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Characterisation of DNA Binding and Transcriptional Regulatory Function of an Endogenous Mutant p53 in MDA-468 Human Breast Cancer Cells

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Tumour suppressor p53, the most frequently altered gene among human cancers, is known to function by modifying gene transcription. Transcriptional regulatory activity of p53 has been established using transfected cell culture models and *in vitro* systems. Here, we report the characterisation of transcriptional function of an endogenous mutant p53 ($p53^{273.His}$) in MDA-468 human breast cancer cells, in terms of DNA-binding and transactivation assays. We have detected specific complexes of $p53^{273.His}$ with a high affinity consensus sequence (CON) and with Fragment A (FRA) sequences, two of the known p53 DNA binding sites. Furthermore, these sequences mediated p53-specific transcriptional modulation of a reporter gene. While CON conferred 10 fold transactivation of transcription, FRA, on the other hand, suppressed transcription to a similar extent in MDA-468 cells. These experiments indicate a sequence-dependent novel transcriptional regulatory function for endogenous $p53^{273.His}$ in MDA-468 cells.

The tumour suppressor gene, p53, is one of the most frequently affected genes among a variety of human cancers (1-3). A large body of evidence indicate a role for p53 in G1-S cell cycle transition and for progression of DNA replication in normal and tumour cells (2, 3). Further, p53 operates as a 'gate-keeper', managing a check point operating in late G1, and is thought to be responsible for maintaining genomic integrity following DNA damage (4-6). A p53-inducible gene, waf1 mediates p53-dependent cell cycle arrest by inhibiting cyclin dependent kinases (7, 8). Further, p53, at least in some cell types, is shown to mediate apoptosis upon extensive DNA damage induced by various agents (9, 10). Transcription of one of the apoptosis promoting gene, bax, is also known to be regulated by p53 (11). While, WAF1 and Bax could also be induced in a p53-independent manner, a role for p53 in transcriptional regulation of these genes provides clues into the possible molecular mechanisms of p53 function in cell cycle regulation.

MDA-468 breast cancer cells express a single allele for p53 with a point mutation at codon 273 resulting in an amino acid change from arginine to histidine ($p53^{273.His}$) (12, 13). Several studies have clearly demonstrated a role of $p53^{273.His}$ in proliferation and tumourigenicity of MDA-468 cells. Exogenously introduced wild-type p53 abolished the focus formation ability of MDA-468 cells (14, 15) . Down regulation of endogenous p53, on the other hand, by a flavonoid compound, quercetin, was reported to cause cell cycle arrest (16). We are interested in understanding the molecular mechanisms underlying EGF-mediated G1 arrest in MDA-468 cells (17). We have previously observed several immediate effects of EGF on $p53^{273.His}$ such as decreased phosphorylation and altered protein conformation. Briefly, in EGF-treated cells nuclear p53 displayed loss of mutant-specific, PAb 240 epitope, while retaining wild-type-specific, PAb 1620 reactivity as indicated by immunofluorescence studies (18). We have hypothesised that EGF may be shifting the $p53^{273.His}$ conformation into a wild-type form, in turn, causing G1 arrest. As a part of our study to elucidate possible role for $p53^{273.His}$ in EGF-induced G1 arrest, it was necessary to obtain information regarding the function of endogenous $p53^{273.His}$ in MDA-468 cells, given the ambiguous reports from transfection and *in vitro* systems. The DNA sequence first identified as p53-response element, fragment A (FRA), was not bound by $p53^{273.His}$ (19, 20). Moreover, the authors observed that $p53^{273.His}$ interfered with FRA-binding and FRA-dependent transcriptional activation function of wild-type p53 under

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in vitro conditions (21). In contrast, a *con*sensus sequence (CON) was later shown to be bound by $p53^{273.His}$ similar to wild-type p53 (22). In addition, $p53^{273.His}$ was demonstrated to activate transcription through specific-binding to CON sequence, once again, to an extent similar to that of wild-type p53 (23).

To this end, we have carried out a detailed study of specific DNA binding and transcriptional activation by $p53^{273.His}$ in MDA-468 cells using the two different p53-response sequences, FRA and CON, described above. In the literature, it has been documented that only wild type p53 binds to and transactivates from FRA, while both wild-type p53 and $p53^{273.His}$ have been shown to interact with the CON sequence. We have further tested the transcriptional activity from promoters containing p53 response elements, CON and FRA, in MDA-468 breast cancer cells.

MATERIALS AND METHODS

Antibodies used. PAb 1801 (Ab 2), a human-specific antibody, reactive with both wild-type and mutant p53 (24), PAb 240 (Ab 3), a mutant-specific antibody (25), and PAb 421 (Ab 1), a mammalian p53-specific antibody reactive with both wild-type and mutant forms of p53 (26), were obtained from Oncogene Science. PAb 1620, human wild-type p53-specific antibody (27) was provided as a gift by Dr. Heinz-Kurt Hoechkoppel, Ciba-Geigy, Switzerland.

Cell culture. MDA-468, human breast cancer cell line was maintained in Leibovitz-15 modified medium (ICN/Flow) supplemented with 10% FBS (ICN/Flow & Gibco), 50 IU/ml penicillin and 50 μg/ml streptomycin (ICN/Flow). Cells were routinely grown in 100 mm plates and or 75 cm2 flasks. A concentration of 10^{-8} M of EGF (Collaborative Research Inc.) for 6 h was employed in our experiments to study any EGF-mediated changes in p53 DNA binding activity.

Oligonucleotides. The following oligos were employed for electrophoretic mobility shift assays (EMSA) and for construction of p53-responsive elements. The oligos 5′-GTCCGGACATGCCCGGGCAT-3′ and 5′-GGACATGCCCGGGCATGTCC-3′ were annealed to obtain a double stranded fragment with 5′-overhang, corresponding to the CON fragment (22) and the latter oligo 5′-GGACATGCCCGGGCATGTCC-3′ was self-annealed to obtain blunt-ended double-strand-CON. A 33-base sequence 5′-TTCTCCTTGCCTGGACTTGCCTGGCCTTGCCTT-3′ and 5′-AGAAAAGGCAAGGCCAGGCAAGTCCAGGCAAGG-3′ were annealed to obtain a double stranded fragment with 5′ overhang, corresponding to the bases 106-138 of fragment A (FRA) (20). 5′-CCTTGCCTGGACTTGCCTGGCCTTGCCTTTTCT-3′ was annealed with 5′-AGAAAAGGCAAGGCCAGGCAAGTCCAGGCAAGG-3′ to obtain blunt-ended double-stranded FRA. The individual oligos were custom synthesised and obtained from OLIGOS ETC. Inc. A double stranded oligo with sequence corresponding to NF1 binding sequence 5′-AACCTAATTGCATATTTGGCATAAGGTTT-3′, was used as a non-specific competitor in electrophoretic mobility shift assays.

Nuclear extract preparation. Semi-confluent plates (approx. 3×10^6 cells/100 mm dish) after 24 h of plating were further incubated +/− EGF (10^{-8} M) for 6 h. At the end of the treatment, plates were washed 3× with ice-cold PBS. Cells were scraped with 1.5 ml of Buffer A (20 mM Hepes [pH 7.6], 20% glycerol, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 10 μg/ml leupeptin, 100 μg/ml aproteinin, 1 mM PMSF {(28)}) per 100 mm plate. Cell suspension was centrifuged at 2000 rpm at 4°C in a microfuge. The pellet (nuclei) was resuspended in 0.5 ml of Buffer B (essentially identical to Buffer A except 0.5 M NaCl). The suspension was rocked gently for 1 h at 4°C followed by centrifugation at 10,000 rpm for 5 min. Supernatant was recovered and protein concentrations of the extracts were estimated using a Bio-Rad protein assay dye reagent (29).

Electrophoretic mobility shift assays. Double stranded oligos (CON or FRA; 0.5 μg) with 5′-overhang, were incubated with 20 units of AMV reverse transcriptase (Pharmacia), 5 μl dNTP mix (excluding dATP or dCTP, depending on the isotope used; [Nick-translation kit Gibco/BRL]), 5.0 μl [α-^{32}P]-dATP (3000 Ci/mmol; NEN/DuPont), ([α-^{32}P]-dCTP [3000 Ci/mmol; Amersham] was used for CON in some experiments), in reverse transcriptase buffer at 37°C for 1 h. Oligos were then precipitated and were recovered by centrifugation at 14,000 rpm for 30 min at 4°C. Labelled oligos were washed twice with 70% ethanol, air dried for 15-30 min, resuspended with sterile d.H20. Aliquots of the samples were counted in a scintillation counter.

DNA binding reactions were performed with nuclear extracts equivalent of 3 μg of protein in binding buffer (1 mM $MgCl_2$, 0.5 mM DTT, 7% glycerol, 10 mM Hepes [pH 7.4], 1.0 μg sonicated salmon sperm DNA, and 25,000 cpm of end-labelled probe). The reaction was carried out at room temperature for 30 min. Appropriate antibodies, as indicated, were incubated with nuclear extracts prior to the binding reaction for 15 min on ice. The complexes were resolved on a 4% native polyacrylamide gel. A pre-run at 100 V was performed for 2 h at 4°C with buffer recirculation, followed by the electrophoresis of the samples for 3-4 h with 0.5 × TBE (0.045 M Tris borate, 0.001 M EDTA [pH 8.0]) at 4°C with buffer recirculation. At the end of the run, the gels were dried at 80°C for 1 h and exposed to Kodak XAR film at −70°C.

Construction of p53-responsive plasmids and chloramphenicol acetyl transferase (CAT) assay. Equimolar concentrations of appropriate oligos were annealed as described above to obtain blunt-ended double strand oligos. These oligos, corresponding to CON and FRA, were 5′ phosphorylated using T4 kinase (Gibco/BRL) and ATP. 5′-phosphorylated oligos were inserted into the SalI site of the vector pBLCAT2 (30). The ligated plasmids were then used to transform competent E. coli (strain XL-Blue) cells. Ampicillin-resistant colonies were screened for inserts by restriction analysis. Recombinant plasmids were prepared in large scale by caesium chloride centrifugation and amount of DNA was estimated spectrophotometrically.

Semi-confluent plates, 24 h after plating, were transfected by calcium phosphate precipitation method (31) using 5-10 μg DNA (5 μg CAT-constructs, and 2 μg internal control, RSV-β-gal) per 100 mm plate. Transfected cells were harvested by scraping after 72 h with Tris.EDTA.NaCl (0.04 mM Tris.HCl [pH 7.4], 1 mM EDTA, 150 mM NaCl). CAT assays were carried out by thin layer chromatography method (31). CAT activity was measured as percent conversion of chloramphenicol to acetyl-chloramphenicol. This was done by counting radioactivity from portions of TLC plate corresponding to both unconverted and converted forms in a scintillation counter. Aliquots of cell extracts were estimated for protein concentrations by a Bio-Rad protein-estimation kit. CAT activities were standardised to protein concentrations. An internal control, RSV-β gal was used in all the experiments. The β- galactosidase activity, however, seemed to be influenced by p53 status and EGF treatment, therefore was not used for standardisation of values.

RESULTS

Nuclear extracts from MDA-468 cells were tested for their ability to form specific complexes with the p53-response elements in electrophoretic mobility shift assays. 3 μg of protein from nuclei of MDA-468 cells was incubated with end-labelled double-stranded oligos corresponding to CON and FRA. We have observed mobility shifts for both CON and FRA in MDA-

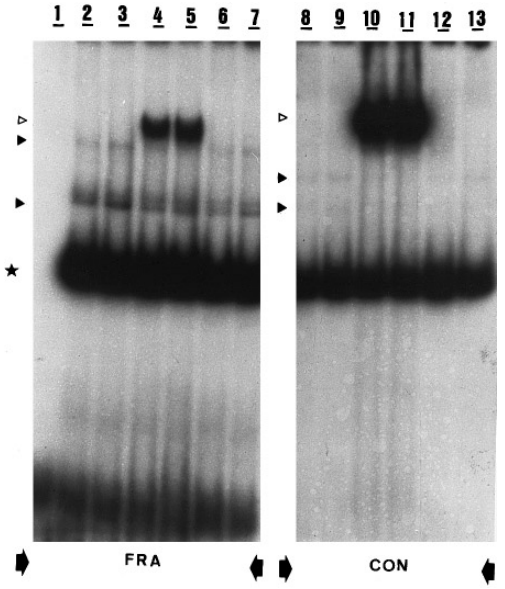

FIG. 1. DNA-binding ability of $p53^{273.His}$. Nuclear extracts (3 μg) from MDA-468 cells were analysed for mobility shifts of end-labelled oligos representing p53-specific sites (left panel FRA oligo, right panel CON oligo). Lane 1, no nuclear extract; lanes 2, 3, 8 & 9, nuclear extract alone; lanes 4, 5, 10 & 11, nuclear extract and PAb 421 (0.1μg); lanes 6, 7, 12 & 13, nuclear extract and PAb 1620 (1.0 μg). The figure is an 18 h exposure of the autoradiograph. * is a non-specific complex. Solid arrowheads indicate specific p53-DNA complexes and antibody-supershifted complexes are denoted by open arrowheads. Free probe has run off the gel. Odd numbered lanes are nuclear extracts from EGF (10^{-8}M)-treated cells.

468 nuclear extracts (Fig. 1). The fastest migrating complex (denoted by a star) was non-specific since none of the anti-p53 antibodies altered its mobility and was completely competed out with a non-specific competitor corresponding to NF1 binding site (data not shown). This complex has been consistently observed by several others in EMSA experiments with p53 binding sites (32-34). The two slower migrating complexes (denoted by solid arrow heads) contained p53 molecules, as indicated by their reactivity with anti-p53 antibodies. PAb 421 not only supershifted the complexes (denoted by open arrow heads) but also significantly enhanced the DNA binding of p53 (Fig. 1, lanes 4 & 5; lanes 10 & 11). This increase in DNA binding was more pronounced with CON than with FRA. On the other hand, PAb 1620 (wild-type specific/conformation-dependent), reduced the specific complexes (Fig. 1, lanes 6 & 7; lanes 12 & 13). Increased concentrations, up to 5 μg, of PAb 1620 totally prevented the specific p53-DNA complex formation (data not shown). We have further carried out a series of EMSAs in the presence of different anti-p53 antibodies used, alone or in combination with PAb 421, to understand the nature of p53 DNA binding in MDA-468 cells.

PAb 240, mutant-specific antibody, prevented specific p53-CON interaction resulting in decreased complex formation (Fig. 3, compare lanes 2 & 3 with 10 & 11). No supershift was observed with PAb 240. This was confirmed with longer exposures (data not shown). Interestingly, there was no interference with p53-FRA complexes by PAb 240 (Fig. 2, compare lanes 2 & 3 with 10 & 11). PAb 1620 had the interfering effect on p53-DNA complexes with both CON and FRA (Fig. 1, compare lanes 2 & 3 with 6 & 7 and lanes 8 & 9 with 12 & 13). In the presence of PAb 1801, reduced formation of primary complexes were observed as well (Fig. 2 & 3, compare lanes 2 & 3 with 12 & 13). Taken together, these results indicate specific interaction of p53 molecules with two of the characterised p53 recognition sites, FRA and CON sequences.

When PAb 240, PAb 1620, or PAb 1801 were included along with PAb 421 in the binding reaction, interesting observations were made. There was near total abolition of the FRA-p53 complexes by PAb 1620 and PAb 421 in combination (Fig. 2, lanes 4 & 5). A considerable amount of FRA-p53 complex was present in the presence of PAb 240 and 421 (Fig. 2, lanes 8 & 9). Comparable amounts of CON-p53 complexes were detected using either PAb 1620 (Fig. 3, lanes 4 & 5) or PAb 240 (Fig. 3, lanes 8 & 9) along with PAb 421. PAb 1801 supershifted CON-p53 complexes without further increasing the binding in the presence of PAb 421. (Fig. 2 & 3, lanes 6 & 7).

A majority of the information concerning the function of $p53^{273.His}$ is from transfection studies employing exogenous p53 constructs. Therefore, we wished to estab-

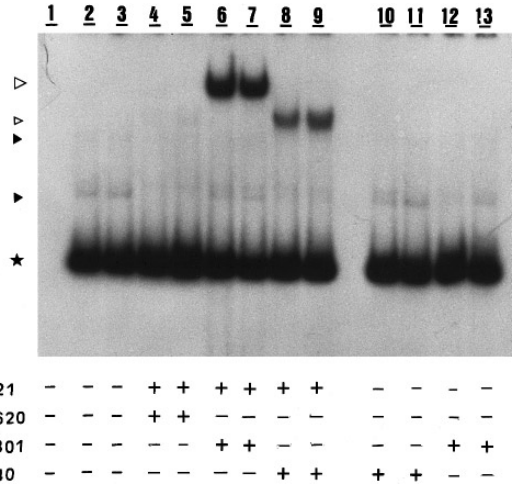

FIG. 2. Effect of anti-p53 antibodies on p53-FRA interaction. Nuclear extracts (3 μg) from MDA-468 cells were analysed for interaction with FRA. Lane 1, no nuclear extract; lanes 2 & 3, nuclear extract alone. The anti- p53 antibodies included in the binding reaction are PAb 421 (0.1 μg), PAb 1620 (1.0 μg), PAb 1801 (0.1 μg), and PAb 240 (0.1 μg). The figure is a 24 h exposure of the autoradiograph. * is a non-specific complex. Solid arrowheads indicate specific p53-DNA complexes, and antibody-supershifted complexes are denoted by open arrowheads. The free probe has run off the gel. Odd numbered lanes are nuclear extracts from EGF (10^{-8}M)-treated cells.

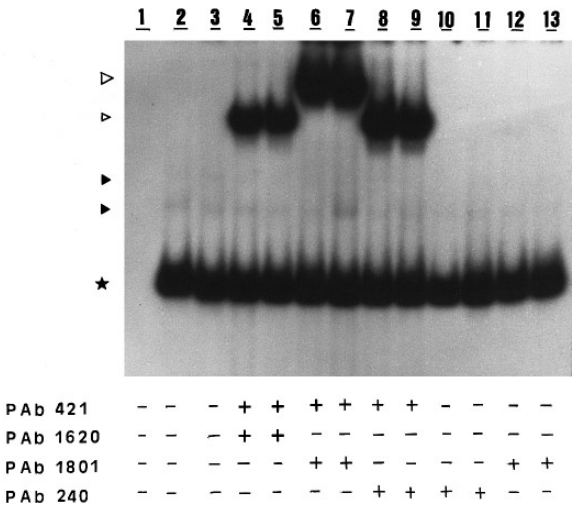

FIG. 3. Effect of anti-p53 antibodies on p53-CON interaction. Nuclear extracts (3 μg) from MDA-468 cells were analysed for interaction with CON. Lane 1, no nuclear extract; lanes 2 & 3, nuclear extract alone. The anti- p53 antibodies included in the binding reaction are PAb 421 (0.1 μg), PAb 1620 (1.0 μg), PAb 1801 (0.1 μg), and PAb 240 (0.1 μg). The figure is a 24 h exposure of the autoradiograph. * is a non-specific complex. Solid arrowheads indicate specific p53-DNA complexes, and antibody-supershifted complexes are denoted by open arrowheads. The free probe has run off the gel. Odd numbered lanes are nuclear extracts from EGF (10^{-8}M)-treated cells.

lish the transcriptional activation function of endogenous $p53^{273.His}$ in MDA-468 cells. Since we have detected comparable levels of DNA binding to both CON and FRA in the absence of PAb 421, contrary to the previous reports, it was essential to determine the functional consequences of the observed DNA binding activity. To this end, the p53 response DNA elements were cloned upstream of a minimal promoter regulating a chloramphenicol acetyl transferase (CAT) reporter gene (pBLCAT2). This vector contains a minimal promoter with a TATA box from the herpes simplex virus thymidine kinase gene (30). In transient transfection assays carried out in MDA-468 cells, CON sequences conferred about 11-fold activation of transcription. Unexpectedly, FRA sequences mediated about 10-fold suppression of transcription compared to that from vector alone (Fig. 4). This was an interesting observation given that no FRA-mediated modulation of transcription by $p53^{273.His}$ has been reported before. Co-transfection of SV 40 T antigen cDNA driven by the SV 40 enhancer/promoter blocked p53-response element mediated-effect, both activation and repression, confirming the involvement of p53 in this process. SV 40 T antigen co-transfection with the minimal promoter alone containing no p53 binding sites, interestingly , resulted in about 3 fold increase in transcription.

DISCUSSION

We have been interested in EGF-mediated growth inhibition of MDA-468 breast cancer cells. Earlier, we had reported that EGF induces a late G1 arrest (17) and that EGF-induces shift in the conformation of endogenous $p53^{273.His}$ protein in MDA-468 cells (18). This conformation shift mediated through altered phosphorylation, we hypothesised, may induce a reversible late G1 arrest. In this article, we report a part of our study involving the characterisation of a novel transcriptional regulatory function of $p53^{273.His}$ in MDA-468 cells. We provide evidence that endogenous $p53^{273.His}$ forms specific complexes with two of the p53-response DNA elements (CON and FRA). Moreover, these DNA sequences had opposite effect on p53-mediated transcription. Briefly, in MDA-468 cells, $p53^{273.His}$ activates transcription from CON and represses through FRA.

Electrophoretic mobility shift assays provided interesting clues regarding the nature of p53 molecules in MDA-468 cells. It was observed earlier, $p53^{273.His}$ has unusual properties unlike a majority of other mutants. These include PAb 1620 reactivity, ability to bind SV 40 T antigen, transcriptional regulatory function, longer half life, and PAb 240 reactivity. Previous attempts to characterise the nature of p53 molecules in MDA-468 cells offered two explanations. The first one, suggested a 'pseudo-wild-type' form for $p53^{273.His}$ distinct from both wild-type and mutant conformations. The second proposed the existence of two conformationally distinct, wild-type and mutant p53 molecules in MDA-468 cells. However, our observations with EMSA can be satisfactorily explained by the presence of two conformationally distinct wild-type and mutant molecules in MDA-468 cells. While only wild-type-like molecules may able to bind to FRA, both mutant- and wild type-like molecules may bind to CON as suggested by previous studies employing p53 expression vectors. PAb 1620, then conceivably, sequesters the wild-type

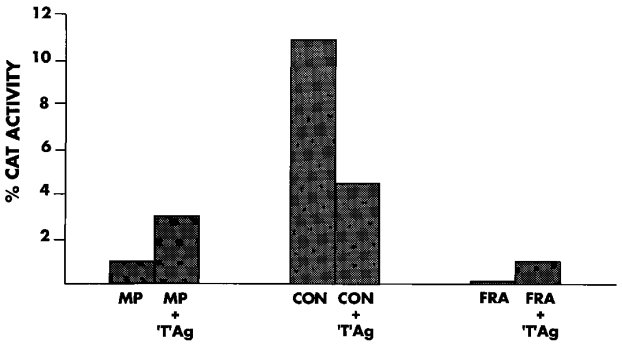

FIG. 4. p53-response element-mediated transcriptional activity. MDA-468 cells (2×10^6 cells/100mm plate) were transfected with 5 μg of minimal promoter-CAT (MP-CAT/pBLCAT2), CON-CAT, or FRA-CAT constructs as described in Materials and methods. SV 40 T antigen (5 μg/100mm plate) was co-transfected as a control. CAT activity was measured 48 h post-transfection and was equalised to protein concentrations. The % conversion (CAT activity) by MP-CAT was standardised to 1.0 and the remaining values were correspondingly adjusted.

like molecules leaving mutant-like molecules free, which can form complexes only with CON and not with FRA. This explains the marked interference that was observed with FRA-p53 complexes (Fig. 2, lanes 4 & 5) and persistence of CON-p53 complexes in the presence of PAb 1620 and PAb 421 (Fig. 3, lanes 4 & 5). PAb 240, on the other hand, when used alone, had no effect on FRA-p53 complex formation (Fig. 2, lanes 10 & 11), but prevented p53-CON interaction (Fig. 3, lanes 10 & 11). In combination with PAb 421, PAb 240 did not significantly prevent either FRA complex formation by the wild-type-like molecules or CON-p53 interaction (Fig. 2 & 3, lanes 8 & 9). Taken together, these experiments clearly indicate the co-existence of conformationally distinct wild-type-like and mutant-like p53 molecules in MDA-468 cells which differ in their DNA - binding specificity.

Our EMSA studies have indicated that $p53^{273.His}$ can bind specifically to both FRA and CON sequences. It is interesting to note that most of the previous work describing the lack of binding of $p53^{273.His}$ to FRA has been performed using p53 expression vectors *in vitro,* while we have utilised an endogenous protein along with its natural host setting. This could be a reasonable explanation for the apparent discrepancy between our findings and the previous reports. The carboxyl-terminal domain of p53 which is required for DNA-binding activity (35), was found to be influenced by a variety of post-translational mechanisms including phosphorylation, tryptic digestion, peptide and antibody (PAb 421)-binding (36, 37). The latter authors suggested that these and perhaps other, as yet unknown modifications, may convert the latent DNA-binding activity of cellular p53 into an active one (36). Intriguingly, it was also observed that cellular p53 in nuclear extract preparations bound to DNA, whereas *in vitro* translated-p53 failed to do so (22). These observations strengthen the notion of Hupp et al. (36)) that transcriptional activity of p53 may be influenced by interaction with other cellular factors. This view is further supported by a recent finding that a cellular protein activated DNA binding activity of p53 through direct physical interaction (38).

Furthermore, we have observed a unique transcriptional activity of $p53^{273.His}$ in MDA-468 cells. Our experiments clearly demonstrated activation of transcription from CON and interestingly, an active suppression from FRA sequences inserted in pBLCAT2. Taken together, these data indicate that $p53^{273.His}$ can mediate specific and unique effects in a sequence-dependent manner. Inclusion of SV 40 T antigen in the transfection studies was aimed at sequestering p53 from transactivation and DNA binding. It has been demonstrated that T antigen abrogates DNA-binding and transcriptional activity of p53 (39), and $p53^{273.His}$ is known to possess T antigen binding ability similar to that of wild-type p53 (2). In MDA-468 cells, SV 40 T antigen co-expression abrogated transcription from CON and FRA and from the minimal promoter as well (Fig. 4). This indicate that $p53^{273.His}$ is involved in transcriptional modulation from both minimal promoter and the p53-responsive elements. It has been demonstrated that wild-type p53 suppresses transcription from minimal promoters in the absence of p53-response elements(40). In this context, our observation that SV 40 T antigen-induced activation of transcription by a minimal promoter could then be interpreted as release of p53-induced suppression upon binding of p53 by T antigen.

Taken together, these novel transcription regulatory functions of $p53^{273.His}$ may be responsible for the observed *gain-of-function* phenotype of this mutant. Alternatively, $p53^{273.His}$ may be excerting a critical function through an as-yet-unidentified sequence. This suggestion is particularly important because EGF, in growh inhibitory concentrations, apparently had modest increase in DNA binding activity at best (compare odd numbered, EGF-treated, lanes with even numbered, untreated, lanes in Fig. 1 to 3) and further had little effect on the transcriptional activity through CON and FRA (data not shown). Recent findings suggest an alternate function of high affinity DNA interaction with nuclear matrix/scaffold attachment regions (MAR/SAR) for mutant p53 that may be responsible for gain-of-function phenotype of some mutants (41). Another report documents that some mutants of p53, including $p53^{273.His}$, are capable of stimulating transcription from promoter for Insulin-like growth factor I receptor gene, while wild-type p53 acts as repressor of transcription (42). Taken together, evidence is compelling for an altered functioning of mutant p53 molecules in tumour cells. In this context, our findings suggest a novel transcriptional regulatory function for $p53^{273.His}$ in MDA-468 cells, a combination of sequence specific transactivation and repression functions. While, $p53^{273.His}$ clearly possess sequence-dependent DNA binding and transcription regulatory functions, the cellular environment may determine the ultimate effect that would be essential for a particular cell cycle stage. Identification of specific DNA sequences mediating the EGF-mediated growth arrest and characterisation of putative altered molecular interaction involving p53 and transcriptional apparatus will further our knowledge of biological activities of p53 in tumour cells.

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