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THE REGION 3' TO THE MAJOR TRANSCRIPTIONAL START SITE OF THE MDR1 DOWNSTREAM PROMOTER MEDIATES ACTIVATION BY A SUBSET OF MUTANT P53 PROTEINS

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Abstract. We have examined the response of the human multidrug resistance gene-1 (MDR1) downstream promoter to various mutants of human p53 in a reporter assay system. Our findings indicate that mutant 175H inhibits reporter activity driven by the MDR1 downstream promoter (base pairs -189 to +133 relative to the major transcriptional initiation site) in a dose-dependent manner in cotransfection assays in the BHK and the Saos-2 cell lines. A 123 base-pair segment of DNA (-119 to +4 relative to the major transcriptional initiation site) and a 193 base-pair segment (-189 to +4) have been isolated from the MDR1 downstream promoter which, like the full promoter, are negatively controlled by mutant 175H. However, a 135 base-pair segment (-2 to +133) of the promoter is activated by mutant 175H as well as mutant 248Q, but not by mutants 213Q and 234H. Thus some mutants of p53 are able to activate transcription from the 3' region of the MDR1 downstream promoter, an activity that characterizes these p53 mutants as "gain of function" mutants.

Introduction. The role of mutants of p53 in the regulation of transcription is poorly understood. Most promoters are either unresponsive to or activated by mutants of p53. Typically, viral promoters and long terminal repeats (LTRs) are activated by a subset of mutant p53 proteins (1, 2, 3). Some human promoters have also been shown to be activated by mutant p53, such as MDR1 (4, 5).

The MDR1 gene encodes a membrane-bound, energy-dependent efflux pump which is known to transport lipophilic compounds out of the cell (6, 7). The MDR1 gene product, p-glycoprotein (pgp), is responsible, in part, for the resistance of cancer cells to treatment with certain chemotherapeutic agents (8, 9). The refractoriness of tumors to treatment with chemotherapeutic agents is a concern of utmost importance and the regulation of pgp expression may play a critical role in this resistance.

In this communication we report that in cotransfection assays using mutant p53 expression constructs and luciferase reporter constructs driven by various segments of the MDR1

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downstream promoter, mutant p53 suppressed the transcription driven by the entire downstream promoter or by the 5' portion relative to the major site of transcriptional initiation. However, when the reporter gene was driven by only the 3' portion of the promoter, certain mutants of p53 were able to activate transcription strongly. In addition, these mutants do not necessarily possess dominant negative activities as assayed by an independent reporter system, but may display gain of function activities.

Materials and Methods: Plasmid construction. The pCisp53 expression vectors were constructed by inserting the wild-type or mutant p53 cDNAs into the Xba1 restriction site of the pCis vector (figure 1). The MDR1 reporter constructs were made by first PCR-amplifying the MDR1 downstream promoter from normal human placenta DNA. The primers used were 5'-TCTAGAGGTGCAACGGAAGCC-3' and 5'-TTCGAAGGAACGGCCACCAAG-3'. Thermocycling conditions were 35 cycles of 93° C, 1 min., 64° C 1 min, and 72° C 1 min. The 322 bp PCR product was gel isolated and blunt-end ligated into the Sma1 site of pBluescript KS- to make pBS-MDR. Positive clones were sequenced and exactly match published sequences for the MDR1 downstream promoter (10). The full promoter or fragments of it were isolated from pBS-MDR and ligated into pGL2Basic (Promega) luciferase reporter construct at the appropriate sites to produce pMDR-lux, pΔ3'-MDR-lux, pΔ5'-MDR-lux, and pΔΔMDR-lux (figure 1). Positive clones were sequenced to insure positive orientation, single fragment insertion, and correct sequence.

Cell culture and cotransfections. BHK cells were maintained in high glucose DME supplemented with Fungizone and gentamycin at 37° C, 10% CO_2 and 10% O_2. BHK cells were plated to an approximate density of 50% confluence in 60 mm tissue culture dishes for the transfections. All cotransfection assays were performed in duplicate and on at least three different occasions. A total of 8 μg of DNA was used in each cotransfection: 1 μg of lux reporter construct and 0.5 or 1.5 μg of pCis vector. The remainder of the DNA concentration was made up with sonicated calf thymus DNA. A standard calcium-phosphate precipitation technique was used to transfect the cells. The DNA was mixed in 170 μl of 0.25 M $CaCl_2$ before being added to 170 μl of 2x BES buffered saline (BBS), pH 6.96. The cells were washed once with complete medium, and the medium removed. The DNA precipitate was mixed with 4 mls of complete medium, then added to the plates. The plates were incubated at 35° C, 2.5% CO_2 for 10-12 hours before the medium was removed, washed with complete medium, and replaced with 4 mls of complete medium. The cells were then incubated at 37° C, 10% CO_2 and 10% O_2 for an additional 24 hours.

Cells were harvested by trypsinization, washed once with phosphate buffered saline, and once with 100 mM KPO_4, 1 mM EDTA, pH 7.8. The cells were lysed by a thrice repeated freeze-thaw-vortex cycle in 100-200 μl of 100 mM KPO_4, 1 mM EDTA, pH 7.8. Protein concentration of each lysate was determined by the Bradford-Bio-Rad method.

Reporter assays. Luciferase assays were performed using 10 μl of cell lysate. Lysate was added to 250 μl of luciferase assay buffer (22 mM $MgSO_4$, 37 mM glycylglycine, pH 7.8) and assayed in a Monolight 2010 luminometer (Analytical Luminescence) with 100 μl 17.5 mM ATP, pH 7.0 and 100 μl 1 mM potassium-D-luciferin (Analytical Luminescence). CAT assays were performed by a standard protocol with 25 to 50 μg of cell lysate. The acetylated [^{14}C]-chloramphenicol was separated by thin layer chromatography, recovered from the TLC plate, and quantitated by liquid scintillation counting.

Results and Discussion: Cotransfection of MDR1 reporter constructs and p53 expression vectors. To analyze the MDR1 promoter region, the MDR1 downstream promoter (-189 to +133)-luciferase reporter construct, pMDR-lux (Fig 1B) was used in cotransfection assays with CMV-promoted p53 expression vectors (Fig 1A) using the BHK cell line. The p53 expression constructs and pMDR-lux construct were sequenced to ensure that the DNA exactly matched published DNA sequences (10, 11).

Mutant 175H was found to suppress expression from the MDR1 downstream promoter in a dose-dependent fashion (Figure 2A). These results obtained with the p53 mutant 175H are

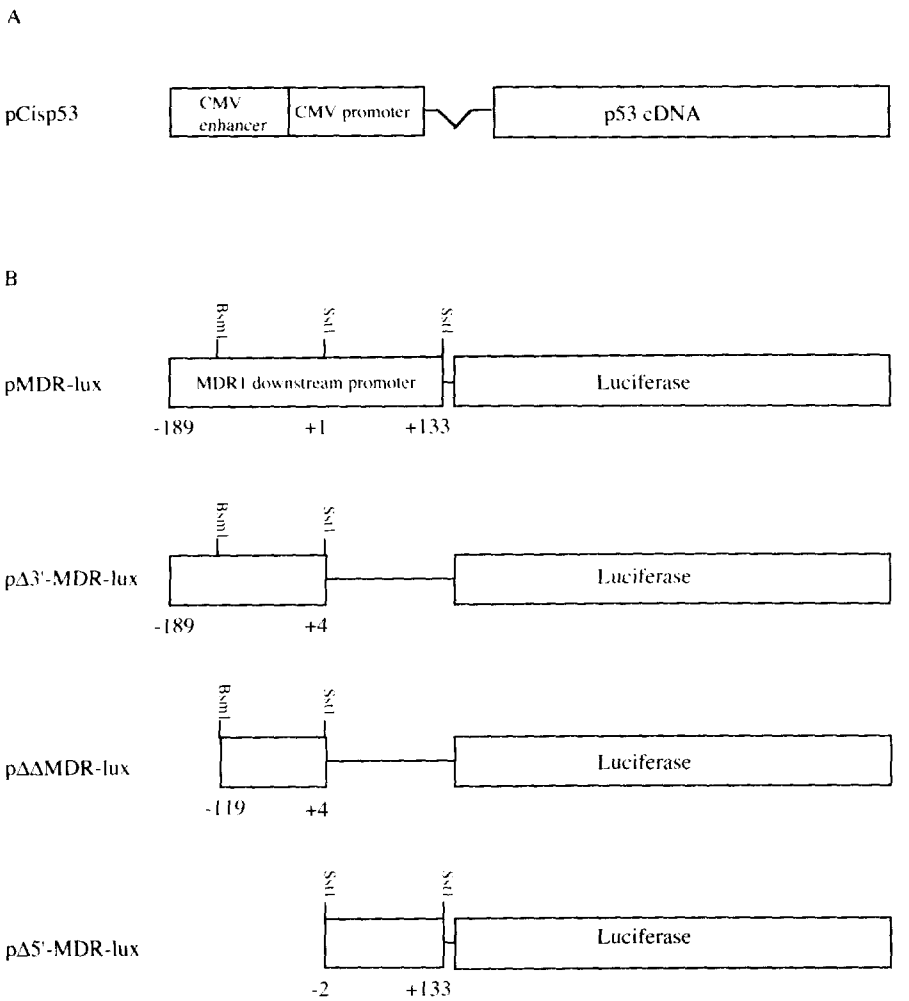

Figure 1. Schematic representation of vectors used in cotransfection assays. (A) Representation of the pCis expression vectors. p53 cDNA encoding the wild-type (Arg at codon 72), or mutants (His at codon 175, Gln at 213, His at 234, or Gln at 248) were cloned into the pCis expression vector to form pCisp53(wt), pCisp53(175H), pCisp53(213Q), pCis(234H), and pCis(248Q), respectively. (B) Reporter constructs were made by linking the MDR1 downstream promoter or the indicated segments to the luciferase gene as described in the Materials and Methods. The major site of transcriptional initiation is located at (+1).

different from those presented by Chin *et al* (4) and by Zastawny *et al.* (5) who showed suppression by wild-type p53 and activation by mutant p53(175H) of the MDR1 promoter.

The MDR1 downstream promoter was truncated at the 3' end and the resulting fragment (-189 to +4 relative to the major site of transcriptional initiation) was used to drive the luciferase gene, yielding the pΔ3'-MDR-lux construct (Figure 1B). The mutant 175H suppressed expression from the pΔ3'-MDR-lux reporter construct in a dose-dependent manner (Figure 2B). These results closely match those obtained with the non-deleted pMDR-lux reporter construct. However, when the pΔΔMDR-lux (Figure 1B) reporter construct (-119 to +4) was used in the

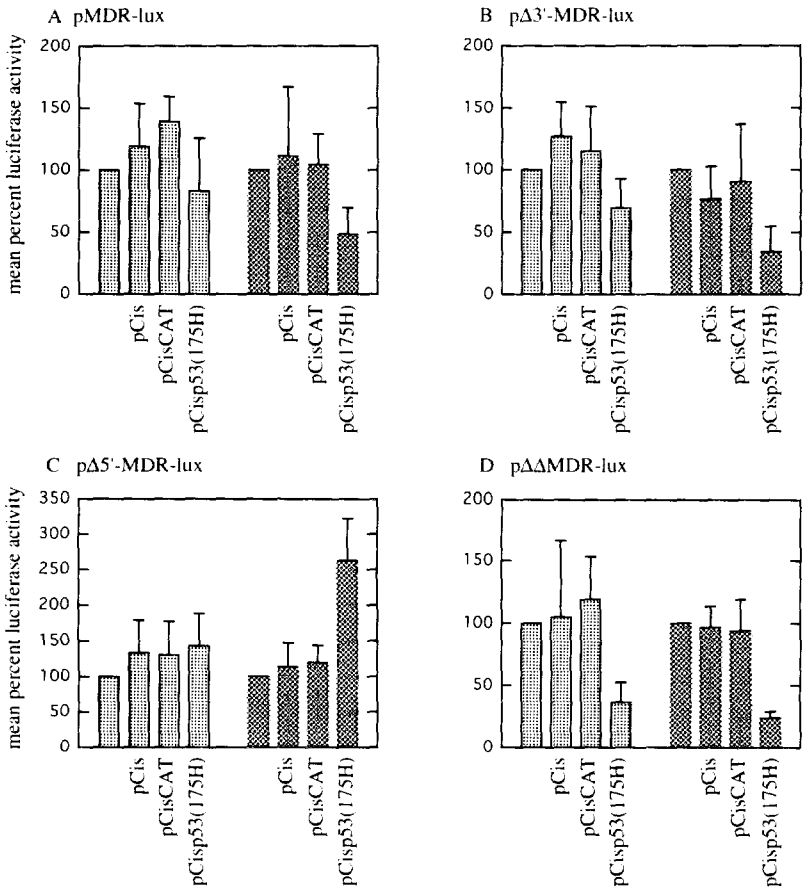

Figure 2. Results of cotransfection assays performed in the BHK cell line reveal an activation domain in the MDR1 downstream promoter. Data are presented as the mean percent activity of at least three independent experiments, where the basal activity of the reporter construct is defined as 100%. Each experimental point was performed in duplicate and luciferase values were adjusted for differences in protein concentration. 1.0 μg of each reporter construct (A) pMDR-lux, (B) pΔ3'-MDR-lux, (C) pΔ5'-MDR-lux, or (D) pΔΔMDR-lux (lanes 1 and 5) was cotransfected along with pCis parental vector (lanes 2 and 6), pCisCAT (lanes 3 and 7), or pCisp53(175H) (lanes 4 and 8). Light shading indicates 0.5 μg of pCis vector was used, dark indicates 1.5 μg.

cotransfection assays, the transcription was suppressed by p53 mutant 175H more efficiently as compared to the results obtained with either the pMDR-lux or the pΔ3'-MDR-lux reporter constructs (Figure 2D).

The MDR1 downstream promoter was truncated at the 5' end and the resulting fragment (-2 to +133) was used to drive the luciferase gene (Figure 1B). Mutant 175H consistently activated the transcription from the pΔ5'-MDR-lux reporter construct (Figure 2C). The activity of the mutant 175H was interesting and surprising, therefore a broad titration of mutant p53 expression vector quantities was cotransfected along with the pΔ5'-MDR-lux construct to verify that at least some mutant proteins are capable of significant up-regulation of reporter activity. Mutants 175H and 248Q each activated the reporter construct in a concentration-dependent manner (Figure 3).

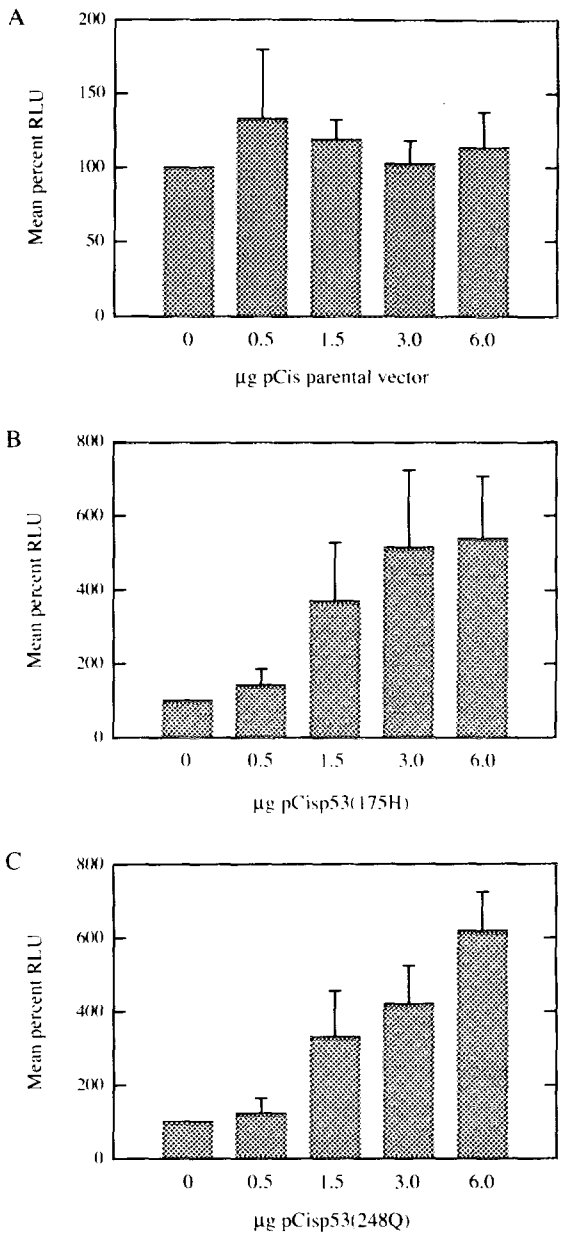

Figure 3. Results of cotransfection assays performed in the BHK cell line show a subset of p53 mutants which activate the MDR1 downstream promoter. Data are presented as the mean percent relative luciferase (RLU) activity of at least three independent experiments, where the basal activity of the reporter construct is defined as 100%. Each experimental point was performed in duplicate and luciferase values were adjusted for differences in protein concentration. 1.0 µg of the pΔ5'-MDR-lux reporter construct was cotransfected with A. pCis parental vector, B. pCisp53(175H), and C. pCisp53(248Q) in the amounts noted.

The 3' segment of the MDR1 promoter was also activated by mutants 213Q and 234H, although to a lower extent (a two-fold increase, data not shown) than that found with mutants 175H and 248Q. Thus, various p53 mutants may each have a specific affect on the pΔ5'-MDR-lux

construct. In summary, the pΔ5'-MDR-lux construct can be activated in the presence of some mutant p53 proteins, activation that is independent of wild-type p53 protein, and hence represents a bona fide "gain of function" activity.

The possibility exists that sequences found in the pCis vector affect the transcription from the MDR1 downstream promoter. To address this concern, the pCis parental vector was cotransfected along with each reporter construct. The results show that the pCis parental vector alone had no affect on the reporter activity (Figure 2). Another possibility to be considered was that the *expression* of an exogenous gene in the BHK cell line may alter the basal transcriptional rate of the reporter constructs. This concern was addressed by cotransfecting the pCisCAT vector along with each reporter construct. The results show that the exogenous gene expressed from the pCisCAT vector did not alter the basal transcription of the reporter constructs (Figure 2). Thus, we conclude that neither the pCis vector nor the expression of an exogenous gene affected the results obtained in the cotransfection assays.

In conclusion, the MDR1 downstream promoter or its 5' region relative to the major site of transcriptional initiation are suppressed by mutant 175H. However, the 3' region is activated by a subset of mutants of p53 (including 175H and 248Q). Thus, the 3' region seems to confer mutant p53-mediated transcriptional activation. Note that wild-type p53 suppressed reporter activity driven by all fragments of the MDR1 downstream promoter used here (Strauss et al, manuscript submitted).

The subset of mutants which activate the MDR1 downstream promoter 3' region is independent of the subset of mutants which act in a dominant negative manner. We have shown that certain mutants (175H and 248Q) of p53 can activate the 3' region of the MDR1 downstream promoter. This activity may represent a gain of function (an activity not associated with the wild-type protein) since the wild-type protein does not activate the MDR1 promoter in our hands (Strauss et al, manuscript submitted, 12). The gain of function role that is expressed by the 175H and 248Q mutants might be associated with a dominant negative activity over the wild-type protein (13). To assay for dominant negative activity with respect to transcriptional functions, we cotransfected BHK cells with a constant amount of the wild-type p53 expression vector along with an increasing amount of the various mutant p53 expression vectors in the presence of the PG13CAT reporter construct. The PG13CAT construct contains the known, wild-type p53 binding site, RGC, repeated 13 times to drive the expression of the CAT gene (14). Dominant negative activity would be observed as the ability of the mutant p53 to ablate the transactivation mediated by the wild-type, resulting in reduced CAT activity.

Figure 4 shows that mutant 175H does display a dominant negative activity since it is able to lessen the wild-type p53-mediated transactivation(Figure 4a), however mutant 248Q does not (Figure 4d), even though both of these mutants displayed a gain of function with respect to the MDR1 3' promoter region. Mutant 234H displayed a dominant negative activity (Figure 4c), but did not show any gain of function on the 3' region of the MDR1 downstream promoter. Mutant 213Q retained its ability to transactivate the PG13CAT reporter construct, thus 213Q activated the reporter gene independently of the wild-type protein (Figure 4d). Thus there was no clear correlation between the gain of function mutants of p53 as assayed on the MDR1 downstream promoter and the dominant negative activities as assayed on the RGC consensus sequence.

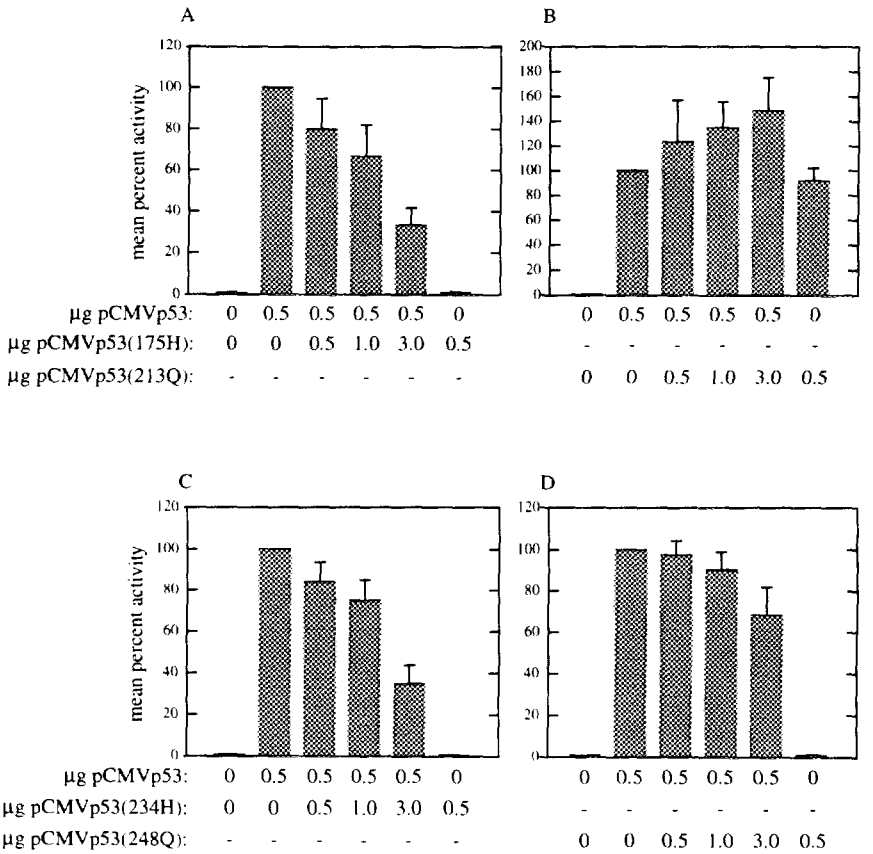

Figure 4. Results of cotransfection assays performed in the BHK cell line show a lack of correlation between gain of function and dominant negative mutations. Data are presented as the mean percent CAT activity of at least three independent experiments, where the activity of the reporter construct in the presence of wild-type p53 is defined as 100%. Each experimental point was performed in duplicate and CAT values were adjusted for differences in protein concentration. 1.0 μg of the PG13CAT reporter construct was cotransfected along with the pCMVp53(wt) vector as indicated as well as A. pCMVp53(175H), B. pCMVp53(213Q), C. pCMVp53(234H) and D. pCMVp53(248Q). Note that the expression vector used is derived from the pRc/CMV vector.

The ability of a subset of mutant p53 proteins to activate transcription driven by the 3' portion of the MDR1 downstream promoter may be relevant to the acquisition of the drug resistant phenotype in vivo, although this point remains to be tested. Clearly, some mutants of p53 have activities which are distinct from the wild-type protein. These distinct activities represent a possible gain of function (12). However, we have shown that the putative gain of function may not be correlated with the dominant negative activity of mutant p53 (13). Thus, each mutant of p53 may have a distinct set of biochemical functions.

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