An intron binding protein is required for transformation ability of p53

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ABSTRACT

Regulatory elements in intron sequences have been identified for several eukaryotic genes. The fourth intron of p53 is known to increase expression of p53 in a position dependent manner. We asked whether p53 intron 4 sequences interacted with DNA binding proteins to exact their effect. Three overlapping DNA fragments spanning the 5' end of p53 intron 4 were determined to specifically interact with protein in nuclear extracts from several cell lines by band shift analysis. Methylation interference experiments were used to identify purine residues involved in this protein-DNA interaction. Two G nucleotides were identified at intron 4 positions 33 and 44 and these were replaced by T and C, respectively. These two single base pair substitutions in the intron resulted in 1) lack of protein binding and 2) decreased expression of p53 as measured by a transformation assay. Thus the binding of protein to p53 intron 4 was shown to have functional significance. These experiments demonstrated a specific protein binding region in the 5' end of intron 4 critical for p53 expression and distinct from those elements already known to be involved in splicing.

INTRODUCTION

One of the functions of the nuclear protein p53 is to suppress transformation. Addition of wild-type p53 to rat embryo fibroblasts inhibits the transformation of these cells by E1A and *ras* or by *myc* and *ras* (1,2). In addition, mutations, deletions, and rearrangements of the p53 gene have been found in colorectal tumors, lung cancer, breast cancer, osteosarcomas and leukemias (3–10). In many of these cases, both p53 alleles are altered. These tumors do not make wild-type p53, suggesting expression of a normal p53 gene product is incompatible with tumor growth. The addition of wild-type p53 to a cell line established from a colorectal tumor that did not express a wild-type p53 protein drastically decreased the ability of these cells to grow in culture (11).

Interestingly several critical mutations within the p53 coding sequence result not only in the loss of suppression but in the gain

of transforming ability. When p53 gene constructs bearing mutations in the coding region were cotransfected with the *ras* oncogene into rat embryo fibroblasts, mutant p53 cooperated with activated *ras* to transform these cells (12–15).

p53 appears to suppress transformation by activating transcription. Fusion proteins between p53 and the GAL4 DNA-binding domain that anchor p53 to a DNA target sequence indicate that the p53 protein activates transcription (16,17). In the assay, two transforming mutants of p53 could not activate transcription (16). Thus wild-type p53 that results in transformation suppression may activate transcription of a class of genes necessary to suppress transformation, and mutants which no longer suppress these genes are transcriptionally inactive and lead to transformation.

Since deletions and rearrangements of p53 seen in many tumors often result in the lack of p53 expression, the factors involved in p53 regulation are also important denominators in p53 tumor suppression. The analysis of several wild-type and mutant p53 cDNA/genomic constructs *in vitro* and *in vivo* have shown the importance of introns in p53 expression (15,18). Tissue culture experiments indicate that the presence of introns 2–9 in their normal position within the p53 DNA results in high levels of p53 protein (15), owing to an increase in p53 mRNA levels (19). Similarly in transgenic mice, p53 gene constructs with introns expressed greater than 100-fold the p53 mRNA of cDNA constructs without introns (18). This observation is also true for other transgenes expressed in mice (20). The presence of a single p53 intron, intron 4, was also very efficient at increasing mRNA levels (15,19). This observation initially suggested that intron 4 may contain a classical enhancer. Precedents include both the immunoglobulin heavy chain and the β globin genes, which contain enhancers within their introns (21–23). Placement of p53 intron 4 sequences 5' or 3' of an expressing p53 cDNA or of a CAT reporter plasmid had no effect on mRNA or protein levels (18,19).

To understand how p53 intron 4 affects expression of p53 *in vitro* and *in vivo*, we first sequenced the intron and looked for protein-DNA complexes that affect its transformation ability. We present here evidence that such a protein exists in different cells.

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METHODS

Sequencing

The NcoI-NcoI fragment of the mouse p53 gene, which encompasses all of intron 4 and parts of exons 4 and 5, was subcloned into the SmaI site of the PGEM3 vector (Promega). Restriction endonuclease analysis allowed subsequent subcloning into M13 vectors and sequencing using the Sequenase system (US Biochemicals). Based on the nucleotide sequence we developed a complete restriction map of intron 4.

In vitro DNA binding assay

In the band shift assay (24–25) DNA-protein complexes were separated from free DNA by electrophoretic fractionation on non-denaturing polyacrylamide gels. Crude nuclear protein extracts were prepared (26) from mouse myeloma cells (S194), p53 and *ras* transformed rat embryo fibroblasts (P53T10A1) and NIH-3T3 cells. Convenient restriction sites within intron 4 were used to generate overlapping DNA fragments of approximately 150–350 base pairs that together spanned the entire intron. Binding conditions were as described (27). In addition, wild-type and mutant double-stranded oligonucleotides containing sequences of the intron 4 binding site were synthesized. Methylation interference assay located two guanine residues in the binding site which we replaced with pyrimidines in the two mutant oligonucleotides.

Methylation interference

Methylation interference was performed as previously described (28). An intron 4 DNA fragment carrying sequences of the intron 4 binding site was end-labeled with [³²P]dATP and incubated with dimethyl sulphate for 7 min at 20°C. Protein-bound and unbound DNA fractions were separated by gel shift, eluted from the gel and resuspended in 1.0 M piperidine. The protein-bound and unbound DNA fractions were lyophilized, resuspended separately in loading buffer and run on a denaturing polyacrylamide gel. Differences in the electrophoretic patterns of the two fractions were used to identify guanine residues in the intron 4 binding site which participated in the protein-DNA interaction.

Northern analysis

Northern analysis was performed as described (29). Twenty micrograms of total RNA was loaded on a formamide gel and blotted. An XhoI-SacII fragment from a p53 cDNA clone was labeled and used as probe.

Plasmids

Mutant p53 gene constructs were created that contained two single base pair substitutions at the guanine residues of the intron 4 binding site. Mutant single strand oligonucleotides containing intron 4 binding site sequences were used to generate templates for obtaining altered DNA sequences by the method of Kunkel (30). Having identified two guanine residues in the intron 4 binding site that participate in the intron 4-protein interaction, we made mutant p53 gene constructs with base pair substitutions at these sites. A triple ligation was performed using a NcoI-XbaI fragment of the M13 construct, carrying the intron 4 base pair substitutions, the XhoI-NcoI fragment of pLTRcG and the XhoI-XbaI fragment of MSV-AB (15). The SacI-SacI fragment of this clone containing introns 2 and 3 of the mouse p53 gene, was subsequently replaced with a similar fragment from pMSVcl-val,

which did not contain introns 2 and 3 (15). The final construct pMSVcl-val I4, contained a p53 cDNA with only intron 4 in its appropriate position and two base pair substitutions at the intron 4 protein binding site. Restriction analysis and limited sequencing confirmed that the construct was correct.

Transformation assay

Using a p53 and *ras* transformation assay, p53 cDNAs with wild-type or mutant intron 4 were compared for tumorigenic potential. Gene constructs containing a cDNA/genomic p53 sequence (LTRp53cG) or a p53 cDNA (MSVcl-val) were also used for comparison. All p53 DNA constructs encode a mutant mouse p53 with an ala to val substitution at amino acid 135. For the transformation assay, primary rat embryo fibroblasts (REF) were prepared from 14 day old Fisher 344 rat embryos. On day 1 of the assay, 5×10^5 secondary REF were transfected by the calcium phosphate procedure (31) with 10 μ g of both a mutant p53 gene construct and the Ha-*ras* oncogene (32). Fourteen days after transfection, cells were stained with crystal violet and the foci counted.

Figure 1. Nucleotide sequence of intron 4 of the mouse p53 gene. **A.** The nucleotide sequence of intron 4 of the mouse p53 gene. Underlined sequences are 5' and 3' signals necessary for splicing. Methylation interference was used to identify those guanine residues that participated in an intron 4-protein interaction and are designated by *. Sequences in bold are oligonucleotides synthesized for subsequent study. **B.** The DNA fragments from intron 4 used in gel shift assays are shown (F1 to F7). A * designates those fragments specifically bound by protein and shifted. **C.** Mutant oligonucleotides synthesized for binding experiments.

RESULTS

A 5' DNA sequence from intron 4 binds protein specifically

The presence of p53 intron 4 in its normal position within the context of the p53 gene enhances the levels of p53 RNA *in vitro* and *in vivo*. Because this intron does not contain a classical, orientation- and position-independent enhancer, how it accomplishes this enhancement is not known. To decipher the mechanism, we first sequenced the intron. As shown in figure 1, p53 intron 4 contains canonical 5', 3', and 3' branch point splicing signals.

Since the presence of intron 4 enhances the levels of p53 mRNA, we postulated that this enhancement might occur through a protein-DNA interaction not unlike those in which transcription factors bind to promoter sequences. We therefore used the sequence of intron 4 to generate a restriction map by which we could divide intron 4 into several overlapping DNA fragments (Fig. 1B). These fragments were end labeled, mixed with nuclear extract from S194 cells, and subjected to band shift analysis (Fig. 2). Fragment F1, containing the 5' end of p53 intron 4, bound a protein present in S194 cells. This protein-DNA interaction was specific as binding to labeled DNA fragment F1 could be eliminated by a 75 fold molar excess of unlabeled fragment F1, but not by the same molar excess of an unlabeled

DNA fragment from another part of the intron, fragment F2 (Fig. 2, lanes 6 and 7). Likewise, DNA fragments F4 and F7, which overlap F1, bound the same protein and competed for binding to fragment F1 in competition experiments (data not shown). Thus fragments F1, F4, and F7 which share intron 4 nucleotides 11–88 must contain a nucleotide sequence that binds a specific nuclear protein in band shift analysis.

Protein binding to intron 4 is independent of p53 mRNA levels

We next asked whether this protein-DNA interaction was cell type specific. For this experiment we extracted nuclear proteins from three divergent cell lines for band shift analysis. These cell lines expressed various levels of p53 mRNA (Fig. 3A). p53T10A1 cells were made by transfecting mutant p53 and activated *ras* into secondary rat embryo fibroblasts (33). NIH-3T3 cells were also chosen for this analysis since they are only partially transformed and express p53 mRNA but at approximately 1/20th the level of p53T10A1 cells (Fig. 3A). Mouse myeloma cells, S194, were selected because we could not detect any p53 mRNA expression by Northern analysis. Band shift analysis using fragment F1 and nuclear extracts from these three cell lines showed they contained comparable levels of intron 4 binding protein (Fig. 3B). Competition with specific (F1) and non-specific DNA fragments again revealed the specificity of the binding. Thus the intron 4 binding protein was not specific to transformed cells or to cells overexpressing p53.

Identification of the purine residues in the DNA binding site

These data indicated that a protein from nuclear extracts specifically bound a sequence within nucleotides 11–88 of p53 intron 4. In order to define precisely the DNA contact made by this protein, methylation interference was performed. Labeled DNA fragment F1 was mixed with nuclear extract from S194 cells and separated by band shift assay. Both bound and free DNA fragments were isolated and treated with dimethyl sulfate. The binding of protein to the DNA fragment protected from degradation those purine nucleotides involved in the protein-DNA interaction (Figure 4). Although the sample with bound protein was slightly underloaded, we concentrated on the region of overlap of the gel shift fragments. Figure 4 indicates that two Gua nucleotides at position 33 and 44 within the intron 4 binding site were protected. Subsequent site direct mutagenesis confirmed our interpretation.

Figure 2. Band shift assay of intron 4 binding activity. Labeled DNA fragment F1 was mixed with S194 nuclear protein extract in a binding reaction. The mix was run on a 6% non-denaturing polyacrylamide gel to separate free DNA from bound DNA. Lane 1 shows protein-bound DNA migrating slower than free DNA. In lanes 2–9, unlabeled DNA fragment F1 as a specific competitor or unlabeled DNA fragment F2 as a non-specific competitor were added to the binding reaction at the molar excess indicated at the top of the gel.

Figure 3. Band shift assays of intron 4 binding activity using nuclear protein extracts from three different cell lines. A. Northern analysis of total RNA isolated from three cell lines is shown. The Northern was hybridized with a labeled p53 cDNA probe, washed and exposed for 5 hours. B. Labeled DNA fragment F1 was mixed with nuclear protein extract from three different cell lines in binding reactions. The mixes were run on 6% non-denaturing polyacrylamide gels. Lanes 1–4 show intron 4 binding activity using nuclear extract from S194 cells, lanes 5–8 show binding activity using nuclear extract from P53T10A1 cells, and lanes 9–12 show activity using nuclear extract from NIH-3T3 cells. In all cases, equal amounts of nuclear protein were used. Specific binding activity was seen using each of the different nuclear extracts. Unlabeled DNA fragment F1 was used as a specific competitor in lanes 2–3, 6–7, and 10–11 at the molar excess indicated. A synthetic double-stranded oligonucleotide containing promoter sequences of the mouse 2(I) collagen gene was used as a nonspecific competitor (NS) in lanes 4, 8, and 12 at 100 molar excess.

Mutation of two Gua residues in intron 4 disrupts protein-DNA binding

Since methylation interference experiments showed that two Gua nucleotides were involved in binding, we asked whether mutation of these two nucleotides would result in lack of binding. Four 32-bp oligonucleotides were synthesized. Two oligonucleotides represented both DNA strands of the wild-type sequence, while the other two oligonucleotides contained two base pair substitutions (Fig. 1C). In band shift assays, using wild-type oligonucleotide as a probe and S194 nuclear extracts, we observed the complex described above (Fig. 5). This complex was competed away by a small molar excess (50-fold) of unlabeled wild-type oligonucleotide. But an oligonucleotide in which the two Gua residues protected in methylation assays were mutated to Thy and Cyt was unable to compete for the binding of the factor to wild-type sequences (Fig. 5, lanes 8 & 9). Furthermore, this mutant oligonucleotide was unable to bind the factor present in S194 nuclear extract (Fig. 5, lane 1) which binds wild-type oligonucleotide (Fig. 5, lane 2).

Mutations in the intron 4 DNA binding site decrease p53 transformation

The transformation of primary rat embryo fibroblasts by mutant p53 and activated *ras* has been studied in detail (12–15). Two conditions are required for efficient p53 cooperation with *ras* and the formation of transformed foci: 1) a mutation (ala to val at amino acid 135 in this case) within the p53 coding sequence and 2) the presence of introns within the p53 cDNA (15). Analysis

of protein and mRNA levels of cells established from foci have shown that the presence of introns in their normal position within p53 coding sequences increases p53 mRNA levels (19).

To address the functional significance of our p53 intron 4 binding protein, we used the p53 and *ras* transformation assay to measure the effect of mutations in p53 intron 4 on the number of foci produced. The activated *ras* oncogene was cotransfected with a mutant p53 construct containing p53 introns 2–9, intron 4 alone, a mutated intron 4 or no introns. We showed that mutation of the intron 4 protein-binding site inhibited the ability of p53 to cooperate with *ras* in transformation (Table 1). In three separate experiments, the presence of two single base pair substitutions at the intron 4 protein-binding site caused a marked decrease in the number of foci formed.

DISCUSSION

Since their discovery, the presence and role of introns have puzzled scientists. Numerous genes contain introns and their presence increases the expression of these genes *in vitro* and *in*

Table 1. Number of cell foci in p53 and *ras* transformation assays

plasmid	intron #	# of foci		
		exp #1	II	III
LTRp53cG	2–9	34 [†]	46	18
MSVcl-val I4	4	14	12	3
MSVcl-val I4*	4*	2	1	0
MSVcl-val	–	1	0	0
Ha-ras	–	0	0	1

*This intron 4 contains T and C substitutions at intron 4 positions 33 and 44, respectively.

[†]The numbers shown are the total number of cell foci detected in separate experiments.

Figure 4. Methylation interference pattern of the intron 4 binding protein. Protein binding protected the intron 4 binding site from digestion by 1.0 M piperidine (lane B). Free DNA was completely digested (lane F). Guanine residues that participated in the intron 4-protein interaction (arrows) were determined by comparison to a Maxam–Gilbert sequencing ladder (G+A). Sequences of the intron 4 binding site, designated O, were subsequently synthesized as oligonucleotides.

Figure 5. Band shift assay of the binding activity of wild-type and mutant oligonucleotides containing sequences of the intron 4 protein binding site. Labeled wild-type and mutant oligonucleotides were mixed with nuclear protein extract from P53T10A1 cells in separate binding reactions. The mixes were run on 6% non-denaturing polyacrylamide gels. In lane 1 the mutant (M) oligonucleotide was used as a probe in a binding reaction. In all other lanes, the wild-type oligonucleotide was used as a probe. Unlabeled wild-type (WT) oligonucleotide (lanes 3–6) or unlabeled mutant (M) oligonucleotide (lanes 8–11) was used as a competitor of the binding reaction at the molar excess indicated at the top of the gel.

vivo, yet some genes lacking introns are still efficiently expressed. Thus genes have been classified as intron-dependent or intron-independent (34). The p53 gene falls into the intron-dependent category. Efficient expression of p53 in tissue culture or in transgenic mice can be achieved only with introns (15,18), but not all introns are required for efficient expression and not all introns are equal in their effect on expression. In p53 the presence of a single intron, intron 4, resulted in high levels of mRNA production, but intron 9 had no effect (Hinds and Levine, unpublished observations). The variations in effect could be due either to differences among intron sequences themselves or to differences in position within the p53 gene. Introns within the immunoglobulin and β globin genes are known to contain enhancer sequences and placement of these introns 5', 3' or in reverse orientation adjacent to the gene allowed efficient expression (21–23). Alteration of the distance of a single intron from the promoter has not been analyzed.

Studies using intron 4 as an enhancer of the chloramphenicol acetyltransferase gene (CAT) in tissue culture cells and in transgenic mice have suggested that intron 4 is not an enhancer as classically described (18). In another set of experiments using constructs containing p53 DNA, intron 4 was placed in two orientations upstream of the murine sarcoma virus long terminal repeat and also downstream of the p53 termination codon (19). These constructs, as well as a p53 cDNA construct with intron 4 inserted in its proper position, were each transfected along with activated *ras* into primary rat embryo fibroblasts. The ability of the constructs to produce transformed foci was monitored. The results of these experiments showed that the effect of intron 4 was position dependent. Only those constructs that had intron 4 in its proper position cooperated in transformation. From these two studies, it thus appears that p53 gene regulation by intron 4 is achieved through a mechanism different from the classical enhancer model.

Consequently in this study we examined intron 4 for protein-DNA interactions and found that three overlapping DNA fragments, each containing intron nucleotides 11–88, bound protein specifically. The exact Gua nucleotides in contact with the protein were determined by methylation interference. Experiments using the band shift assay showed that oligomers that contained sequences of the AP1, CCAAT, NF1 and SP1 binding sites did not compete for binding with a wild-type DNA fragment (data not shown). In addition, the intron 4 binding site did not match published binding sites of other DNA-binding proteins. We believe, therefore, that the intron 4 binding protein has not been previously characterized.

Having identified the site of protein-DNA interaction, we established a biological significance for this interaction. We made two identical plasmids containing a wild-type intron 4 or incorporating 2 bp substitutions at intron 4 nucleotides 33 and 44. These two bp substitutions eliminated protein-DNA interaction and furthermore resulted in decreased p53 expression as measured by focus-forming ability, a direct correlate of p53 mRNA levels (19).

Point mutations in other introns have been shown to affect gene expression by preventing accurate splicing (35–37). One notable exception is a point mutation in *ras* intron 4. An A to G transition approximately 160 bp into the intron results in a 10 fold increase in mRNA levels and in transformation potential (38). The mechanism by which this occurs in *ras* is not understood. Others have found that proteins interact with intron sequences (39, 40). In the example of the β -globin gene, protein-binding to intron

2 results in DNase I-hypersensitivity (39). In our study, we show that a protein binds to a specific sequence in p53 intron 4 and that mutation of this sequence which results in lack of binding has functional significance. Taken together, these examples suggest that introns can encode critical regulatory sequences, but the mechanism by which these function is poorly understood.

Having determined that intron 4 protein interaction is functionally significant, we must still clarify how the interaction affects mRNA expression. Several possibilities of regulating gene expression include: 1) participation of the DNA binding protein in a transcription complex, 2) partial unwinding of the DNA helix resulting in formation of nuclease hypersensitive sites or 3) stability and perhaps transport of the precursor RNA to the splicing machinery. Only further experimentation will allow us to determine how intron 4 protein binding interactions affect gene expression.

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REFERENCES

1. Finlay, C.A., Hinds, P.W. & Levine, A.J. (1989) *Cell* **57**, 1083–1093.
2. Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O. & Oren, M. (1989) *Proc. Natl. Acad. Sci., U.S.A.* **86**, 8763–8767.
3. Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., vanTuinen, P., Ledbetter, D.H., Barker, D.F., Nakamura, Y., White, R. & Vogelstein, B. (1989) *Science* **244**, 217–221.
4. Takahashi T, Nau, M.M., Chiba, I., Birrer, M.J., Rosenberg, R.K. Vinocour, M., Levitt, M., Pass, H., Gazdar, A.F. & Minna, J.D. (1989) *Science* **246**, 491–494.
5. Bartek, J.R., Iggo, R., Gannon, J. & Lane, D.P. (1990) *Oncogene* **5**, 893–899.
6. Masuda, H., Miller, C., Koeffler, H.P., Battifora, H. & Cline, M.J. (1987) *Proc. Natl. Acad. Sci., USA* **84**, 7716–7719.
7. Mulligan, L.M., Matlashewski, G.J., Scrable, H.J. & Cavenee, W.K. (1990) *Proc. Natl. Acad. Sci., USA* **87**, 5863–5867.
8. Cheng, J. & Haas, M. (1990) *Mol. Cell. Biol.* **10**, 5502–5509.
9. Ahuja, H., Bar-Eli, M., Advani, S.H., Benchimol, S. & Cline, M.J. (1989) *Proc. Natl. Acad. Sci., USA* **86**, 6783–6787.
10. Mowat, M., Cheng, A., Kimura, N., Bernstein, A. & Benchimol, S. (1985) *Nature* **314**, 633–636.
11. Baker, S.J., Markowitz, S., Fearon, E.R., Willson, J.K.V. & Vogelstein, B. (1990) *Science* **249**, 912–915.
12. Eliyahu, E., Raz, A., Gruss, P., Givol, D. & Oren, M. (1984) *Nature* **312**, 646–649.
13. Parada, L.F., Land, H., Weinberg, R.A., Wolf, D. & Rotter, V. (1984) *Nature* **312**, 649–651.
14. Jenkins, J.R., Rudge, K. & Currie, G.A. (1984) *Nature* **312**, 651–654.
15. Hinds, P., Finlay, C. & Levine, A.J. (1989) *J. Virol.* **63**, 739–746.
16. Raycroft, L., Wu, H. & Lozano, G. (1990) *Science* **249**, 1049–1051.
17. Fields, S. & Jang, S.K. (1990) *Science* **249**, 1046–1049.
18. Lozano, G. & Levine, A.J. (1991) *Mol. Carcin.* **4**, 3–9.
19. Hinds, P.W. (1989) Thesis. Princeton University.
20. Brinster, R.L., Allen, J.M., Behringer, R.R., Gelinas, R.E. & Palmiter, R.D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 836–840.
21. Gillies, S.D., Morrison, S.L., Oi, V.T. & Tonegawa, S. (1983) *Cell* **33**, 717–728.
22. Banerji, J., Olson, L. & Schaffner, W. (1983) *Cell* **33**, 729–740.
23. Queen, C. & Baltimore, D. (1983) *Cell* **33**, 741–747.
24. Garner, M.M. & Revzin, A. (1981) *Nucleic Acids Res.* **9**, 3047–3060.
25. Fried, M. & Crothers, D.M. (1981) *Nucleic Acids Res.* **9**, 6505–6525.
26. Dignam, J.D., Lebovitz, R.M. & Roeder, R.G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
27. Karsenty, G. & de Crombrughe, B. (1990) *J. Biol. Chem.* **265**, 9934.
28. Siebinlist, U. & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 122–126.

29. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) in *Molecular Cloning* (Cold Spring Harbor Laboratory Press), 2nd Ed., pp. 743–748.
30. Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488–492.
31. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752.
32. Goldfarb, M., Schinizu, J. & Wigler, M. (1982) *Nature* **296**, 404–409.
33. Finlay, C.A., Hinds, P.W., Tan, T.-H., Eliyahu, D., Oren, M. & Levine, A.J. (1988) *Mol. Cell. Biol.* **8**, 531–539.
34. Buchman, A.R. & Berg, P. (1988) *Mol. Cell. Biol.* **8**, 4395–4405.
35. Rees, D.J.G., Rizza, C.R. & Brownlee, G.G. (1985) *Nature* **316**, 643–645.
36. DiLella, A.G., Marvit, J., Lidsky, A.S., Güttler, F. & Woo, S.L.C. (1986) *Nature* **322**, 799–803.
37. Arpaia, E., Dumbille-Ross, A., Maler, T., Neote, K., Tropak, M., Troxel, C., Stirling, J.L., Pitts, J.S., Bapat, B., Lamhonwah, A.M., Mahuran, D.J., Schuster, S.M., Clarke, J.T.R., Lowden, J.A. & Gravel, R.A. (1988) *Nature* **333**, 85–86.
38. Cohen, J.B. & Levinson, A.D. (1988) *Nature* **334**, 119–124.
39. Galson, D.L. & Housman, D.E. (1988) *Mol. Cell Biol.* **8**, 381–392.
40. Jost, J.P., Saluz, H. & Moncharmont, B. (1987) *J. Cell. Biochem.* **35**, 69–82.