P. (1994) *Science* **265,** 346–355.
11. Freeman, J., Schmidt, S., Scharer, E., and Iggo, R. (1994) *EMBO J.* **13,** 5393–5400.
12. Chène, P., Mittl, P., and Grütter, M. (1997) *J. Mol. Biol.* **273,** 873–881.
13. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) *Cell* **75,** 817–825.
14. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992) *Cell* **71,** 875–886.
15. Milner, J., and Medcalf, E. A. (1990) *J. Mol. Biol.* **216,** 481–484.
16. Stephen, C. W., and Lane, D. P. (1992) *J. Mol. Biol.* **225,** 577–583.
17. Milner, J., Cook, A., and Sheldon, M. (1987) *Oncogene* **1,** 453–455.
18. Ory, K., Legros, Y., Auguin, C., and Soussi, T. (1994) *EMBO J.* **13,** 3496–3504.
19. Suzuki, M. (1994) *Structure* **2,** 317–326.
20. Zamyatin, A. A. (1972) *Prog. Biophys. Mol. Biol.* **24,** 107–123.
21. Beroud, C., and Soussi, T. (1998) *Nucleic Acids Res.* **26,** 200–204.