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Topological Control of p21$^{WAF1/CIP1}$ Expression in Normal and Neoplastic Tissues[1]

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ABSTRACT

The p53-regulated gene product p21$^{WAF1/CIP1}$ is the prototype of a family of small proteins that negatively regulate the cell cycle. To learn more about p21$^{WAF1/CIP1}$ regulation *in vivo*, monoclonal antibodies were developed for immunohistochemistry. These revealed that p21$^{WAF1/CIP1}$ expression followed radiation-induced DNA damage in human skin in a pattern consistent with its regulation by p53. A detailed comparison of the human, rat, and mouse p21$^{WAF1/CIP1}$ promoter sequences revealed that this induction was probably mediated by conserved p53-binding sites upstream of the transcription start site. In unirradiated tissues, p21$^{WAF1/CIP1}$ expression was apparently independent of p53 and was observed in a variety of cell types. Moreover, there was a striking compartmentalization of p21$^{WAF1/CIP1}$ expression throughout the gastrointestinal tract that correlated with proliferation rather than differentiation. As epithelial cells migrated up the crypts, the Ki67-expressing proliferating compartment near the crypt base ended abruptly, with the coincident appearance of a nonproliferating compartment expressing p21$^{WAF1/CIP1}$. In colonic neoplasms, this distinct compartmentalization was largely abrogated. Cell cycle inhibitors are thus subject to precise topological control, and escape from this regulation may be a critical feature of neoplastic transformation.

INTRODUCTION

The cell cycle and the genetic alterations that drive tumorigenesis are inextricably linked. Examples include the amplification of cyclin and CDK[4] genes, the phosphorylation of Rb by CDKs, the control of the CDK inhibitor p21$^{WAF1/CIP1}$ by p53, and the tumor suppressor activity of the CDK inhibitor p16 (reviewed in Refs. 1 and 2).

A turning point in cell cycle research was realized with the discovery that cyclin complexes had different constitutions in transformed and nontransformed cells (3, 4). In particular, the complexes in the nontransformed cells were associated with small proteins different from those found in transformed derivatives. A variety of approaches subsequently determined that these small proteins not only bound to the cyclin complexes but were potent inhibitors of the associated CDKs (p21, Refs. 5–7; p16, Refs. 8 and 9; p18, Ref. 10; p27, Refs. 11 and 12; p57, Refs. 13 and 14).

Six CDK inhibitors have thus far been identified. The first to be cloned, *p21*, also known as *WAF1*, *CIP1*, *SDI1*, *MDA6*, or *CAP20*, is encoded by a gene on chromosome 6p and can be directly regulated by p53 (15). It inhibits a broad range of cyclin-CDK complexes and may be involved in cellular senescence as well as in neoplasia (16). *In vitro*, *p21*$^{WAF1/CIP1}$ also complexes with the proliferating cell nuclear antigen, resulting in an inhibition of DNA replication (17, 18). A related gene, *p27*^{KIP1}, is located on chromosome 12p and may play a role in a subset of leukemias (19–21). A third family member, *p57*^{KIP2}, is on chromosome 11p15.5 and is a candidate tumor suppressor gene (14). The *p16*, *p15*, and *p18* genes are unrelated to the *p21/p27/p57* family and have a more selective inhibitory activity, affecting only CDK4 and 6. The *p16* and *p15* genes are homozygously deleted in many human cancers, and *p16* mutations result in familial melanoma (22–26).

Although examination of nontransformed cells in culture provided the first key insights about these CDK inhibitors, such cells cannot be considered "normal." Neither can the conditions existing in a Petri dish be equated to those occurring in the intact animal. Therefore, we attempted to study the CDK inhibitor p21$^{WAF1/CIP1}$ *in situ* to learn more about its regulation and expression in normal and neoplastic states.

MATERIALS AND METHODS

Analysis of the p21$^{WAF1/CIP1}$ Regulatory Region. A *Bam*HI restriction endonuclease digest of a P1-*WAF1/CIP1* clone was analyzed by Southern blot, and the region upstream of human p21$^{WAF1/CIP1}$ was identified as a 4.5-kb *Bam*HI fragment. The fragment was cloned, and its sequence was determined from clones generated by transposon insertion (27). The human p21$^{WAF1/CIP1}$ promoter deletion-CAT reporters were constructed by first PCR amplifying progressively smaller segments of the region upstream of the *p21*$^{WAF1/CIP1}$ gene and then subcloning into pJFCAT1 (28). To obtain the mouse and rat p21$^{WAF1/CIP1}$ promoters, we first isolated the mouse and rat p21$^{WAF1/CIP1}$ cDNAs by probing appropriate cDNA libraries with the human cDNA at low stringency. The sequences of these cDNA clones was used to design primers for PCR screening of mouse and rat genomic P1 libraries (Genome Systems, St. Louis, MO). Restriction fragments containing 4.6 kb (mouse) and 4.7 kb (rat) of the p21$^{WAF1/CIP1}$ upstream region were subcloned into the pJFCAT1 vector, and their sequence was determined with the aid of nested deletions, generated by exonuclease III and mung bean exonuclease digestion. Transfections and CAT assays were performed as described previously (29).

The DNA-binding immunoprecipitation assays were performed as described (30), except that binding was quantitated by liquid scintillation counting of the ^{32}P-labeled immune complexes. DNA fragments tested for binding were generated by PCR amplification of sites 1 and 2 from the rat promoter. Site 1 contained nt −3273 to −3112, and site 2 contained nt −2333 to −2160 relative to the TATA box. Mutated sites 1 and 2 (Fig. 1*c*) were comprised of PCR-generated fragments of the same length as the wt sites but with substitutions at position 4 of the p53-recognizing pentamers. Thus, mutant site 1 contained the sequence 5′-GGAATATATCTTGAATTCTTC-3′, and mutant site 2 contained the sequence 5′-ATCCTGGGAATTTCTGGGAA-3′ at positions corresponding to nt −3194 to −3178 and nt −2255 to −2236, respectively. The promoter sequences of human, rat, and mouse p21$^{WAF1/CIP1}$ were compared to each other by using a computer program designed to detect any contiguous identities of 7 bp or greater, regardless of position within the promoter.[5]

mAbs. Female (BALB/c × C57Bl/6) F_1 mice were immunized by i.p. injection of purified GST-*WAF1/CIP1* fusion protein in Ribi adjuvant (Ribi

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[4] The abbreviations used are: CDK, cyclin-dependent kinase; nt, nucleotide; wt, wild type; CAT, chloramphenicol acetyltransferase.

[5] K. W. Kinzler, unpublished data.

Immunochem Research, Inc.). The fusion protein, containing amino acids 1 to 164 of p21$^{WAF1/CIP1}$, was expressed as an insoluble protein in *Escherichia coli*, collected by centrifugation following cell lysis by passage through a French pressure cell (SLM Instruments) and purified by electroelution using SDS-PAGE. Hybridomas were produced as described (31), except that test bleeds and hybridomas were screened by ELISA for anti-p21$^{WAF1/CIP1}$ reactivity using GST-p21$^{WAF1/CIP1}$- and GST-MDM2-coated microtiter plates. Hybridoma supernatants that were positive by ELISA were tested by immunoblot using lysates of GM cells treated with dexamethasone to induce p53-dependent p21$^{WAF1/CIP1}$ expression as described (15). Five hybridomas were isolated that appeared to specifically detect p21$^{WAF1/CIP1}$ and were subcloned twice by limiting dilution. Monoclonal antibody EA10, isotype IgG1, gave the strongest signal in immunoblotting and immunostaining of p21$^{WAF1/CIP1}$ and was chosen for further study.

The DO7 anti-p53 monoclonal antibody was obtained from Novacastra (Newcastle upon Tyne, United Kingdom). Clone MIB-1 anti-Ki67 monoclonal antibody was obtained from Immunotech (Westbrook, ME).

UV Treatment of Human Skin. Biopsies of skin were obtained from the lower back of healthy male volunteers, with informed consent, following UVB radiation as described previously (32). The UV source was an irradiation monochromator (Applied Photophysics, Ltd., Surrey, United Kingdom) optically coupled to a high pressure Zenon arc lamp with a central wavelength of 300 nm and a band width of ±5 nm. Doses ranged between 2.5 and 112 mJ/cm^2, and erythemal intensities were measured using a reflectance instrument (32). Biopsies were taken from normal unirradiated skin, skin with no visibly perceptible erythema (subminimal erythema dose) and skin with an erythema index nearest to 0.1 above baseline. As an additional control, comparisons were made with sites covered with sunscreen prior to irradiation (Sun E45 SPF 25 containing 18% titanium dioxide; Crookes Healthcare, Nottingham, United Kingdom). Biopsies were taken 24 h following irradiation, fixed in paraformaldehyde, embedded in paraffin, and sectioned for immunohistochemical analysis.

Cell Lines and Tissues. The GM cell line was obtained from Ed Mercer (Thomas Jefferson University, Philadelphia, PA) (33). REF-112 cells were obtained from Moshe Oren (Weizman Institute, Rehovot, Israel) (34). Normal human tissues were obtained from the Department of Pathology of The Johns Hopkins Hospital or from Biogenex Laboratories (San Ramon, CA). p53 −/− and +/+ mice were obtained from GenPharm (Mountain View, CA).

Immunohistochemical Methods. Paraffin sections were treated with xylenes for 30 min and hydrated using graded alcohol concentrations. Antigen retrieval was routinely performed on paraffin sections by incubation in prewarmed 100 mM sodium citrate (pH 6.0) at 90°C for 30 min. Frozen sections were fixed for 5 min in Histochoice (Ameresco, Solon, Ohio), followed by incubation in PBS for 10 min. After blocking nonspecific antigens with filtered goat serum for 1 h, cryopreserved or paraffin-embedded sections were incubated with primary antibodies for 12 to 16 h. In some experiments, endogenous peroxidase activity was reduced by treating sections with 3% hydrogen peroxide-50% methanol in PBS for 5 min. Staining was achieved using a Biotin-conjugated goat anti-mouse secondary antibody (Pierce) and the ABC horseradish peroxidase method (Vector laboratories). For double staining, sections were first probed with the anti-Ki67 antibody. Following development of brown nuclear staining with a DAB chromogen (Sigma Chemical Co.), sections were treated with 2.2 M glycine (pH 2.0) for 1 h. The sections were then blocked with goat serum and probed with anti-p21$^{WAF1/CIP1}$ antibody. Nickel chloride was added to the chromogen to produce a black staining pattern, which was distinguishable from the brown stain of the Ki67 antigen. Tissue specimens were counterstained for 2 min in 0.5% methyl green, followed by sequential incubation in 100% ethanol and xylenes, and then mounted in Cytoseal 60 (Stephens Scientific, Riverdale, NJ). The ApopTag kit (Peroxidase detection) for *in situ* detection of DNA fragmentation was obtained from Oncor (Gaithersburg, Md) and used as described (35).

Northern Blot Analysis of Colonic Epithelium. Colonic epithelium was prepared by scraping the colonic lumen of mice with a rubber policeman 10 min after incubation in HBSS containing 30 mM EDTA. Phase microscopy demonstrated that over 98% of the scraped cells consisted of epithelium. Total cellular RNA was isolated from the crypts, and Northern blot analysis was performed as described previously (15). A 2.0-kb mouse p21$^{WAF1/CIP1}$ cDNA was used as the probe following random primer labeling (36).

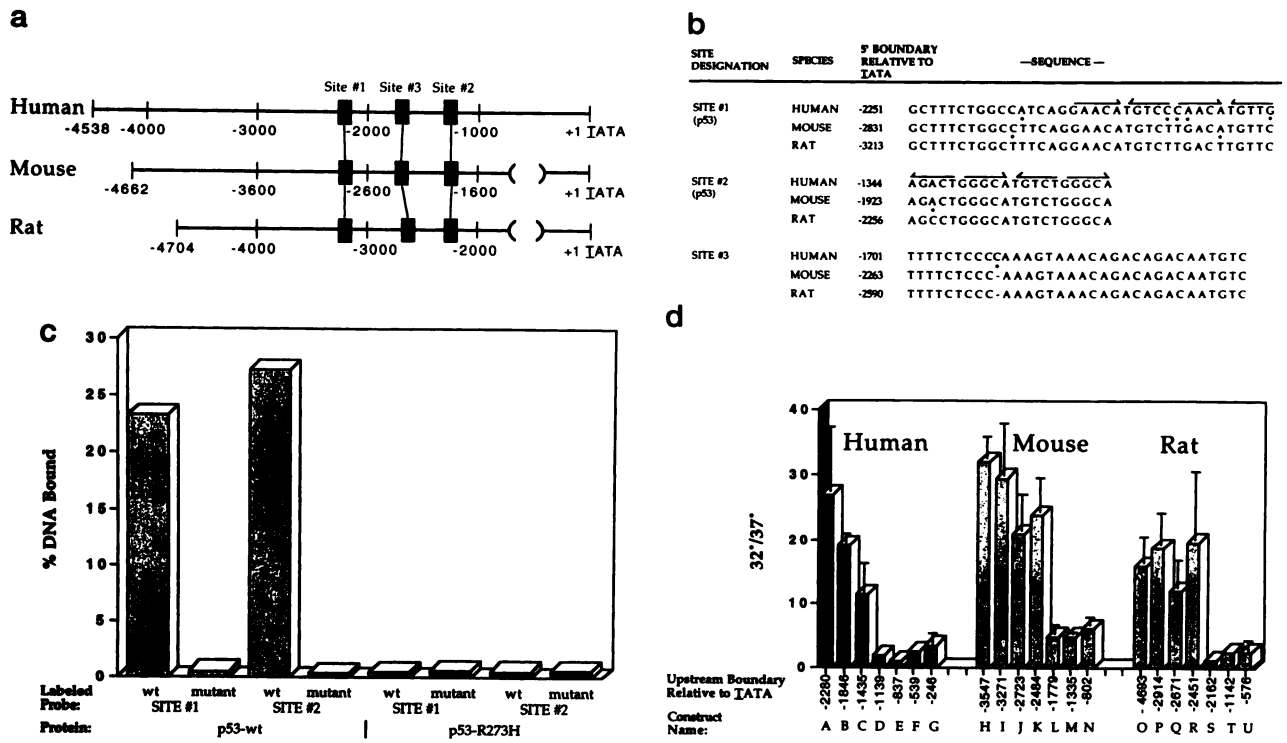

Fig. 1. Analysis of the p21$^{WAF1/CIP1}$ promoter. *a*, arrangement of sites 1, 2, and 3 in human, mouse, and rat with respect to the TATA box. *b*, sequence of sites 1–3 in the three species. *Arrows*, the p53-binding consensus tetramers. *, nucleotide positions that are not conserved among the three species. *c*, binding of sites 1 and 2 to wt and mutant p53 proteins. Mutant binding sites contained substitutions at position 4 of the pentamer (see "Materials and Methods"). *d*, p53-dependent transcriptional activity of deletion constructs. All constructs contained the transcription start site, TATA box (designated as position 1), and varying amounts of promoter sequence, as indicated. The level of p53-dependent CAT activity was defined as the activity at 32°C (wt p53) divided by that at 37°C (mutant p53 conformation). The accession numbers for the human, mouse and rat p21$^{WAF1/CIP1}$ upstream regulatory regions are U24170, U24171, and U24172, respectively. The accession numbers for the human, mouse, and rat p21$^{WAF1/CIP1}$ cDNAs are U03106, U24173, and U24174, respectively.

RESULTS

Analysis of the p21$^{WAF1/CIP1}$ Regulatory Region. As previously demonstrated, p21$^{WAF1/CIP1}$ expression can be transcriptionally regulated by wild-type p53 (15). However, p21$^{WAF1/CIP1}$ can also be regulated in a p53-independent manner (37–40). To learn more about the regulatory elements of p21$^{WAF1/CIP1}$, we determined the sequence of the human *p21*$^{WAF1/CIP1}$ gene upstream of the coding region and compared it to analogous sequences of the mouse and rat. Because the structure, function, and p53 inducibility of p21$^{WAF1/CIP1}$ is conserved among these three species, we assumed that important regulatory elements might also be conserved.

The amino acid sequences of rat and mouse p21$^{WAF1/CIP1}$ were 90% identical to each other, and 75% identical to human p21$^{WAF1/CIP1}$. The sequences upstream of the coding region, however, were remarkably divergent. Even when compared for short regions of conserved sequence, only a few stretches of homology were observed within the 4.5 kb of sequence determined for each species. The longest of these were three 20–36-bp blocks located 1.3, 1.7, and 2.2 kb upstream of the first exon of human p21$^{WAF1/CIP1}$. These sites were not only conserved in sequence but also in relative position (Fig. 1, *a* and *b*). For example, there were 891 bp separating sites 1 and 2 in the human, and 892 bp separating these sites in the mouse. Two of these three sites (sites 1 and 2) contained p53 recognition elements consisting of four tandem PuPuPuC(A/T) pentamers (30, 41). Site 1 had been identified previously in a screen for sequences that could confer p53-inducibility to reporter genes in *Saccharomyces cerevisiae* (15). Both these sites bound to wt p53, as determined by immunoprecipitation assays, but they did not bind to mutant p53 protein (Fig. 1*c*). When the C residues at position 4 of each p53-binding site consensus pentamer were substituted with A residues, the sites no longer bound to wt p53 (Fig. 1*c*).

To determine the relationship of these sites to p53 inducibility, a series of deletion constructs was generated. In each case, a segment of the *p21*$^{WAF1/CIP1}$ gene encompassing the beginning of the transcribed sequences and various lengths of upstream sequence was placed adjacent to a CAT reporter gene. These constructs were transfected into rat fibroblasts containing a temperature-sensitive *p53* gene and assayed for expression at the permissive (32°C for wt p53) and nonpermissive (39°C for mutant p53) temperatures (34). Deletion of site 1 alone reduced p53 responsiveness in the human constructs, as described previously (15), but had little effect on the rat and mouse genes (Fig. 1*d*). However, wt p53 inducibility was largely eliminated when the constructs excluded both sites 1 and 2 (Fig. 1*d*: compare constructs *C* and *D* in human, *K* and *L* in mouse, and *R* and *S* in rat). Although it is possible that other sequences between bp 2162 and 2451 (in the rat, for example) mediated p53 responsiveness, the sequence and binding results (Fig. 1, *b* and *c*) strongly suggested that site 1 was important for p53 inducibility.

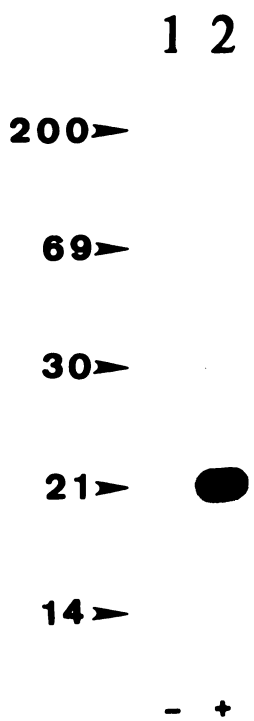

Fig. 2. Specificity of the anti-p21$^{WAF1/CIP1}$ mAb. Western analysis of GM cell lysates either in the absence (*Lane 1*) or presence (*Lane 2*) of dexamethasone using the EA10 antibody. The sizes of coelectrophoresed molecular weight markers are shown on the *left* in kilodaltons.

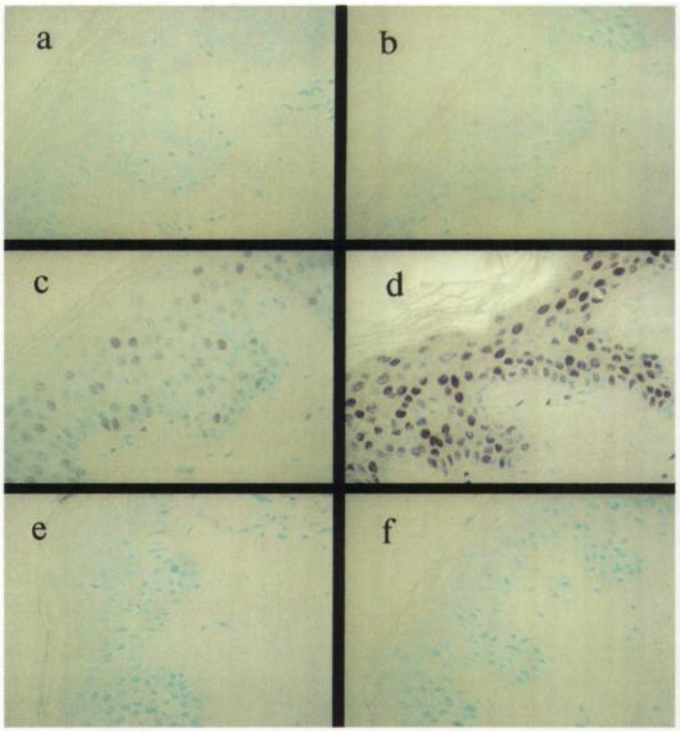

Fig. 3. UV induction of p21$^{WAF1/CIP1}$ protein *in vivo*. Human skin was either untreated (*a* and *b*) or treated (*c-f*) with UV light either without (*c* and *d*) or with (*e* and *f*) pretreatment with sunscreen. Sections were analyzed for p53 (*a, c,* and *e*), or p21$^{WAF1/CIP1}$ (*b, d,* and *f*) expression using immunohistochemical methods.

Site 3 was of interest in that it contained a palindromic sequence similar to the MyoD recognition site at its 3′ end (GACAATGTC). Transcripts of p21$^{WAF1/CIP1}$ have been shown to be regulated by MyoD in a p53-independent manner (39, 38). There was strong conservation of the positions of this site with respect to the p53-binding sites in all three species examined (Fig. 1*a*). Only a few other short stretches of identity among the three species were observed in the upstream sequence comparisons. Of these, the only one with more than 12 bp of contiguous identity was the sequence 5′-AGAATT-GTCCTTTAT-3′ at position −1067, −1166, and −1245 of the human, mouse, and rat promoters, respectively.

mAbs. A series of mAbs was generated to human p21$^{WAF1/CIP1}$. Among several reactive hybridomas tested, the monoclonal EA10 showed the strongest reactivity to denatured p21$^{WAF1/CIP1}$ protein, with no cross-reacting proteins visible on Western blot analysis (Fig. 2). This antibody was further tested for its ability to recognize p21$^{WAF1/CIP1}$ via immunohistochemical methods. The GM cell line contains a wild-type *p53* gene whose expression is inducible by

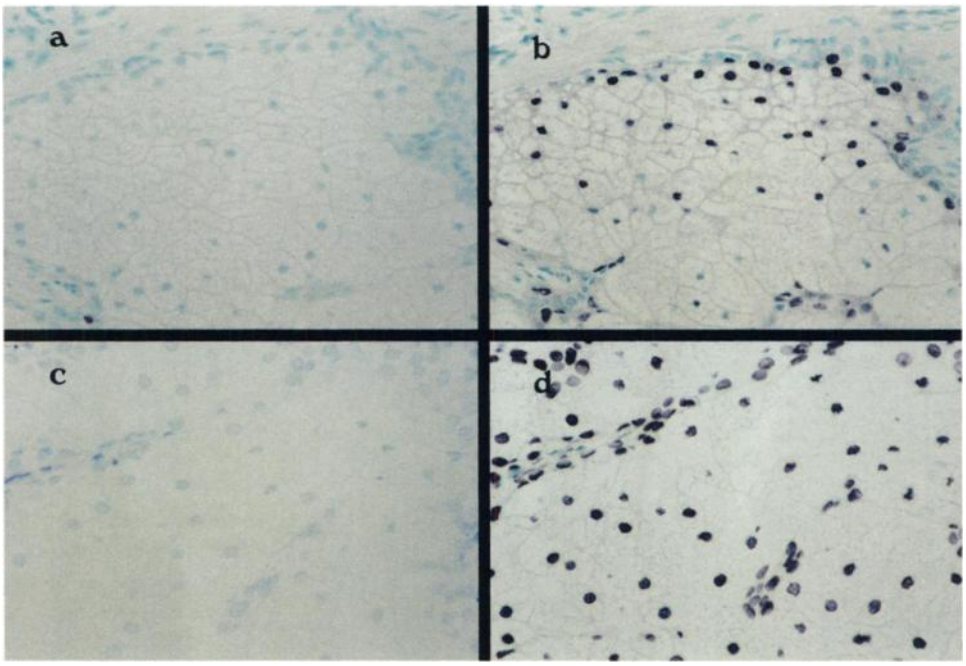

Fig. 4. p21$^{WAF1/CIP1}$ expression in sebaceous glands. Expression of p53 (*a*) or p21$^{WAF1/CIP1}$ (*b*) was detected using the DO7 and EA10 antibodies, respectively. *In situ* DNA fragmentation analysis was carried out either in the absence (*c*) or presence (*d*) of terminal deoxynucleotidyl transferase.

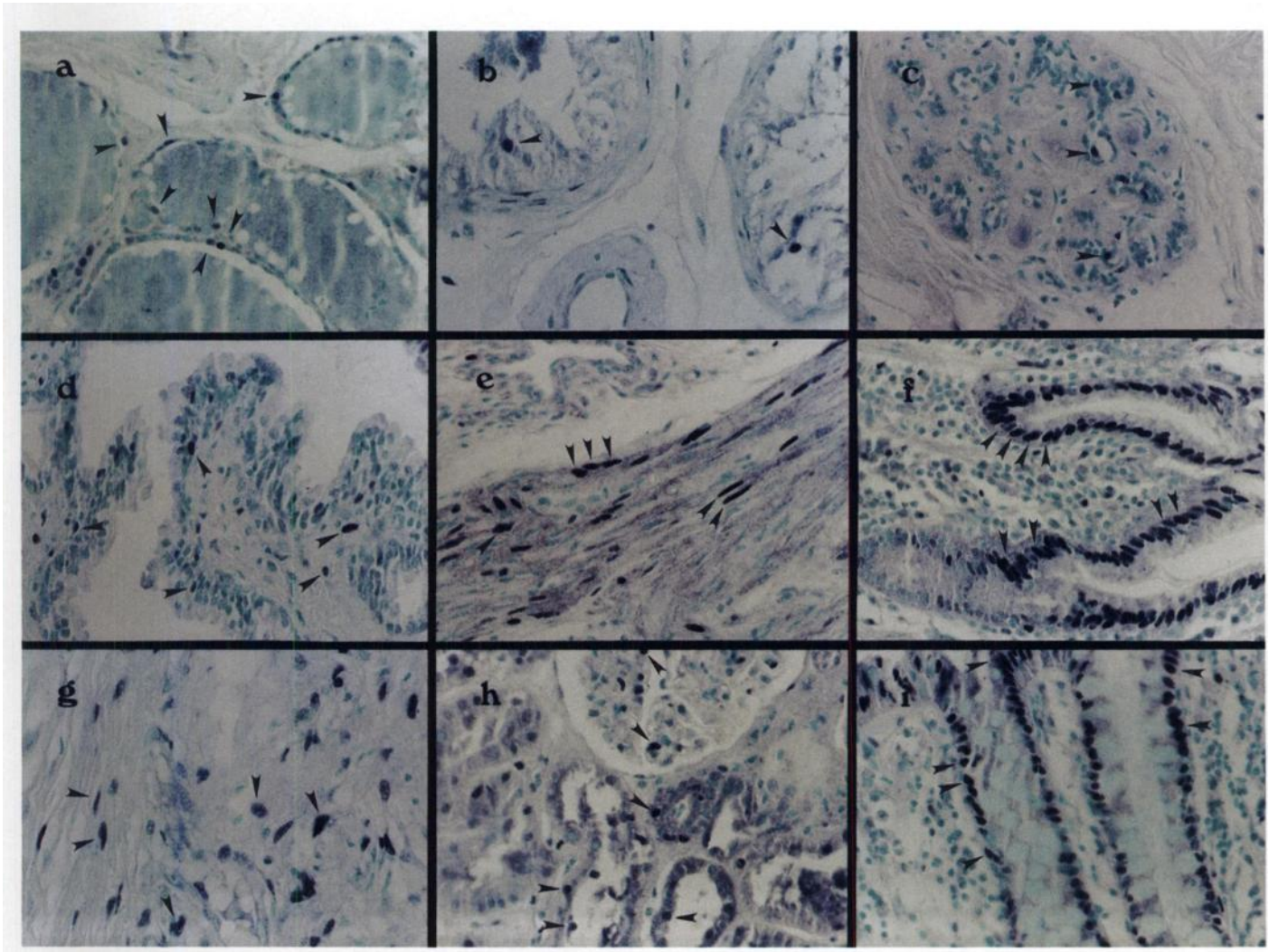

Fig. 5. p21$^{WAF1/CIP1}$ expression in adult human tissues. The EA10 monoclonal antibody was used to detect p21$^{WAF1/CIP1}$ expression in thyroid (*a*), testes (*b*), breast (*c*), prostate (*d*), uterus (*e*), stomach (*f*), tongue (*g*), kidney (*h*), and colon (*i*). *Arrowheads,* some of the p21$^{WAF1/CIP1}$-expressing nuclei.

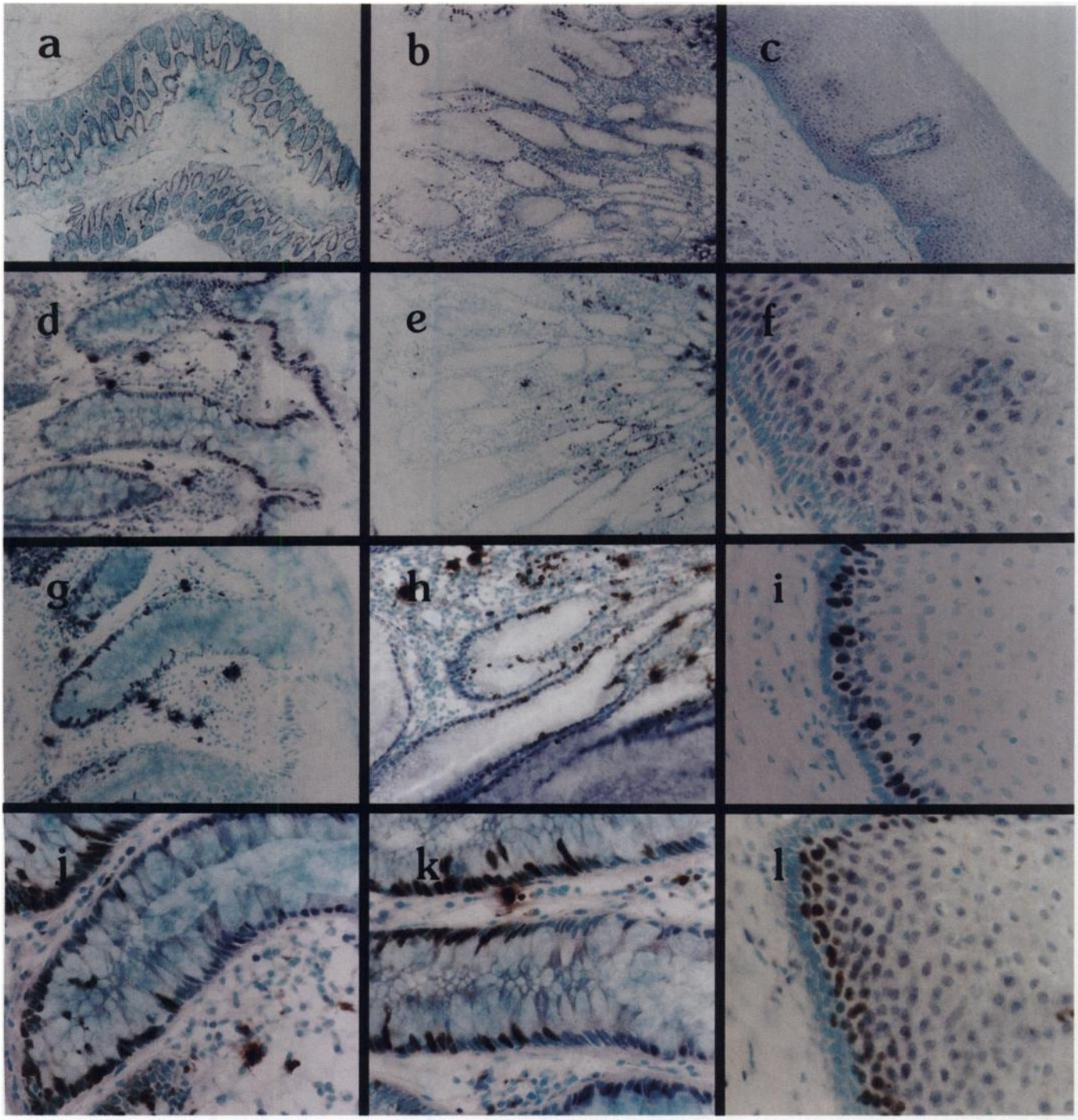

Fig. 6. Proliferating and nonproliferating compartments in the gastrointestinal tract. Colonic mucosae (*a, d, g, j,* and *k*), stomach (*b, e,* and *h*) and esophagus (*c, f, i,* and *l*) were analyzed for expression of p21$^{WAF1/CIP1}$ (*a, b, c, d,* and *f*), Ki67 (*e, g,* and *i*), or both p21$^{WAF1/CIP1}$ (*black*) and Ki67 (*brown*; *h, j, k,* and *l*). Nuclei of cells not expressing Ki67 or p21$^{WAF1/CIP1}$ are stained *green* by the counterstain. The nonepithelial cells that appear stained represent tissue macrophages with endogenous peroxidase activity.

dexamethasone (33). Little p21$^{WAF1/CIP1}$ expression was observed in GM cells in the absence of dexamethasone, but in its presence, intense reactivity confined to the nucleus was observed (data not shown).

It has been demonstrated that DNA-damaging agents cause a substantial increase in p53 expression (32, 42, 43). The increased p53 is soon followed by the induction of p21$^{WAF1/CIP1}$, at least *in vitro* (44, 45). To determine whether this regulatory circuit functioned *in vivo*, volunteers were exposed to low doses of UV radiation, and skin biopsies were obtained and examined for their p53 and p21$^{WAF1/CIP1}$ expression. In the absence of radiation, little p53 or p21$^{WAF1/CIP1}$ expression was detectable (Fig. 3, *a* and *b*). Following irradiation, marked increases in both p53 and p21$^{WAF1/CIP1}$ expression were observed (Fig. 3, *c* and *d*). As *in vitro*, p21$^{WAF1/CIP1}$ expression was localized to the nucleus. The dependence of this response on UV light was documented by its absence when skin was pretreated with sunscreen (Fig. 3, *e* and *f*).

p21$^{WAF1/CIP1}$ Expression in Adult Human Tissues. During the examination of UV-irradiated skin, we noted intense p21$^{WAF1/CIP1}$ staining in the nucleus of sebaceous glands, whether or not the skin had been irradiated (Fig. 4*b*). Sebaceous glands are holocrine, *i.e.*,

they release their contents through total cellular disintegration (46). The proliferating sebaceous cells line the periphery of the gland, and maturing cells can be observed in its center. Staining for p21$^{WAF1/CIP1}$ was observed in the maturing cells, whereas p53 staining was usually not detectable (Fig. 4*a*). We suspected that the process of cellular disintegration was a form of apoptosis. This was supported by a terminal deoxynucleotidyl transferase-based assay to detect DNA fragmentation *in situ* (Fig. 4, *c* and *d*).

A survey of p21$^{WAF1/CIP1}$ expression in adult human tissues is shown in Fig. 5. A subset of nuclei in thyroid, prostate, muscle, kidney, breast, testes, and gastrointestinal tract epithelia stained intensely, whereas little or no staining was observed in lymph nodes, liver, spleen, brain, heart, or lung. There was a striking compartmentalization of p21$^{WAF1/CIP1}$ expression throughout the gastrointestinal tract. In the colon, for example, p21$^{WAF1/CIP1}$ staining was confined to the upper half of the crypts (Fig. 6, *a* and *d*). In order to confirm that the nuclear staining observed in colonic epithelium was due to p21$^{WAF1/CIP1}$, a total cell lysate from the same human colon sample probed with the same EA10 monoclonal antibody used for immunohistochemistry revealed only one detectable protein band at 21,000 daltons by Western blot analysis.[6]

On the basis of previous studies of crypt cell maturation (47, 48), this topography suggested that p21$^{WAF1/CIP1}$ expression might be confined to the postreplicative compartment. To test this idea, we stained adjacent sections with an antibody reactive with Ki67, a 300,000 daltons nuclear protein that is widely used as a marker of replicating cells (49–51). Ki67 is expressed during both the G_1 and S phases of proliferating but not quiescent cells (52). There was a remarkable and mutually exclusive pattern of staining for Ki67 and p21$^{WAF1/CIP1}$ in gastrointestinal epithelia. In the colon, Ki67 was expressed in cells near the base of the crypt and extended about one-half way up the crypt columns (Fig. 6*g*). Ki67 expression then abruptly stopped, and p21$^{WAF1/CIP1}$ expression began, continuing up to the lumen (Fig. 6 *d* and *g*). Staining of serial sections, as well as double staining of the same section, showed that cells at mid-crypt expressed either Ki67 or p21$^{WAF1/CIP1}$ but not both (Fig. 6, *j* and *k*). There were a few nonstaining cells separating the Ki67- and p21$^{WAF1/CIP1}$-expressing compartments (Fig. 6 *j* and *k*). No indication of apoptosis was observed in p21$^{WAF1/CIP1}$-positive cells of the colon by the terminal deoxynucleotidyl transferase-based assay (data not shown).

A few cells at the very base of the crypt did not exhibit p21$^{WAF1/CIP1}$ or Ki67 expression (Fig. 6*j*). These might represent stem cell reserves in "G_0" phase. The expression of p21$^{WAF1/CIP1}$ and Ki67 had little relationship to cellular differentiation *per se*. Fully differentiated, mucous-secreting goblet cells, for example, expressed either Ki67 or p21$^{WAF1/CIP1}$, depending only on their position within the crypt.

A similar inverse relationship between Ki67 and p21$^{WAF1/CIP1}$ staining was observed in the small intestine (data not shown). In the oxyntic gastric mucosa, p21$^{WAF1/CIP1}$ expression was observed in the surface epithelium and the gastric pits (Fig. 6*b*). Ki67 was expressed in a band-like fashion across the neck of the gastric glands (Fig. 6*e*). Neither Ki67 nor p21$^{WAF1/CIP1}$ was expressed in the body of the gastric glands (Fig. 6, *b* and *e*). Serial sections and double staining indicated that Ki67 and p21$^{WAF1/CIP1}$ expression in the stomach were mutually exclusive (Fig. 6, *b*, *e*, and *h*).

In the stratified squamous epithelium of the esophagus, compartmentalized patterns were also observed. The layer of cells closest to the basement membrane did not express p21$^{WAF1/CIP1}$ or Ki67 (Fig. 6, *f* and *i*). Most of the cells in the second layer expressed Ki67 but not p21$^{WAF1/CIP1}$ (Fig. 6*i*). The next two to three layers expressed p21$^{WAF1/CIP1}$ but not Ki67 (Fig. 6, *c* and *f*). Serial sections and double staining of esophageal sections revealed the mutually exclusive character of Ki67 and p21$^{WAF1/CIP1}$ expression (Fig. 6*l*).

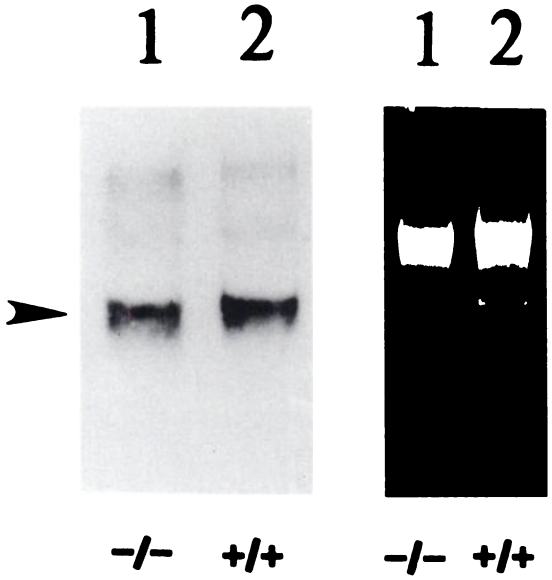

Fig. 7. Northern blot analysis of p21$^{WAF1/CIP1}$ in p53 −/− (*Lane 1*) and +/+ (*Lane 2*) mouse colonic crypts. Crypts were isolated and RNA purified from them as described in "Materials and Methods." An autoradiogram depicting the hybridization is shown on the *left*, and the corresponding ethidium bromide stain of the RNA, showing equivalent loading, is shown on the *right*. *Arrowhead*, the hybridization to p21$^{WAF1/CIP1}$ mRNA.

In contrast to p21$^{WAF1/CIP1}$, there was no p53 expression detectable at the immunohistochemical level in gastrointestinal tract epithelia. This suggested that the observed p21$^{WAF1/CIP1}$ expression might not be dependent upon p53 expression. To test this hypothesis, we examined p21$^{WAF1/CIP1}$ expression in normal mice and in p53-null animals generated by homologous recombination. Our monoclonal antibodies did not react with mouse p21$^{WAF1/CIP1}$. Therefore, we isolated colonic crypt epithelium, using a physical fractionation technique, from p53 −/− and +/+ animals and probed for p21$^{WAF1/CIP1}$ expression by Northern blot analysis (Fig. 7). The expression of p21$^{WAF1/CIP1}$ in such crypt cells was similar in the normal and p53-null mice, consistent with recent *in situ* hybridization results (38).

p21$^{WAF1/CIP1}$ Expression in Tumors. We next determined whether the normal pattern of p21$^{WAF1/CIP1}$ expression was preserved in colorectal adenomas. Adenomas are benign tumors representing the early stages of neoplasia (53). The crypts of these tumors, although composed of cells that often differentiated and migrated in normal fashion, had abnormal patterns of p21$^{WAF1/CIP1}$ expression in two respects: (*a*) there was a global decrease in p21$^{WAF1/CIP1}$ expression. In normal colon, virtually every cell in the top half of each crypt stained intensely for p21$^{WAF1/CIP1}$ (Fig. 6*a*). In the adenomas, less than 20% of the crypts had any observable p21$^{WAF1/CIP1}$ expression, and in those that did, the expression was confined to cells near the surface (Fig. 8*a*); and (*b*) the distinct separation between Ki67- and p21$^{WAF1/CIP1}$-expressing compartments observed in normal colonic epithelium was abrogated in the adenomas. Serial sections and double staining for p21$^{WAF1/CIP1}$ and Ki67 demonstrated a seemingly random mix of p21$^{WAF1/CIP1}$ and Ki67-expressing cells at the surface of the adenomatous crypts (Fig. 8*b*). The abnormal pattern of p21$^{WAF1/CIP1}$ expression observed in the benign tumors was not a result of p53 mutation, as no p53 expression was observed in these adenomas, including the cells expressing p21$^{WAF1/CIP1}$. High p53 expression

[6] Y-X. Zeng and W. S. El-Deiry, unpublished observation.

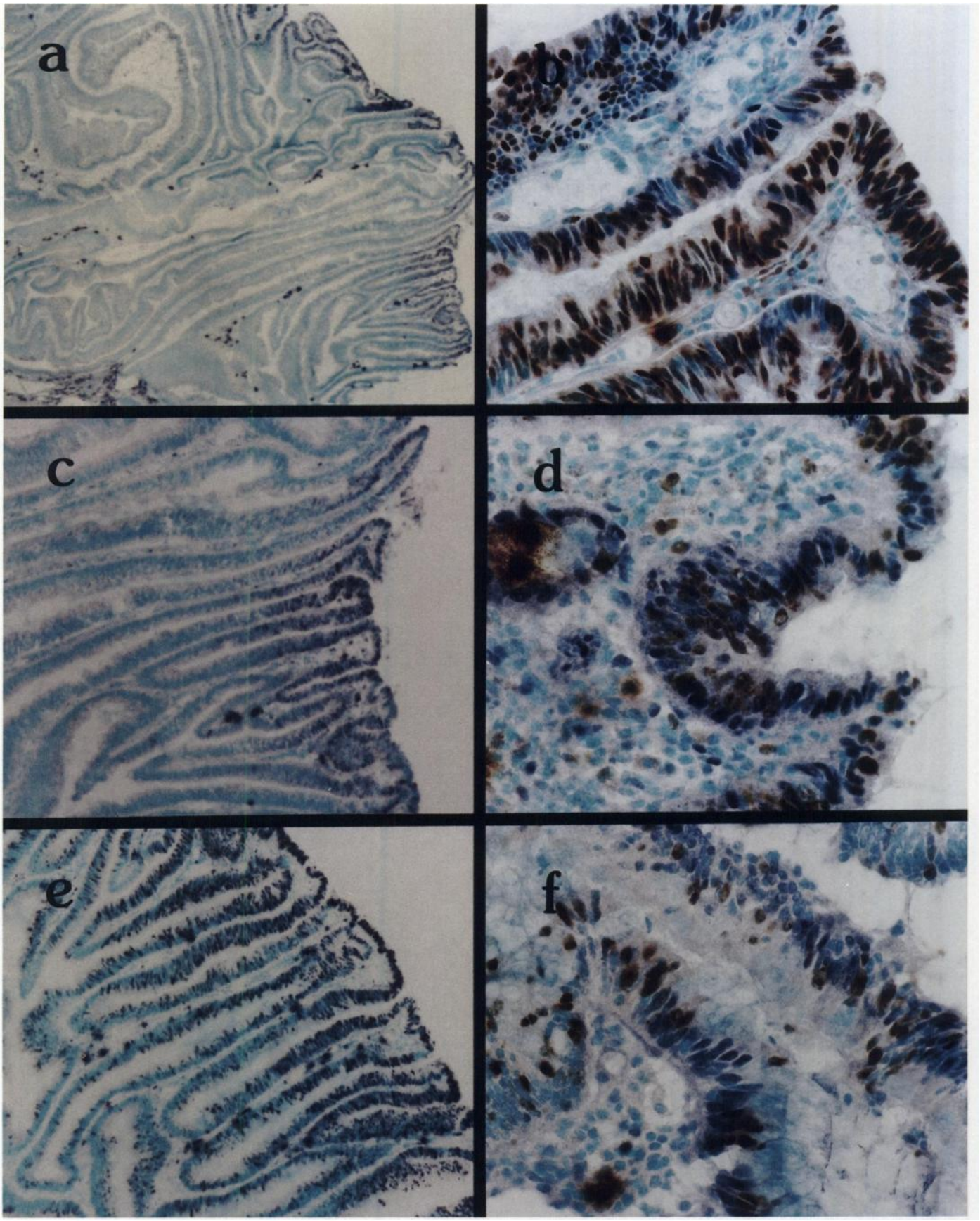

Fig. 8. Loss of topological control in benign colonic tumors. *a*, a low power view (× 40) of the p21$^{WAF1/CIP1}$-expressing portion of an adenoma stained with the EA10 mAb. *c*, a higher power view (× 100) of the same adenoma. *e*, Ki67 staining of a serial section from the same adenoma (× 100). *b*, double staining of this adenoma with Ki67 (*brown*) and p21$^{WAF1/CIP1}$ (*black*) at × 400. The p21$^{WAF1/CIP1}$-expressing portions of two other adenomas, double stained for Ki67 and p21$^{WAF1/CIP1}$ expression, are shown in *d* and *f* (× 400). The nonepithelial cells that appear stained represent tissue macrophages with endogenous peroxidase activity.

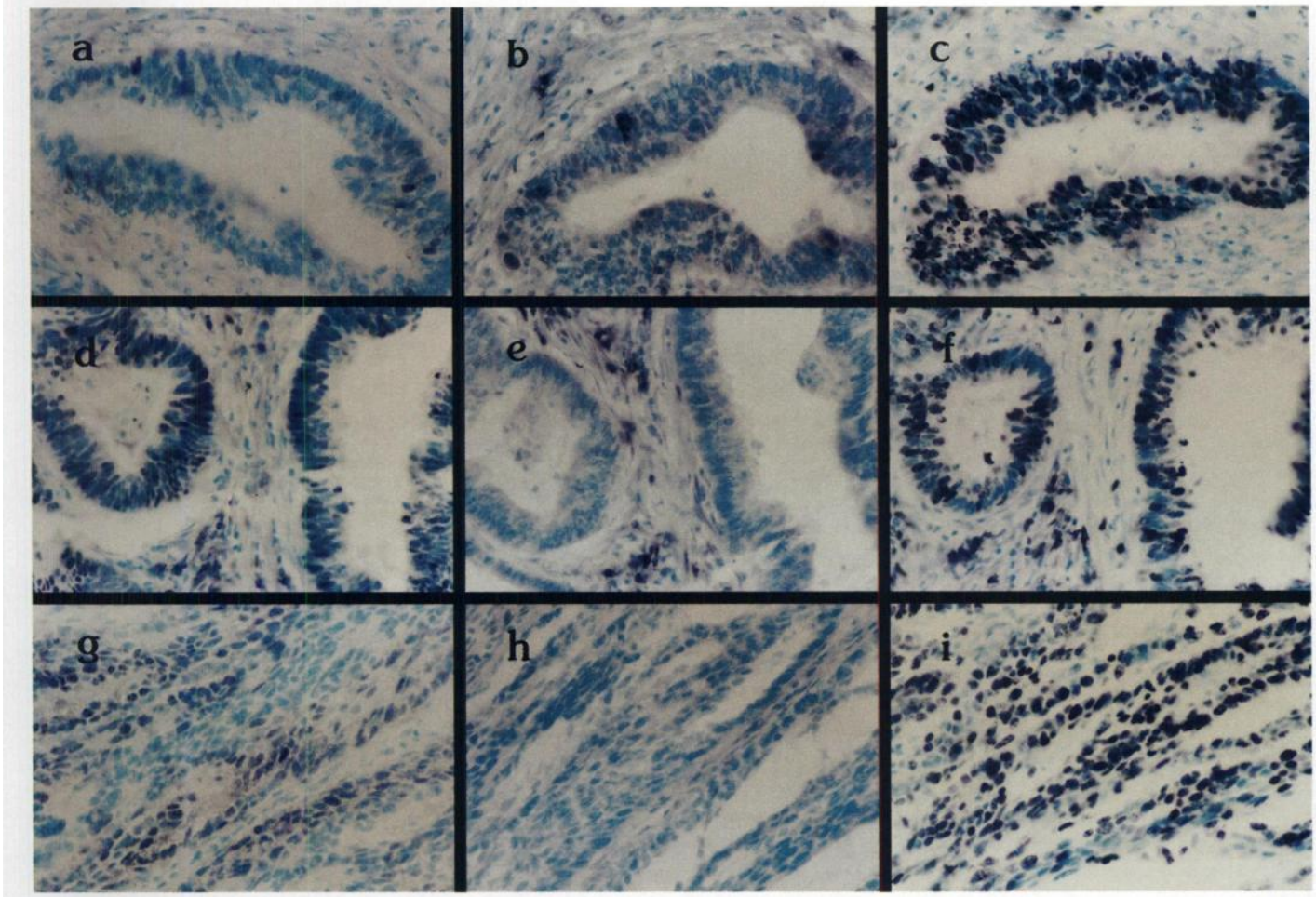

Fig. 9. Patterns of $p21^{WAF1/CIP1}$ and p53 expression in malignant colonic tumors. Coincident p53 and $p21^{WAF1/CIP1}$ expression was observed in a portion of the colorectal carcinoma shown in *a*, *b*, and *c*. This tumor contained wild-type p53. Prominent p53 expression without detectable $p21^{WAF1/CIP1}$ expression was observed in two colorectal cancers with p53 mutation (one in *d*, *e*, and *f*, and the other in *g*, *h*, and *i*). Expression of $p21^{WAF1/CIP1}$ was observed in some of the stromal cells in *e* but not in the neoplastic epithelium. The anti-p53 antibody was used in *a*, *d*, and *g*; the anti-$p21^{WAF1/CIP1}$ antibody was used in *b*, *e*, and *h*; and the anti-Ki67 antibody was used in *c*, *f*, and *i*. The nonepithelial cells that appear stained represent tissue macrophages with endogenous peroxidase activity.

would be expected if p53 mutations existed (54, 55). The immunohistochemical data on p53 were consistent with previous sequencing studies, which showed that p53 mutations are rare in adenomas (56). Each of six adenomas from six different patients exhibited the same disorganized patterns (examples in Fig. 8). The altered pattern of $p21^{WAF1/CIP1}$ expression in the adenomas was not simply a manifestation of increased crypt length, as the elongated regenerating crypts found in patients with ulcerative colitis revealed a normal compartmentalization of $p21^{WAF1/CIP1}$ and Ki67-expressing cells (data not shown).

We next examined five malignant colorectal tumors, one with wild-type p53 and four with mutations ($175^{arg>his}$, $220^{tyr>cys}$, $248^{arg>gln}$, and $286^{glu>lys}$; Ref. 56). In such advanced neoplasms, crypts with defined bases and lumens are not observed, but gland-like structures composed of malignant epithelium infiltrate the layers of the colonic wall. In the colorectal carcinoma with wt p53, neither $p21^{WAF1/CIP1}$ nor p53 expression could be observed in the majority of the epithelial cells (as in most adenoma cells). However, a subset of glands expressed both $p21^{WAF1/CIP1}$ and p53 (Fig. 9, *a* and *b*). Serial sections revealed that the same cells expressed both $p21^{WAF1/CIP1}$ and p53, suggesting that the former was dependent on the latter. In the four colorectal cancers with p53 mutations, high levels of p53 were observed, as expected, but low or undetectable $p21^{WAF1/CIP1}$ expression was found (examples in Fig. 9, *d*, *e*, *g*, and *h*). Ki67 was found to be expressed throughout the cancers, regardless of p53 status (Fig. 9, *c*,*f*, and *i*).

DISCUSSION

The results described above emphasize two important concepts relating to normal and abnormal growth control. In continually renewing cell populations such as in the gastrointestinal tract, precise control of cellular division is imperative. Our data suggest that this control may in part be due to a tightly regulated pattern of cell cycle inhibition by $p21^{WAF1/CIP1}$. The cell cycle is abruptly terminated as cells leave the Ki67 replicative compartment and $p21^{WAF1/CIP1}$ expression is turned on. It was notable that this topological control of $p21^{WAF1/CIP1}$ expression was probably independent of p53, both by immunohistochemical and genetic criteria.

Previous analyses of $p21^{WAF1/CIP1}$ expression are largely consistent with the results presented here, but there are some important differences. The induction of $p21^{WAF1/CIP1}$ expression following radiation or chemically induced DNA damage has been shown to be dependent on wt p53 in tissue culture models (37, 44, 45). From a detailed analysis of the upstream regulatory region of $p21^{WAF1/CIP1}$, it was clear that at least two highly conserved p53-binding sites exist and likely mediate transcriptional activation of $p21^{WAF1/CIP1}$ following DNA damage. However, expression of $p21^{WAF1/CIP1}$ is independent

of p53 in other circumstances, such as during the differentiation of leukemia cells following phorbol ester treatment or during the MyoD-dependent formation of myotubes from myoblasts (38, 39, 40). This p21$^{WAF1/CIP1}$ expression has been interpreted as a manifestation of cellular differentiation. Our observations in the gastrointestinal tract suggest that p21$^{WAF1/CIP1}$ expression is not an effect or cause of differentiation *per se*. Thus, p21$^{WAF1/CIP1}$ expression was not detected in fully differentiated cells in the bottom one-half of the crypts but was expressed in the same cells, once they left the replicative compartment and migrated upwards. This suggests that p21$^{WAF1/CIP1}$ plays a critical role in negatively controlling the proliferative compartment, in line with its known biochemical activities. This inhibition may be required for differentiation to take place in some cell types (*e.g.*, muscle), but the process of differentiation and p21$^{WAF1/CIP1}$ expression could clearly be separated in the intestine. It remains to be determined which regions of the promoter mediate p53-independent regulation of p21$^{WAF1/CIP1}$. One attractive candidate is site 3, which is conserved in sequence as well as position relative to p53-binding sites (Fig. 1, *a* and *b*). The complete sequences of the promoters of the human, mouse, and rat genes determined here should facilitate efforts to identify important regulatory features of p21$^{WAF1/CIP1}$ in the future.

A related point concerns the topological organization of p21$^{WAF1/CIP1}$ expression, a phenomenon that could not have been appreciated from cell culture studies. The striking distribution of p21$^{WAF1/CIP1}$ expression in colonic crypts and squamous epithelium suggests that cell-cell or cell-stroma interactions may be contributing to cell cycle control. As cells migrate up the crypt, they presumably make contacts that influence the replicative machinery negatively (loss of Ki67 expression) as well as positively (induction of p21$^{WAF1/CIP1}$). In colonic neoplasms, there was a disorganization of this precisely ordered topological relationship between cycling and cycle-inhibited cells. This form of molecular disorganization occurred quite early in the neoplastic process, as it was present in benign adenomas, long before malignant transformation.

Our data are consistent with a two-stage model for the abrogation of cell cycle control in neoplasia. The first stage involves a loss of the highly ordered spatial separation between proliferating and nonproliferating compartments. In the case of colonic adenomas, this might be due to mutations in the APC gene (57), which could result in disruption of cell contact signaling of growth as cells migrate up the crypts. APC normally is expressed at higher levels as cells travel up the crypt (31) and influences the cadherin-catenin network linking cell-cell contacts to the cytoskeleton (58, 59). As tumors progress towards more malignant forms, other signals in the abnormal microenvironment may induce wt p53 expression with consequent p21$^{WAF1/CIP1}$ induction. Such abnormal microenvironments were presumably present in the subset of glands in the wt p53-containing cancers that exhibited coincident p53 and p21$^{WAF1/CIP1}$ expression (Fig. 9, *a* and *b*). The *p53* gene may function in these circumstances as a kind of "emergency brake," checking further uncontrolled proliferation. However, once p53 is induced in this way, the stage is set for selective outgrowth of a p53-mutant cell, relieving the final check on cell cycle control (Fig. 9, *d, e, g,* and *h*).

In summary, there is a precisely ordered topological pattern of p21$^{WAF1/CIP1}$ expression in normal cells that becomes disordered during neoplasia. We expect that other CDK inhibitors will be analogously affected in specific neoplasms and that their topographic analysis will provide a new dimension to the study of naturally occurring tumors.

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