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Growth suppression induced by wild-type p53 protein is accompanied by selective down-regulation of proliferating-cell nuclear antigen expression

(gene expression/cell cycle regulation/tumor suppressor)

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ABSTRACT **The p53 gene is a frequent target of mutation in a wide variety of human cancers. Previously, it was reported that conditional expression of wild-type p53 protein in a cell line (GM47.23) derived from a human glioblastoma multiform tumor had a negative effect on cell proliferation. We have now investigated the effect that induction of wild-type p53 protein in this cell line has on the expression of the proliferating-cell nuclear antigen gene. The proliferating-cell nuclear antigen gene encodes a nuclear protein that is an auxiliary factor of DNA polymerase δ and part of the DNA replication machinery of the cell. We show that inhibition of cell cycle progression into S-phase after induction of wild-type p53 protein is accompanied by selective down-regulation of proliferating-cell nuclear antigen mRNA and protein expression.**

The nuclear phosphoprotein p53 was first identified through its association with the transforming protein of simian virus 40, termed the large tumor antigen (1). Since its initial identification, a wide variety of studies have implicated the p53 protein in cell cycle regulation and neoplastic transformation (for reviews, see refs. 2–4). Recent interest in p53 was greatly stimulated by the observation that alterations (deletions and/or missense mutations) in the gene encoding p53 are the most frequent genetic abnormalities found in malignant cells of human tumors; including colon, breast, lung, bone, and brain tumors (5–9). The frequency with which genetic abnormalities of the p53 gene are found in human tumors has led to the suggestion that p53 is a potential tumor suppressor gene, similar to the retinoblastoma susceptibility gene Rb (10).

Consistent with this suggestion is the finding that wild-type (wt) mouse p53 can inhibit focus formation when introduced into primary rat embryo fibroblasts with various combinations of transforming oncogenes including, *myc* plus *ras*, adenovirus *E1A* plus *ras*, or mutant p53 plus *ras* (11, 12). In addition, we have demonstrated (13) that constitutive expression of wt human p53 is antiproliferative in simian virus 40-transformed hamster cells, whereas mutant forms of p53 have no antiproliferative effect (13). Similar results have been reported in cell lines derived from colon cancer after transfection of wt-p53 or mutant p53 constructs (14). Thus these studies support the hypothesis that the inhibitory effect of wt-p53 on oncogene-mediated transformation may be directly linked to its growth-suppressing activity and that this function can be inactivated by mutation.

Direct experimental evidence for a growth-suppressing function of wt-p53 was provided by the demonstration that conditional expression of wt-p53 protein in a cell line derived from a human glioblastoma multiform tumor blocks cell cycle progression (15). In this system, inhibition of cell cycle progression was associated with a marked decrease in histone H3 mRNA expression, an S-phase marker gene whose regulation is tightly linked to DNA replication. This finding suggested that the wt-p53 protein induced in these cells may specifically effect a function (or functions) required for progression from G_1 into S phase. To gain further insight into the possible mechanism(s) of growth suppression induced by wt-p53, we studied the effect of wt-p53 protein on the expression of the gene encoding proliferating-cell nuclear antigen (PCNA). The PCNA gene is also growth-regulated (16, 17) and encodes a protein that is a component of the DNA replication machinery of the cell. PCNA is a nuclear protein of M_r 36,000 that has been identified as a cofactor of DNA polymerase δ (18, 19), an important component of cellular DNA replication (for review, see ref. 20).

In this communication, we provide evidence that expression of human wt-p53 (form II) protein correlates with inhibition of cell cycle progression and that cell cycle inhibition is accompanied by selective down-regulation of PCNA mRNA and protein expression.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. T98G and GM47.23 cells were maintained in Earle's minimal essential medium containing 10% (vol/vol) fetal calf serum (GIBCO) at 37°C. GM47.23 cells are a derivative of T98G cells, which contain an inducible wt-p53 cDNA driven by the hormone-inducible mouse mammary tumor virus promoter (15). For cell cycle studies, cells were arrested in G_0/G_1 phase by allowing them to grow to confluency followed by incubation for 3 days in medium containing 0.5% fetal calf serum. For stimulation, cells were treated with trypsin and replated at a 1:4 split ratio in medium containing 10% fetal calf serum. To induce wt-p53 protein expression, GM47.23 cells were treated with 1 μM dexamethasone (Dex) as described (15). Aphidicolin was obtained from SIGMA and dissolved in dimethyl sulfoxide.

Plasmids and Probe Preparation. The probes used for hybridization were from plasmids digested with the appropriate restriction enzyme and followed by gel purification. They include a human p53 cDNA *Xba* I fragment of p53H (21), a β-actin *Bam*HI fragment of pHFβA-1 (22), a human histone H3 *Eco*RI fragment of pFO422 (23), a human PCNA cDNA fragment of pPCNA-G3, a human thymidine kinase (TK) cDNA fragment of pTK11 (kind gifts of Renato Baserga,

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Abbreviations: wt, wild type; PCNA, proliferating-cell nuclear antigen; Dex, dexamethasone; TK, thymidine kinase.

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Temple University School of Medicine), and the *Hin*dIII–*Eco*RI fragment of mouse β_2-microglobulin of pG1-muβ-2 (a kind gift of Bruno Calabretta, Temple University School of Medicine). Radioactive probes were prepared from these fragments by oligolabeling to high specific activity (24).

RNA Isolation and Northern Blot Analysis. Total RNA was extracted from cells by the method of ref. 25. RNA (10 μg per lane) was denatured with 6.3% (vol/vol) formaldehyde/50% (vol/vol) formamide and then size-fractionated on a 1.2% agarose gel containing 6.6% formaldehyde. Blotting of RNA to nitrocellulose filters was by standard procedures (26). Prehybridization, hybridization, and posthybridization washes were as described (21). The relative intensity of RNA signals on the Northern blots was determined by soft-laser densitometry (Zenith, Biomed Instruments, Fullterton, CA) of autoradiographs.

Metabolic Labeling and Immunoprecipitation. Cells were metabolically labeled with [^{35}S]methionine [0.75 mCi (1 Ci = 37 GBq) per 100-mm culture plate] for 3 hr in methionine-free medium containing 10% dialyzed fetal calf serum. Cell lysates were prepared and immunoprecipitations were performed using p53-specific monoclonal antibodies, PAb421 and -1801 (Oncogene Science, Manhasset, NY), as described (13, 15) except lysates contained 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and 1 mM sodium orthovanidate. Immunoprecipitates were analyzed by SDS/PAGE (27).

Indirect Immunofluorescence and Quantitation. Cells grown on glass coverslips (22 × 22 mm) were washed twice with Hanks' balanced salts solution before fixation. Indirect immunofluorescence was performed and quantitated as described (29). Fluorescence intensity of individual cell nuclei was quantitated using UV epiillumination by computer-operated microfluorimetry.

RESULTS

PCNA Protein Levels Are Decreased in Cells Expressing wt-p53. In quiescent (G_0 phase) cells, the amount of PCNA protein is 30–40% of that found in proliferating cells (28) and PCNA mRNA is undetectable (17). Stimulation of quiescent cells with serum or purified growth factors induces the coordinate synthesis of PCNA mRNA and protein, which reach maximum levels of expression in late G_1/early S phase (17, 18, 28). Furthermore, inhibition of PCNA expression indicates that an increase in PCNA is critically important for cellular DNA synthesis and hence for cell cycle progression (29, 30). It was therefore of interest to investigate PCNA expression in GM47.23 cells whose progression into S phase was inhibited by induction of wt-p53 protein. For these experiments, GM47.23 cells and parental T98G cells were made quiescent and then serum-stimulated with or without Dex as described above. PCNA protein in the nuclei of cells was examined 24 hr after stimulation by indirect immunofluorescence staining using a PCNA-specific monoclonal antibody, ABT-151 (29). The results are presented in the representative fluorescent photomicrographs shown in Fig. 1. In uninduced GM47.23 cells (Fig. 1*A*) and parental T98G cells untreated or treated with Dex (Fig. 1 *C* and *D*), 85–90% of the cells exhibited nuclear staining, some with an intense granular pattern confined to specific areas of the nucleus; a finding very similar, if not identical, to that reported by Bravo and MacDonald-Bravo (28). In contrast, in GM47.23 cells induced to express wt-p53 protein, nuclear staining of PCNA protein was barely detectable in most cells; although a few cells (<10%) showed a slight staining that appeared to be localized around nucleoli. Under the fixation and staining conditions employed, PCNA protein was undetectable in quiescent GM47.23 and parental T98G cells (data not shown).

We quantitated the relative level of PCNA protein in the nucleus of these cell lines under each experimental condition

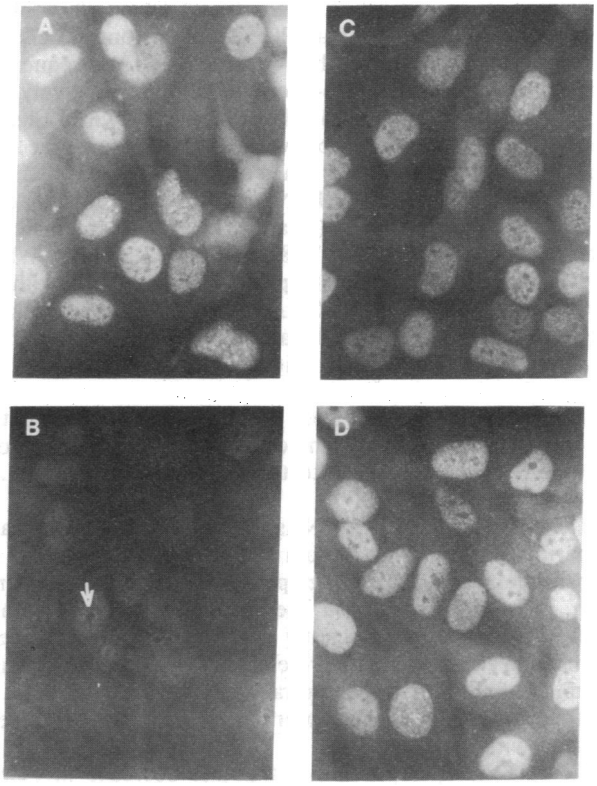

FIG. 1. Fluorescent photomicrographs of PCNA protein. PCNA protein in the nuclei of parental T98G and GM47.23 cells stimulated in the presence (Dex$^+$) or absence (Dex$^-$) of 1 μM Dex was examined by indirect immunofluorescence staining using monoclonal antibody ABT151. (*A*) GM47.23 cells, Dex$^-$. (*B*) GM47.23 cells, Dex$^+$. (*C*) T98G cells, Dex$^-$. (*D*) T98G cells, Dex$^+$. The arrow in *B* shows an example of PCNA staining located around nucleoli. (×280.)

using computer-operated microfluorimetry (31) (Table 1). In uninduced GM47.23 cells (Dex$^-$) and parental T98G cells untreated or treated with Dex, the amount of PCNA protein per cell nucleus ranged from about 35 to 49 arbitrary units of fluorescence. However, in GM47.23 cells induced to express wt-p53 protein (Dex$^+$), the fluorescence intensity per cell nucleus was only 4 arbitrary units. Thus, the amount of PCNA protein in these cells was reduced by at least 10-fold relative to control cells.

PCNA mRNA Expression Is Selectively Down-Regulated. PCNA expression is growth-regulated (17, 18, 28) and the regulation of PCNA mRNA expression during the G_0-to-S phase transition occurs at both transcriptional and post-transcriptional levels (32). Expression of PCNA mRNA in growth-arrested parental T98G and GM47.23 cells and in cells stimulated to proliferate 24 hr with and without Dex treat-

Table 1. Fluorescence intensity of PCNA protein

Cell line	Fluorescence, arbitrary units	
	− Dex	+ Dex
T98G	35.6 ± 2.1	48.9 ± 5.2
GM47.23	46.1 ± 3.8	4.1 ± 2.6

Cells were stimulated from growth arrest with (+) and without (−) the addition of 1 μM Dex. At 24 hr after stimulation under each condition, the cells were fixed in methanol and first stained with mouse monoclonal antibody to PCNA (ABT151) followed by goat anti-mouse IgG conjugated with fluorescein isothiocyanate. Fluorescence intensity of nuclear staining was determined by computer-operated microspectrofluorimetry using a 520- to 540-nm barrier filter as described (31). The nuclear fluorescence intensity per cell (n = 100 cells) in arbitrary units (mean ± 1 standard deviation) is shown for each condition.

ment was examined by Northern blot analysis. The blots were first probed with human PCNA and histone H3 cDNA probes. The blots were then stripped and subsequently probed for β-actin and β_2-microglobulin, respectively. A representative composite Northern blot is shown in Fig. 2. The β_2-microglobulin probe was used to normalize the amount of RNA among the lanes of the blot, since it is not growth regulated (33, 34). PCNA, histone H3, and β-actin mRNAs are not detectable (or are only barely detectable) in growth-arrested (G_0 phase) T98G and GM47.23 cells. When T98G cells are stimulated to proliferate for 24 hr with or without Dex treatment, PCNA, histone H3, and β-actin mRNA levels increase significantly. In GM47.23 cells stimulated to proliferate with or without Dex treatment, β-actin mRNA levels were also increased; however, in GM47.23 cells induced to express wt-p53 protein by Dex treatment, the level of PCNA mRNA is significantly decreased. In addition, histone H3 mRNA is not detectable in Dex-treated GM47.23 cells, as observed (15).

The relative level of mRNAs for these genes under each experimental condition in both cell lines was quantitated by densitometry of autoradiographs from three experiments (Fig. 3). In each experiment, the levels of PCNA mRNA (and histone H3) were significantly decreased in GM47.23 cells induced to express wt-p53 protein relative either to β-actin or β_2-microglobulin. On the contrary, no decrease in PCNA or histone H3 mRNA was observed in parental T98G cells treated with Dex.

To rule out the possibility that the decrease in PCNA mRNA levels in Dex-treated GM47.23 cells is not simply due to inhibition of DNA synthesis *per se*; the drug aphidicolin, a potent inhibitor of DNA polymerase α (35), was added to cells after serum stimulation. Northern blot analysis was performed and, as shown in Fig. 4, histone H3 mRNA levels are decreased in cells treated with this drug in a dose-dependent manner; however, PCNA mRNA levels are not affected. The percentage of S-phase cells was determined by [^{3}H]thymidine labeling and autoradiography. At a drug concentration of 1.0 μg/ml, the percentage of labeled cells in the population was inhibited by greater than 90% relative to untreated controls.

In previous studies employing exponentially growing GM47.23 cells, induction of wt-p53 protein was also shown to significantly decrease the percentage of S-phase cells in the population beginning at 18 hr (15). Therefore, it was of interest to determine whether PCNA mRNA levels were also down-regulated in exponentially growing GM47.23 cells treated with Dex to induce wt-p53 protein. GM47.23 cells were treated with Dex and at various times after wt-p53 induction, RNA was isolated and Northern blots were prepared and probed for PCNA and H3 mRNA levels. A representative composite blot is shown in Fig. 5. PCNA mRNA levels are also significantly decreased in GM47.23 cells at 18 and 24 hr after induction of wt-p53 protein relative to uninduced control cells at time zero. By densitometric measurement the level of PCNA mRNA at 18 hr is 4.5-fold lower and at 24 hr is 3-fold lower than the mRNA level in uninduced control cells. As expected, this is paralleled by a decrease in histone H3 mRNA, which is apparent at 18 and 24 hr. To control for the amount of RNA in each lane of the blot, the blot was subsequently rehybridized with the β-actin probe, which indicated that the decrease in PCNA and histone H3 mRNA levels observed at 18 and 24 hr is not an artifact of improper loading or transfer of RNA. We also examined the effect that wt-p53 induction has on the expression of another late G_1/S-phase gene, TK. As can be seen in the composite Northern blot, the steady-state level of TK mRNA is not decreased in GM47.23 cells after induction of wt-p53 protein.

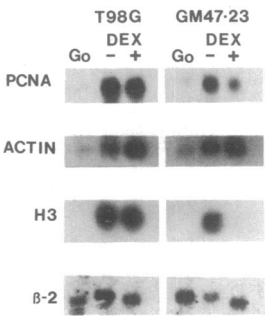

FIG. 2. Composite Northern blot of mRNA expression. RNA was isolated from parental T98G and GM47.23 cells unstimulated (G_0 phase) or stimulated for 24 hr with (+) or without (−) the addition of 1 μM Dex. Northern blots were prepared and hybridized with ^{32}P-labeled probes for PCNA, β-actin, histone H3, and β_2-microglobulin (β-2) as indicated.

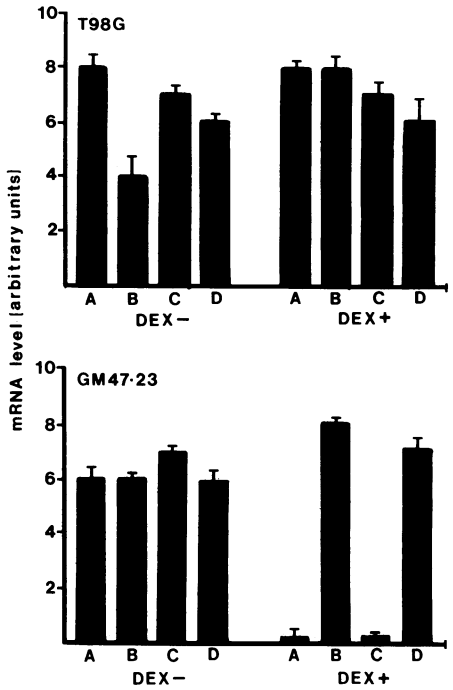

FIG. 3. Relative level of mRNA expression. Northern blots were prepared from RNA isolated from parental T98G and GM47.23 cells and probed for PCNA (bars *A*), β-actin (bars *B*), histone H3 (bars *C*), and β_2-microglobulin (bars *D*), as described in Fig. 2. The relative level of mRNA expression for each gene in three experiments was determined by soft laser densitometric scanning of the resulting autoradiographs. The results are presented in arbitrary units of density with standard errors (error bars).

The Presence of Form II p53 Protein Is Correlated with PCNA Down-Regulation. Very little is known about the various forms of human p53 protein of their biological functions. In previous studies, two monoclonal antibodies,

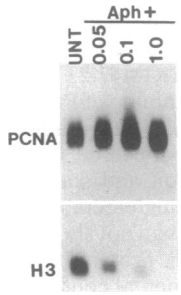

FIG. 4. Northern blot of mRNA expression in GM47.23 cells treated with aphidicolin. Quiescent GM47.23 cells were serum-stimulated. At 10 hr after stimulation, the drug aphidicolin (Aph) was added as indicated (in μg/ml). Lane UNT is untreated cells. Total RNA was isolated at 20 hr and Northern blots were prepared. The blots were probed for PCNA and histone H3 mRNA.

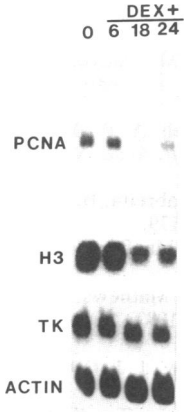

FIG. 5. Northern blot analysis of mRNA in exponentially growing GM47.23 cells induced to express wt-p53 protein. Exponentially growing GM47.23 cells were induced to express wt-p53 protein by Dex treatment. RNA was prepared at various times as indicated in hours and a Northern blot was prepared and probed for PCNA and histone H3 simultaneously followed by β-actin and by TK.

PAb1801 (36) (which recognizes human p53 epitopes localized on the N terminus) and PAb122 (37, 38) (which recognizes epitopes localized on the C terminus) were shown to be capable of preferentially precipitating different electrophoretic forms of p53 protein (15). The PAb122 antibody primarily precipitated a slower migrating form of p53 protein (form I) from parental T98G cells and uninduced GM47.23 cells, whereas this form of p53 protein was poorly precipitated from these cells by PAb1801. When PAb421 (38, 39), which also recognizes epitopes localized to the C terminus, was substituted for PAb122, similar results were obtained (unpublished results). However, when wt-p53 was induced in GM47.23 cells, the PAb1801 antibody primarily precipitated a faster migrating form of wt-p53 protein (form II).

In the present study, we have exploited the different reactivities of these monoclonal antibodies toward p53 proteins to identify the p53 protein preferentially expressed in GM47.23 cells in which PCNA expression is down-regulated. Sequential immunoprecipitation using PAb421 and PAb1801 was performed on lysates from GM47.23 cells (uninduced or induced), metabolically labeled with [^{35}S]methionine, to study the possible interactions of p53 forms I and II (Fig. 6). Sequential precipitation with PAb421 followed by PAb1801 (Fig. 6, lanes a–e) shows that none of the form II p53 protein remains in the lysate of uninduced (Dex$^-$) GM47.23 cells (lane e). On the contrary, in GM47.23 cells induced with Dex (Dex$^+$) and sequentially precipitated with PAb421 followed by PAb1801 (lanes h–l), there is a substantial amount of form II wt-p53 protein present in the lysate (lane l). If sequential precipitation of the lysates from Dex$^+$ GM47.23 cells with PAb1801 followed by PAb421 is performed (lanes m–q), very little, if any, of the form II wt-p53 or endogenous form I p53 protein remains in the lysate (lane q). Therefore, an increased amount of form II wt-p53 protein is present in the lysates of Dex-induced GM47.23 cells and this is correlated with growth inhibition accompanied by selective down-regulation of PCNA expression.

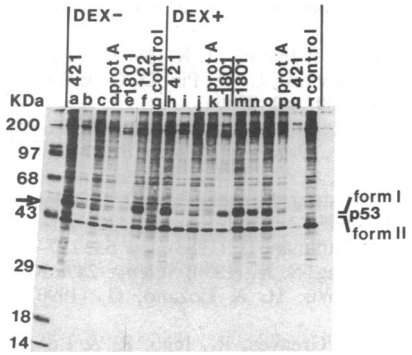

FIG. 6. Sequential immunoprecipitation of p53 proteins expressed in GM47.23 cells. p53 proteins were sequentially immunoprecipitated from [^{35}S]methionine-labeled cell lysates prepared from growth-arrested GM47.23 cells stimulated to proliferate without (Dex−) and with (Dex+) the addition of 1 μM Dex. Dex$^-$ cultures were precipitated sequentially with PAb421 (lanes a–c), protein A (lane d), and PAb1801 (lane e). Dex$^+$ cultures were precipitated sequentially with PAb421 (lanes h–j), protein A (lane k), and PAb1801 (lane l), or Dex$^+$ culture were precipitated sequentially with PAb1801 (lanes m and o), protein A (lane p), and PAb421 (lane q). Controls include precipitation with nonspecific monoclonal antibody (Ma-22) (lanes g and r) or anti-p53 monoclonal antibody PAb122 (lane f).

DISCUSSION

We (13, 15) and others (14) have provided direct experimental evidence that human wt-p53 protein encodes a growth-suppressing activity that is abrogated in mutant forms of the protein. In the present communication, we show that the expression of wt-p53 protein (form II) in GM47.23 cells correlates with inhibition of cell cycle progression and that this inhibition is accompanied by selective down-regulation of PCNA mRNA and protein expression.

Down-regulation of PCNA expression does not appear to be an indirect effect of the hormone inducer because parental T98G cells treated with Dex do not exhibit this decrease in PCNA mRNA or protein. Further, the decrease in PCNA mRNA levels does not appear to be a nonspecific global effect of wt-p53 protein on the general transcriptional machinery of the cell since the steady-state levels of endogenous p53 mRNA (15); β-actin, β_2-microglobulin, or TK mRNAs (present results) are not decreased. Moreover, down-regulation of PCNA expression is not a consequence of inhibition of DNA synthesis *per se* because PCNA mRNA is not down-regulated when DNA replication is inhibited by drugs such as aphidicolin (present results) or hydroxyurea (16). Therefore, unlike histone mRNA expression (40), PCNA expression does not directly depend on DNA synthesis. This implies that the expression of PCNA and histone H3 mRNAs is controlled by different mechanisms. Thus, it appears that down-regulation of PCNA expression may be a direct consequence of the expression of form II wt-p53 protein in these cells.

PCNA has been identified as an auxiliary protein of DNA polymerase δ (18, 19) and is required for simian virus 40 DNA replication *in vitro* (41, 42). Experiments using antisense oligonucleotides or microinjection of anti-PCNA antibodies strongly suggest that PCNA is also essential for cellular DNA synthesis (29, 30). The finding that inhibition of cell cycle progression into S phase in cells induced to express wt-p53 protein is accompanied by down-regulation of PCNA mRNA (but not TK mRNA) levels suggests that wt-p53 protein plays a role in modulating PCNA expression and thus cellular DNA synthesis.

Recent studies have suggested that wt-p53 protein (but not transforming mutant forms) possesses a potent transactivating function (43, 44). The relationship between the transactivating function and the growth-suppressing function of wt-p53 protein is at present unclear. A proposed model for the transactivating function of wt-p53 in controlling cell proliferation is that wt-p53 protein could coordinately activate the transcription of a set of genes that negatively regulate key events in cell proliferation (43). The results presented in this communication are consistent with such a model since it is difficult to argue against entry into S phase (and DNA synthesis) being a key cell cycle regulatory event.

Preliminary evidence suggests that the major endogenous form of p53 protein expressed in parental T98G and uninduced GM47.23 cells is a mutant protein, based on reactivity with monoclonal antibody PAb240, which preferentially recognizes mutant forms of human p53 (ref. 45 and unpublished results). Whatever the case may be, the sequential immuno-

precipitation results indicate that a substantial amount of wt-p53 (form II) protein recognized by PAb1801 is present and is free of endogenous p53 (form I) protein in these cells under induced conditions.

Multiple subspecies of human p53 protein with different isoelectric points have been observed by two-dimensional gel electrophoresis (46), but little is known about the structural properties or biochemical activities of these subspecies. In our model, the presence of form II wt-p53 protein is correlated with inhibition of cell cycle progression (15) accompanied by selective down-regulation of PCNA expression (present study). It is tempting to speculate that post-translational modifications may contribute to the difference in electrophoretic mobilities between the endogenous form I p53 protein and the inducible form II wt-p53 protein. Differential phosphorylation is a likely candidate since recent studies have shown that human p53 protein is phosphorylated by $p34^{cdc-2}$/p60 and $p34^{cdc-2}$/cyclin B complexes (47).

This study provides evidence that expression of wt-p53 protein is associated with the down-regulation of another cellular growth-regulated gene. A great many steps and components are involved in the progression of cells through G_1 into S phase (for review, see ref. 48). It is likely that the expression of other cellular genes could also be modulated (either negatively or positively) by wt-p53 protein. Further studies will be required to elucidate the molecular mechanism by which wt-p53 modulates cellular gene expression. Of particular interest is the determination of whether the effect of wt-p53 protein on PCNA expression is direct or indirect; namely, whether this activity is mediated through transactivation of other cellular suppressor genes or by the direct action of form II p53 protein.

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