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TRANSCRIPTIONAL REGULATION OF THE PCNA PROMOTER BY p53

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Summary. We have examined the ability of p53 to affect transcription from the PCNA promoter in a number of cell types. In HeLa cells, p53 activates the PCNA promoter whereas in CV1, CHO, L929, and Saos-2 cells the same promoter is strongly repressed. By using stepwise deletions of the PCNA promoter, we have identified two potential regions of the promoter which are important for the ability of p53 to activate transcription in HeLa cells.

Much evidence suggests that the function of the p53 tumour suppressor protein is to regulate cell cycle progression in response to DNA damage (1-3). Although the precise biochemical action of p53 is not yet understood, available data suggest that at least part of its mechanism involves transcriptional regulation. Thus p53 can activate and repress transcription from several natural and artificially constructed promoters (4). In addition, two genes, GADD45 (5; which is activated in response to DNA damage) and WAF1/CIP1 (6; which causes growth arrest) have recently been identified as *in vivo* targets for p53 regulation.

Another potential target for p53-dependent regulation is the Proliferating Cell Nuclear Antigen (PCNA) gene. PCNA, also known as DNA polymerase-∂ auxiliary factor, is an essential component in both DNA replication (7) and DNA repair (8). The levels of PCNA expression are also linked to cell growth (9). Consistent with this notion, levels of PCNA mRNA and protein were found to be lowered in glioblastoma cells after growth arrest induced by p53 (10) and transcription from this promoter was shown to be down-regulated in HeLa cells by wild-type (wt) human p53 and up-regulated by mutant forms of p53 commonly associated with human cancers (11).

Using co-transfection studies, we have further analysed the effects of mouse and human p53 on the human PCNA promoter in a variety of cell types. In this study, we confirm that p53 down-regulates transcription from the PCNA promoter in several different cell lines, however, we find in

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our HeLa subline (ATCC CCL2) that the PCNA promoter is activated. We have used this observation in HeLa cells to identify region(s) within the PCNA promoter which are important for the ability of p53 to activate transcription. We report here that activation of the PCNA promoter by p53 is probably not defined by a single sequence.

MATERIALS AND METHODS

Cell Culture, Transfection and CAT Assays: These were carried out as described previously (12) with the following exceptions:

Chinese hamster ovary (CHO) cells were maintained in MEM containing 4% FCS supplemented with 0.1mM non-essential amino acids (Gibco BRL, Grand Island, New York, USA) and for transfection of Saos-2 cells, a $SrPO_4$ method was used as described (13).

Plasmids: The isolation, characterization and cloning of upstream sequences of the human PCNA gene-promoter into pBACAT to create a plasmid encoding sequence between -1265 to +62 of the PCNA promoter fused upstream of the bacterial CAT gene (PCNA CAT) have been described (14).

Control plasmid CMV neo (15; encoding the *neo* resistance gene), CMV Nc9 (16; encoding wt mouse p53 and obtained from Dr. M. Oren, Weizmann Institute, Rehovot, Israel), CMV dl 163 (17; encoding a mutant p53 protein lacking amino acid residues 14-66 and obtained from Dr. J. Jenkins, Marie Curie Research Institute, Oxted, UK), CMV hp53wt (encoding wt human p53 provided by Dr. R. Reddel, Children's Medical Research Foundation, Sydney, Australia) and pCMV $p273_{his}$ (18; encoding a human p53 protein with an R to H mutation at codon 273 also obtained from Dr. J. Jenkins, Marie Curie Research Institute, Oxted, UK) all express their respective encoded proteins from the human cytomegalovirus early promoter/enhancer. SV_2CAT (19) expresses the bacterial CAT gene under the control of the SV40 virus early enhancer/ promoter.

Antibodies and Immunoprecipitations: p53 proteins were precipitated from transfected cells with several different monoclonal antibodies. PAb 122 (20) and PAb 421 (21) recognise wt and mutant mammalian p53 proteins. PAb1620 (22) specifically recognises wt mammalian p53 proteins whilst PAb 240 (23) specifically recognises mammalian mutant p53 proteins.

Preparation of lysates from transfected cells labeled in medium containing 150μCi/ml of ^{35}S-Translabel (1192 Ci $mmol^{-1}$, ICN Biomedicals, Irvine CA, USA) and immuno- precipitations were performed as described previously (12).

RESULTS

Wt mouse and human p53 transactivate the human PCNA promoter in transfected HeLa cells

HeLa cells were co-transfected with a reporter plasmid containing 1265bp of upstream promoter sequence of the human PCNA gene and 62bp of transcribed sequence (14), together with a control plasmid (CMV neo) or plasmids expressing wt and mutant p53 proteins. In a series of ten independent experiments, we found that wt mouse (CMVNc9) and human p53 (CMVhp53wt) consistently transactivated the PCNA promoter. Results from typical experiments are presented in Figs. 1A and 1C. Mean activation of the PCNA promoter by p53 was 3-fold but the range of

activation extended to 7-fold. Moreover, by varying the amounts of CMV Nc9, we observed a clear dose-dependent increase in activation of the PCNA promoter (Fig. 1B). We also found, as previously reported, that the human p53 mutant CMV273$_{his}$ activated the PCNA promoter (Fig. 1C). By contrast, the mouse p53 mutant (CMV dl 163) failed to activate the PCNA promoter (Fig.

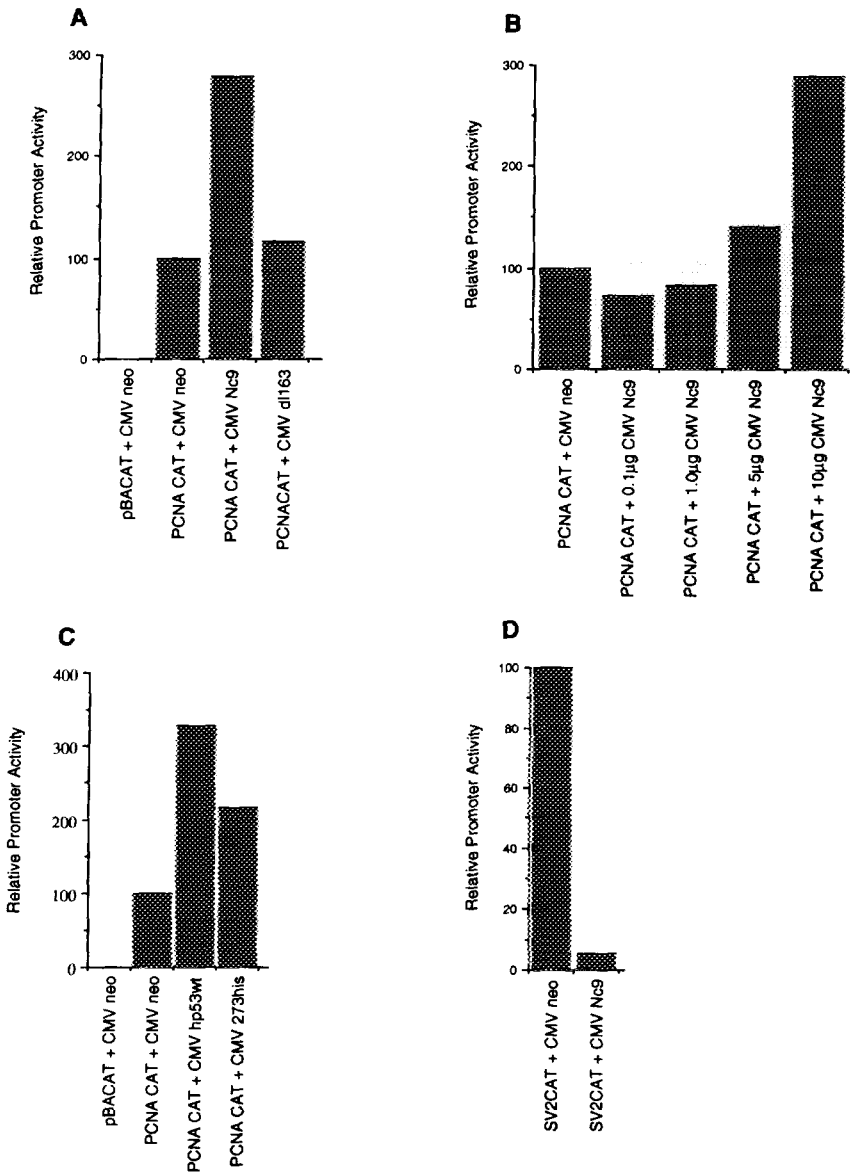

Fig. 1. Activation of the human PCNA promoter by wt mouse and human p53. (**A**) HeLa cells were transfected with the indicated plasmids as described in Materials and Methods. Data presented are representative of ten (with CMV Nc9) and three (CMV dl 163) independent experiments. (**B**) Hela cells were transfected with 10µg of CAT reporter plasmid and increasing amounts of CMV Nc9 as indicated. In all cases, the total amount of DNA transfected was maintained at 20µg by the addition of control plasmid. Data presented are representative of two independent experiments. (**C**) As for (**A**) except that CMVhp53 wt was used. (**D**) HeLa cells were co-transfected with 10µg SV_2CAT and 10µg of either CMV neo or CMV Nc9. Data presented are representative of five independent experiments.

1A), despite the fact that it was highly expressed and showed a wt-like conformation (see Fig. 2 below). As this mutant is deleted between residues 14 and 66, this finding supports a previous report with p53 fusion proteins (24) that the transactivation domain of p53 is located in the N-terminus.

Although the degree of activation of this promoter was quite small, in parallel dishes we could nonetheless obtain a 20-fold repression of the SV40 promoter (Fig. 1D) as previously reported (12). Thus, the limited degree of transactivation of the PCNA promoter is probably not due to poor cotransfection frequencies.

We conclude that both wt mouse and human p53 can transactivate the human PCNA promoter in HeLa subline ATCC CCL2.

Expression of endogenous p53 protein in HeLa cells

Initial studies of HeLa cells indicated that these cells did not express detectable levels of endogenous p53 protein even though they contained p53 mRNA (25). The absence of p53 protein is now thought to be due to the expression in HeLa cells of human papillomavirus type 18 E6 protein which binds to and promotes the degradation of p53 (26). Two recent reports, however, have suggested that endogenous p53 protein is present in HeLa cells (27, 28). In the former study, substantial endogenous p53 protein was detected after transfection with a plasmid expressing the mouse p53 mutant dl 163, whilst in the latter report, endogenous p53 was detected by its ability to bind a consensus DNA motif in gel-shift assays. Significant levels of endogenous p53 protein might well effect the levels of PCNA expression making interpretation of data from our transfection experiments complicated.

To determine if endogenous p53 protein was expressed in the HeLa cells used for this study, we transfected HeLa cells with a non-specific control plasmid CMV neo or CMV dl 163. Cells were then labeled with ^{35}S-methionine, lysates prepared and p53 proteins immunoprecipitated with several different p53 monoclonal antibodies. Results (Fig. 2) show that in cells transfected with the control plasmid, no endogenous p53 protein was detected with PAb1620 (22; specific for wt conformation of p53, lane 1), PAb240 (23; specific for mutant conformation of p53, lane 2),

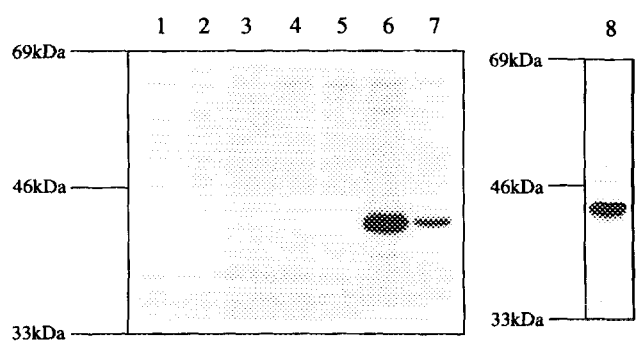

Fig. 2. Expression of endogenous and transfected p53 proteins in HeLa cells. Cells were transfected with 20μg of control plasmid (lanes 1-4) or 10μg each of control plasmid and CMV dl 163 (lanes 5-8). After 72h, cells were labeled for a further 2h with ^{35}S-methionine and cell lysates prepared. Lysates were then immunoprecipitated with either PAb1620 (lanes 1, 6), PAb240 (lanes 2, 5), PAb122 (lanes 3, 7,) or PAb421 (lanes 4, 8,) as described (12).

Table 1. Effect of wt and mutant mouse p53 on the human PCNA promoter after transfection into different cell types

Plasmids Used*	No of expts.	Mean Relative Promoter Activity (range)	Overall effect of p53
CV1 cells			
PCNA CAT + CMV neo	4	100	-
PCNA CAT + CMV Nc9	4	12 (4 - 21)	8-fold repression
PCNA CAT + CMV dl163	4	37 (12 - 71)	3-fold repression
L929 cells			
PCNA CAT + CMV neo	4	100	-
PCNA CAT + CMV Nc9	4	16 (10 - 36)	5-fold repression
PCNA CAT + CMV dl163	4	75 (23 - 134)	no effect
CHO cells			
PCNA CAT + CMV neo	4	100	-
PCNA CAT + CMV Nc9	4	4 (2 - 8)	25-fold repression
PCNA CAT + CMV dl163	4	13 (7 - 21)	8-fold repression
SAOS-2			
PCNA CAT + CMV neo	3	100	-
PCNA CAT + CMV Nc9	3	23 (12 - 33)	4-fold repression
PCNA CAT + CMV dl163	3	215 (168 - 300)	2-fold activation

* For each cell-type, 10μg per plasmid was used for transfection as described (12).

PAb122 (20; recognises wt and mutant p53, lane 3) or PAb421 (21; recognises wt and mutant p53, lane 4). Furthermore, no band corresponding to the size of endogenous wt p53 was detected in cells transfected with the mouse p53 mutant dl 163 using any of the above antibodies (lanes 5-8). Expression of the mouse p53 deletion mutant dl 163, however, was clearly detected using PAb1620 (lane 6), PAb 122 (lane 7) and PAb421(lane 8). Interestingly, dl 163 was not detected with PAb 240 (lane 5) which is specific for the mutant conformation of p53.

We conclude that the HeLa cells used in these studies do not express detectable p53 protein. Thus activation of the PCNA promoter is due to the introduction of mouse p53 into these cells.

Table 2. Effect of mouse p53 on Transcriptional Activities from fragments of the human PCNA promoter

PCNA-CAT Reporter Plasmid	Activity Relative to full promoter (-1265 to +62)	Mean Relative Activity Activity in the presence of p53 (range)	N*	Overall Effect of p53
-1265 to +62CAT	100	3.4 (1.2 - 6.8)	3	activation
-397 to +62CAT	93	3.3 (1.4 - 5.7)	4	activation
-249 to +62CAT	110	1.0 (0.2 - 1.9)	5	no effect
-172 to +62CAT	20	1.0 (0.4 - 2.2)	5	no effect
-87 to +62CAT	2	4.3 (3.9 - 4.8)	2	activation
-47 to +62CAT	0.5	7.9 (3.6 - 16.9)	5	activation

HeLa cells were co-transfected with 10μg of each of the indicated reporter plasmids and 10μg of either a control plasmid or pCMVNc9 and assayed for CAT activity as described (12).
*Number of experiments.

Cell-type dependent regulation of the PCNA promoter

Because a previous report had shown that p53 repressed transcription of the PCNA and other promoters in HeLa cells (11), we decided to determine how general transactivation is of the PCNA promoter by p53. We therefore examined the ability of mouse p53 to regulate transcription of the human PCNA promoter in different cell lines. In all cases, expression of the mouse p53 in transfected cells was confirmed by immunoprecipitation (data not shown). These data (summarized in Table 1) show that wt mouse p53 causes significant repression of the PCNA promoter in all cell types tested, including in the p53-deficient cells, Saos-2. In most cell-types dl 163 was less effective at causing repression of the PCNA promoter and in Saos-2 it caused a slight activation.

We conclude that with this type of assay system, in the generality of cases wt p53 represses transcription of the PCNA promoter.

Location of PCNA promoter sequences required for p53-dependent activation in HeLa cells

To locate PCNA promoter sequences responsive to mouse p53 in HeLa (ATCC CCL2) cells, we made use of several reporter plasmids containing various fragments of the PCNA promoter (14; Table 2). In a series of control experiments (Table 2), we observed relative levels of activity from these plasmids similar to those previously reported (14).

The effect of p53 on the activity from each of the promoter plasmids was then determined by co-transfecting each reporter plasmid with either a control plasmid (CMV neo) or CMV Nc9 expressing wt mouse p53. Results of these experiments are summarized in Table 2. Consistent with our initial findings, p53 activated transcription from the full-length PCNA promoter (-1265bp to +62bp) about 3-fold and still activated to the same extent when the promoter was deleted to -397bp of upstream sequence(-397 to +62, Table 2). When the promoter was deleted to 249bp or 172bp of upstream promoter sequence, p53 was now unable to activate transcription. These data suggest that there is a p53-responsive element in the PCNA promoter between -397bp and -249bp.

However, further deletion of the PCNA promoter to 87bp and 47bp of upstream sequence, which reduced promoter activity to near basal levels (Table 2), now restored activation by p53 (Table 2). This result suggests that there is a second potential p53-response element between -47bp and +62bp, broadly in the region of the 'core' promoter.

DISCUSSION

Recent reports have identified cellular genes whose promoters are regulated by p53, notably, GADD45 (5) and WAF1/CIP1 (6). With a role in both DNA replication (7) and repair (8), PCNA represents another potential target for regulation by p53. Two reports have shown that p53 down-regulates the PCNA promoter (10, 11) whilst a third found that p53 had no effect (29). Interestingly, studies of p53 and PCNA protein levels in cells after UV-irradiation have shown that PCNA levels rise concommitantly with an increase in p53 (2) and parallel changes in p53 and PCNA have been found in a panel of bladder cancer cell lines expressing wt p53 (30).

We have extended these observations by examining the effect of both mouse and human wt p53 on the PCNA promoter after transfection into different cell types. In contrast to a previous report (11), we found that mouse and human wt p53 both activated transcription from this promoter in

our HeLa subline (Fig. 1A and 1C). Interestingly, we also obtained transactivation with human mutant 273_{his} as reported (11; Fig. 1C). In human Saos-2, monkey CV1, hamster CHO and mouse L929 cells however, we found that the introduced wt mouse p53 actually repressed transcription from the same promoter (Table 1). Mouse mutant dl 163 failed to activate the PCNA promoter in HeLa cells and either failed, or was severely reduced in its ability, to repress the PCNA promoter in other cell types. These data support previous conclusions that activation (24) and repression (12) domains are located in the N-terminus of p53.

The explanation for activation of the PCNA promoter in HeLa cells by p53 but repression in all other cell types tested, is not clear. In transient assays with our p53 constructs, we consistently obtain much greater levels of p53 protein expression in HeLa cells than in any other cell type we have examined (12 and unpublished observations). Thus, it is possible that low levels of p53 cause repression but high levels cause activation of the PCNA promoter. The observation that PCNA increases concomitant with p53 stabilisation in UV-treated cells (2) is consistent with this explanation. Some support for this suggestion also comes from studies with p53 mutants which activate the PCNA promoter (11; Fig. 1C) and which are post-translationally stabilised leading to increased protein levels. In this context, studies of certain breast cancer cells have suggested that very high levels of wt p53 may function like mutant p53 (31).

To identify sequences which allow p53 to activate the PCNA promoter in HeLa cells, we analysed the effect of p53 on transcription from a series of PCNA promoter deletions (14). p53 was able to activate promoter sequences containing 1265bp (full promoter) and 397bp of upstream sequence but did not activate transcription from plasmids containing only 249bp or 172bp of upstream sequence (Table 2). These data indicate that there is a p53-responsive element between nucleotides 397 to 249. Examination of the PCNA promoter sequence has not however identified a sequence between -397 and -249 which is homologous to the consensus p53-binding motif (32).

Further deletion of the PCNA promoter to 87bp and 47bp of unstream sequence, surprisingly, restored the ability of p53 to transactivate in HeLa cells (Table 2). These data suggest that there is a second p53-responsive element between -47bp and +62bp, but again there is no consensus binding site within this region. Furthermore, the data also suggest there are DNA elements between -87bp and -249bp that silence this 'proximal' p53 activation region.

In the absence of known p53-binding sites in the PCNA promoter, the mechanism of p53 activation is unclear. Conceivably, these sequences may contain novel p53-binding sites. However, p53 may function through interaction with other transcription factors. Recent reports have shown that p53 can bind directly to TBP (33), CBP (34) and Sp1(35). Moreover, p53 can co-operate with TBP to activate transcription (36). In this context it is important to note that the sequences within the PCNA promoter apparently responsive to p53 contains at least four Sp1-binding sites and two CBP-binding sites (14). We are presently investigating the possible interaction between these factors and p53 in the activation of the PCNA promoter in HeLa cells.

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