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22. We thank D. E. Cool, E. G. Krebs, and E. H. Fischer for providing the T cell PTPase cDNA, N. F. Zander and J. A. Lorenzen for providing baculovirus-expressed T cell PTPase protein, P. Russell for providing the *cdc25*$^+$ deletion strain, and S. Sazer for providing the pMNS21LΔNde1 plasmid. We are also grateful to D. E. Cool, U. Fleig, S. L. Forsburg, G. Hering, and T. Patterson for comments on the manuscript. K.L.G. is a fellow of the Jane Coffin Childs Memorial Fund for Medical Research. Supported by the Imperial Cancer Research Fund and Medical Research Council.

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Genetic Mechanisms of Tumor Suppression by the Human p53 Gene

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Mutations of the gene encoding p53, a 53-kilodalton cellular protein, are found frequently in human tumor cells, suggesting a crucial role for this gene in human oncogenesis. To model the stepwise mutation or loss of both p53 alleles during tumorigenesis, a human osteosarcoma cell line, Saos-2, was used that completely lacked endogenous p53. Single copies of exogenous p53 genes were then introduced by infecting cells with recombinant retroviruses containing either point-mutated or wild-type versions of the p53 cDNA sequence. Expression of wild-type p53 suppressed the neoplastic phenotype of Saos-2 cells, whereas expression of mutated p53 conferred a limited growth advantage to cells in the absence of wild-type p53. Wild-type p53 was phenotypically dominant to mutated p53 in a two-allele configuration. These results suggest that, as with the retinoblastoma gene, mutation of both alleles of the p53 gene is essential for its role in oncogenesis.

TUMOR-SUPPRESSOR GENES ARE DEfined as genes for which loss-of-function mutations are oncogenic (*1*). Wild-type alleles of such genes may thus function to prevent or suppress tumorigenesis. For example, introduction of wild-type copies of the retinoblastoma gene (*RB*), the prototype of this class (*2*), suppressed the neoplastic properties of human tumor cells with mutated endogenous RB, thereby providing direct evidence for tumor suppression by a single gene (*3, 4*). Another gene product, p53, was first identified as a 53-kD cellular protein that binds to SV40 T antigen (*5*), a property that is also shared by RB protein. The gene encoding p53 is commonly affected by deletions, rearrangements, or point mutations in human and murine tumor cells (*6, 7*). p53 was originally considered to be an oncogene because mutated p53 alleles could transform primary rat embryo fibroblasts in concert with an activated *ras* gene (*8*). However, cotransfection of wild-type murine p53 was shown to reduce transformation efficiency by many other oncogenes (*9*). These studies, and the observed diversity of mutations in human tumors, suggested that p53 might be a tumor suppressor gene, that is, a gene that is inactivated by mutation. The dominant transforming effect was presumed to be due to a "dominant negative" activity of mutated p53 protein that somehow blocked the growth-restricting function of wild-type p53 protein in cells. This model suggested that the relative quantity of mutated to wild-type p53 could determine the transformed phenotype, but gene dosages could not be tightly controlled in these transfection studies.

Because of such questions, as well as the possibility of species-specific differences in p53 function (*10*) and the uncertain relevance of transformed animal cells to human neoplasia, we sought to reassess the biological properties of p53 in the human system. The human osteosarcoma cell line Saos-2 was chosen as a host cell because it has no endogenous p53, because of the complete deletion of its gene (*6*). We used recombinant retroviruses derived from Moloney murine leukemia virus (Mo-MuLV) to introduce mutated or wild-type p53 under the long terminal repeat (LTR) promoter control. Cell clones isolated after infection and selection carried only a single integrated provirus of each type, and multiple clones were analyzed to exclude positional effects. A comprehensive assessment of biological properties of these clones included morphology, growth rates and saturation density in culture, colony formation in soft agar, and tumorigenicity in nude mice.

As a reference standard for human wild-type p53, we used the genomic DNA sequence of Lamb and Crawford (*11*). Poten-

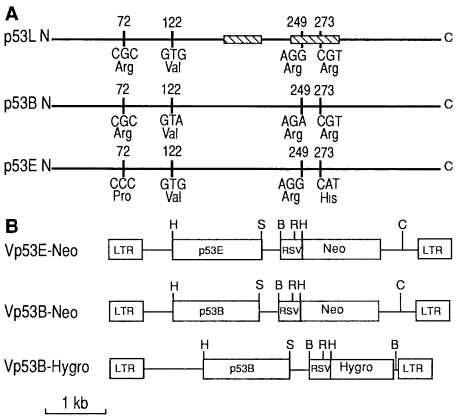

Fig. 1. Comparison of three human p53 cDNAs, and genomic organization of three recombinant retroviruses for expressing p53 protein. (**A**) Three human p53 cDNAs are diagrammed. The sequence reported by Lamb and Crawford (*11*), here labeled as p53L, was derived by sequencing clones from human fetal liver cDNA and genomic libraries, and is considered to be wild type. p53B is a cDNA clone derived from fetal brain RNA by the RT-PCR method (*12*). The deduced amino acid sequences of p53B and p53L were identical despite two silent nucleotide substitutions as indicated. p53E is a cDNA clone (*13*) that has amino acid substitutions at positions 72 and 273 relative to p53L or p53B. The Arg/Pro^{72} replacement represents a common amino acid polymorphism (*15*) without known functional significance, but the substitution of His for Arg at position 273 is found exclusively in tumor cells and is considered to be a mutation. Like many other p53 mutations, Arg^{273} → His lies within one of two conserved regions required for binding to SV40 T antigen (hatched boxes) (*23*). (**B**) Genomic organization of three p53 retroviruses are diagrammed. Vp53E-Neo was constructed by inserting a 1.5-kb Hind III–Sma I DNA fragment containing p53E into the plasmid pLRbRNL (*3*), replacing *RB* cDNA. A 1.35-kb p53B DNA fragment obtained by RT-PCR was inserted into the pLRbRNL vector to form Vp53B-Neo. The insert in one clone was entirely sequenced, as diagrammed in (A). Vp53B-Hygro was constructed by insertion of a Hind III DNA fragment containing p53B and the Rous sarcoma virus promoter into plasmid 477 (a MuLV-Hygro vector provided by W. Hammerschmidt and B. Sugden). These constructs were then used to produce the corresponding viral stocks as described previously (*3*). Some major restriction sites important for construction are indicated. H, Hind III; R, EcoR I; S, Sma I; B, Bam HI; and C, Cla I.

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tially wild-type p53 cDNA was isolated from fetal brain RNA by the method of reverse transcription–polymerase chain reaction (RT-PCR), and was cloned into plasmid (*12*). The insert in one clone (designated p53B) was entirely sequenced (~1300 bp) to reveal a wild-type deduced amino acid sequence despite two silent nucleotide replacements (Fig. 1A). Another p53 cDNA clone (p53E), isolated from epidermoid carcinoma cell line A431 (*13*), was also sequenced, and was found to contain a point mutation at codon 273 that replaced Arg with His (Fig. 1A). This is a functionally significant mutation that has also been identified in p53 from two other tumor cell lines (*14*). In addition, a neutral sequence polymorphism in codon 72 (Fig. 1A) encoded either an Arg (p53B) or a Pro (p53E). This common amino acid polymorphism (*15*), which is without known functional significance, resulted in faster migration of p53B than p53E protein by SDS–polyacrylamide gel electrophoresis (PAGE), and was therefore used to distinguish between these proteins when they were coexpressed in the same cell.

The p53E and p53B fragments were then inserted into a Mo-MuLV–based retroviral vector containing *neo* as a selectable marker gene to form Vp53E-Neo and Vp53B-Neo viral genomes, respectively (Fig. 1B). In addition, to facilitate double replacement, Vp53B-Hygro was made by inserting p53B into a similar vector containing the gene conferring resistance to hygromycin (*16*). Stocks of Vp53E-Neo, Vp53B-Neo, and Vp53B-Hygro viruses were produced as described (*3*) with titers of about 1×10^5, 2×10^4, and 1×10^5, respectively. Expression of p53 proteins from the viruses was initially assessed in the murine NIH 3T3–derived packaging line PA12, which was used for virus production (*3*). Mutated and wild-type human p53 proteins were detected in their respective virus-producing cells, with the expected difference in migration by SDS-PAGE (*17*). Because spontaneous mutation of p53 may occur frequently in cultured cells, we examined two additional biochemical properties of these p53 proteins: their cellular half-lives, and their ability to bind to T antigen. The p53B protein had a half-life of 20 to 30 min compared to 4 to 5 hours for p53E protein (*17*), consistent with published reports on the half-lives of wild-type and mutated p53 proteins (*18*). When virus-producing cells were transfected with a plasmid expresssing large amounts of SV40 T antigen, and lysates were immunoprecipitated with antibody to p53 (anti-p53) or antibody to T antigen (anti-T), T antigen was coprecipitated with p53B but not p53E protein (*17*), indicating that only p53B protein could bind to T. These results suggested that p53B-containing viruses expressed wild-type p53 and that p53E-containing virus expressed mutated p53 (*19*).

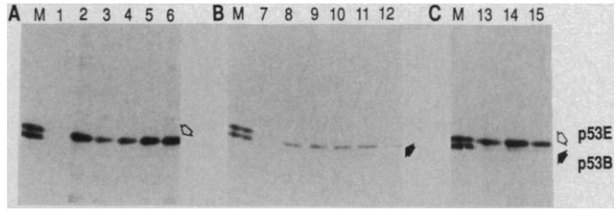

Fig. 2. Expression of human p53 proteins in virus-infected Saos-2 cells. Saos-2 cells (lanes 1 and 7) were infected either with (**A**) Vp53E-Neo to generate p53EN (lanes 2 to 6) or with (**B**) Vp53B-Neo and Vp53B-Hygro to generate p53BN (lanes 8 to 10) and p53BH (lanes 11 and 12) clones, respectively, as described in the text. (**C**) Saos-2 cells were also doubly infected with Vp53E-Neo and Vp53B-Hygro to generate p53EN-BN clones (lanes 13 to 15). Randomly selected clones, and WERI-Rb27 cells (lanes M), were labeled with [^{35}S]methionine and immunoprecipitated with anti-p53 antibody, PAb421 (*24*) as described for (*25*). p53B (filled arrows) and p53E (open arrows) are indicated.

In previous experiments, Saos-2 cells infected with parental viruses containing only neomycin- or hygromycin-resistance genes showed no changes in morphology and growth rate compared to uninfected cells (*3, 17*), suggesting that drug selection did not have a significant influence on their neoplastic properties. Saos-2 cells infected with comparable titers of either Vp53E-Neo, Vp53B-Neo, or Vp53B-Hygro in the presence of the appropriate selective agent each yielded similar numbers of drug-resistant colonies. Most colonies could be individually propagated into mass cultures, with the notable observation that Vp53B-infected cells grew much more slowly than Vp53E-infected cells (see below). Vp53E-infected clones uniformly expressed high concentrations of p53E protein (Fig. 2A). Of 30 Vp53B-infected clones examined, about 80% expressed detectable p53B protein (Fig. 2B). Two each of Vp53E-Neo and

Table 1. Neoplastic properties of p53 virus-infected Saos-2 cells. Soft-agar colony formation: equal numbers (1×10^5 or 2.5×10^4) of cells of the indicated clones were seeded in duplicate in 0.367% soft agar as described (*3*). Total colony numbers were scored after 20 days. Individual colonies contained more than 50 cells. Tumorigenicity: 1×10^7 cells from each clone were injected subcutaneously into flanks of nude mice, and tumor formation was scored at 12 weeks.

Virus-infected cells	No. of soft-agar colonies formed with		Tumorigenicity	p53 expression
	1.0×10^5 cells seeded	2.5×10^4 cells seeded	No. of mice with tumor/no. of mice injected	
Parental	392;388	104;76	10;10	None
p53EN			12;12	Mutated
p53EN-1	928;968	396;372		
p53EN-2	517;593	121;105		
p53EN-3	485;534	96;123		
p53EN-4	445;498	106;121		
p53EN-5	582;441	132;172		
p53BN			0;5	Wild type
p53BN-1	<1;<1	<1;<1		
p53BN-2	<1;<1	<1;<1		
p53BN-3	<1;<1	<1;<1		
p53BN-4	<1;<1	<1;<1		
p53BN-R	414;384	54;48	3;3	None
p53BH			0;6	Wild type
p53BH-1	<1;<1	<1;<1		
p53BH-2	<1;<1	<1;<1		
p53BH-3	<1;<1	<1;<1		
p53EN-BH			0;5	Mutated + wild type
p53EN-1-BH-1	<1;<1	<1;<1		
p53EN-1-BH-2	<1;<1	<1;<1		
p53EN-1-BH-3	<1;<1	<1;<1		
p53EN-2-BH-1	<1;<1	<1;<1		
p53EN-2-BH-2	<1;<1	<1;<1		

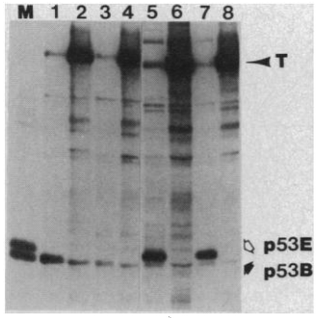

Fig. 3. Formation of p53B-T complexes in Saos-2 cells. Clones p53BN-1 (lanes 1 and 2), p53BH-1 (lanes 3 and 4), p53EN-1-BH-1 (lanes 5 and 6), and p53EN-1-BH-2 (lanes 7 and 8) were transfected with plasmid pRSV40T as described (*25*), and 60 hours later were metabolically labeled with [^{35}S]methionine. Cell lysates were immunoprecipitated with PAb421 (lanes M, 1, 3, 5 and 7) or with PAb419, a monoclonal antibody against SV40 T antigen (lanes 2, 4, 6 and 8). PAb419 coprecipitated only p53B in cells expressing both p53B and p53E.

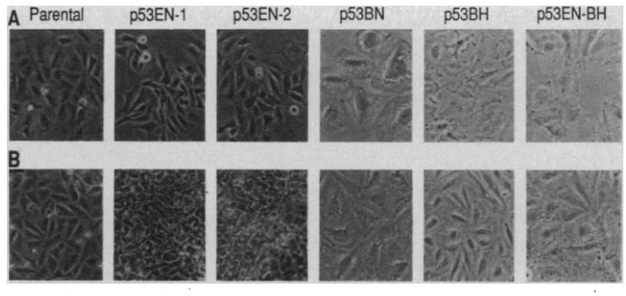

Fig. 4. Morphology in culture of parental Saos-2 cells, and representative virus-infected clones. (**A**) Exponentially growing cells. (**B**) Cells at confluency. Magnification, ×100.

Vp53B-Hygro clones were randomly selected for a second infection by the other virus, and double-infected clones were isolated and propagated as above. These clones coexpressed both p53E and p53B protein (Fig. 2C). In order to again verify that p53B protein in these cells was not secondarily mutated, p53B-expressing clones were transfected with the SV40 T antigen plasmid, and cell lysates were immunoprecipitated as described above (Fig. 3). Anti-p53 coprecipitated T in each clone, but anti-T coprecipitated only p53B, even in cells expressing both p53B and p53E. The half-life of p53B in Saos-2 was also measured and was similar to that of p53B in PA12 cells (*17*). These data again support the notion that Vp53B-infected Saos-2 clones expressed wild-type p53.

Five randomly chosen clones that stably expressed p53E protein (p53EN-1 to p53EN-5) were compared to parental Saos-2 cells in terms of morphology (Fig. 4), growth rate [as doubling time (Fig. 5A)], saturation density (Fig. 5B), soft-agar colony formation, and tumorigenicity in nude mice (Table 1). A difference in morphology was observed only under conditions of cell crowding, where cells of EN clones were far smaller and more refractile than parental cells (Fig. 4B). Correlatively, saturation density of the former was four- to fivefold greater than that of parental cells (Fig. 5B). This relative growth advantage was seen despite similar doubling times as measured under sparse growth conditions (Fig. 5A). Four EN clones and parental cells shared similar efficiencies in soft-agar colony formation and tumorigenicity in nude mice (Table 1). One clone, p53EN-1, had noticeably augmented abilities in both respects; in particular, it reliably formed large tumors from as few as 5×10^5 injected cells (*17*). We considered this discrepancy to be within the range of clonal variability expected among tumor cells. These results suggested that mutated p53 functioned in the absence of wild-type p53 to confer a limited growth advantage (higher saturation density) to Saos-2 cells in culture. In many other aspects of the neoplastic phenotype, the presence of point-mutated p53 was essentially equivalent to complete absence of p53.

In comparison to parental Saos-2 cells, clones expressing p53B (wild-type p53 protein) were invariably enlarged and flattened (Fig. 4) and had prolonged doubling times in culture of about 70 hours rather than 30 to 36 hours for parental or EN cells (Fig. 5A). The efficiency of soft-agar colony formation was reduced to less than the threshold for detecting a single colony, whereas parental cells and EN cells formed hundreds of colonies under the same conditions (Table 1). Injection of 1×10^7 cells of each of seven p53B-expressing clones into the flanks of nude mice resulted in the formation of no tumors after 12 weeks, even while the same number of parental or p53E-expressing cells formed tumors in all contralateral flanks (Table 1). These findings could not be explained by a peculiar effect of viral infection and selection because one clone, Vp53BN-R, derived from Vp53B-Neo–infected cells but lacking detectable expression of p53B, had a phenotype indistinguishable from parental cells (Table 1). The ~50% reduction of growth rate of cultured Saos-2 cells expressing p53B was insufficient to account for the complete loss of tumorigenicity and soft-agar colony formation, implying that wild-type p53 specifically suppressed the neoplastic phenotype of these cells. These results suggest that loss of wild-type p53 was a significant event during the genesis of this tumor line, and, by extension, of other osteosarcomas with mutated endogenous p53 genes (*6*).

Because both mutated and wild-type p53 proteins were apparently functional in Saos-2 cells, we asked whether both activities could be simultaneously coexpressed, whether they canceled out one another, or whether one activity was clearly dominant. The configuration of one wild-type and one mutated allele was most relevant to natural human tumorigenesis, because this is a necessary intermediate step on the pathway toward complete loss of wild-type p53. Infection of two different p53E-expressing clones with Vp53B-Hygro yielded 22 hygromycin-resistant clones, of which 15 coexpressed both p53B and p53E. To determine the number of integrated copies of each virus present in these clones, we analyzed genomic DNA of three clones derived from p53EN-1 cells infected with Vp53B-Hygro by Southern (DNA) blotting (Fig. 6). Hybridization with *neo* as a probe showed a single, common junctional frag-

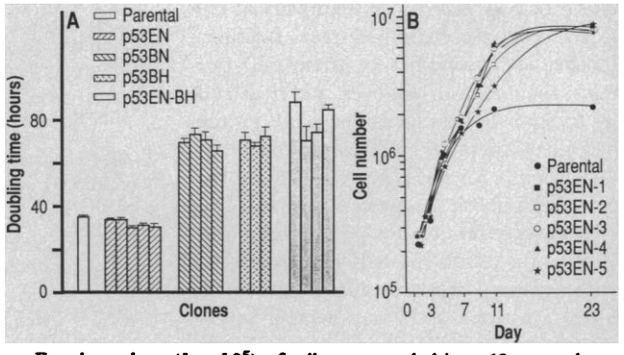

Fig. 5. Growth effects of p53 expression in Saos-2 cells. (**A**) Doubling times of parental Saos-2 cells and virus-infected clones in an exponential growth stage. Equal numbers of each cell type were seeded into 60-mm culture dishes; cells of two dishes were trypsinized and counted at daily intervals for 4 days. Doubling times were derived from lines fitted to log cell numbers. (**B**) Saturation density of parental Saos-2 and EN clones. Equal numbers (1×10^5) of cells were seeded into 60-mm culture dishes; cells of two dishes were trypsinized and counted at the times indicated. Plotted points were mean cell numbers from duplicate dishes. Saturation density of p53E-expressing cells was four- to fivefold greater than parental cells.

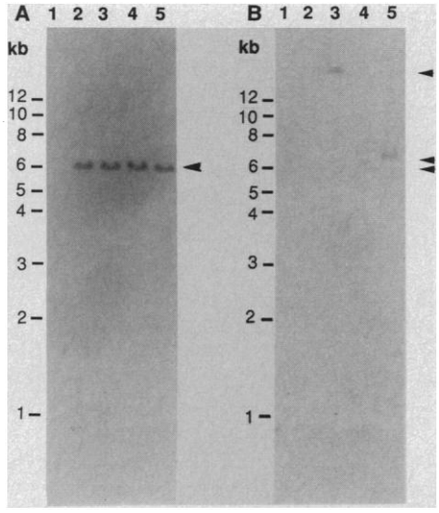

Fig. 6. p53EN-BH cells harbored one copy of Vp53E-Neo and one copy of Vp53B-Hygro. Genomic DNA (10 μg) extracted from parental Saos-2 cells (lanes 1), and clones p53EN-1 (lanes 2), p53EN-1-BH-1 (lanes 3), p53EN-1-BH-2 (lanes 4), and p53EN-1-BH-3 (lanes 5), was digested with Eco RI, and separated in 0.7% agarose gels. Southern transfer was performed, and nylon membranes were hybridized with ^{32}P-labeled *neo* (**A**) or *hygro* (**B**) DNA probes, by standard methods (*26*). A single, unique junction fragment is seen in each clone with each probe, indicating single integrated copies of each virus.

ment in all three clones, indicating the presence of a single integrated copy of Vp53E-Neo in p53EN-1 cells (Fig. 6A). Hybridization with *hygro* showed a single, unique junctional fragment in each clone, indicating the presence of single, independently integrated copies of Vp53B-Hygro in p53EN-BH clones (Fig. 6B). Single integrations were expected, based on previous use of a related recombinant retrovirus at comparable titers (*3*). These findings confirmed that p53EN-BH clones indeed contained one integrated copy of each virus, and that both exogenous p53 genes were expressed (Fig. 2). By criteria of morphology, growth rate, saturation density, soft-agar colony formation, and tumorigenicity in nude mice, double-replacement clones were indistinguishable from clones expressing only p53B (Figs. 4 and 5, Table 1). Cells obtained by infecting in the other order, that is, p53B-expressing cells infected with Vp53E-Neo, had the same phenotype (*17*). Complete dominance of wild-type p53 activity was observed despite the ~10-fold lower quantities of wild-type than mutated p53 in these cells, an expected consequence of the shorter half-life of wild-type p53.

In sum, single copies of wild-type p53 were sufficient to suppress the neoplastic phenotype of human osteosarcoma cells lacking p53 expression, as well as of cells expressing a mutated p53 allele. These results are consistent with the findings of Baker *et al.* (*20*) that transfection of human wild-type p53 suppressed the growth of human colorectal tumor cells, even those with mutated endogenous p53 alleles. These studies suggest that, like *RB*, human p53 has a broad suppression activity in many tumor types. The dominance of wild-type over mutated p53 in a two-allele configuration suggests that both wild-type p53 alleles must be lost for an oncogenic effect of this gene (*21*). Moreover, transfected wild-type p53 failed to suppress the growth of human colorectal adenoma cells containing wild-type p53 alleles (*20*); similarly, exogenous *RB* failed to suppress osteosarcoma cells with wild-type endogenous *RB* (*3*). Thus the suppressive effect of exogenous *RB* or p53 may be limited to tumor cells lacking wild-type endogenous *RB* or p53. These shared properties of *RB* and p53 reinforce the tumor suppressor gene concept, including the possible clinical use of their replacement in appropriate tumor cells.

One question not answered by previous studies is whether point-mutated p53 has some function, or whether it is completely functionless, that is, is equivalent to its complete deletion. The mutated human p53 allele we used retains some function, because its insertion into osteosarcoma cells augmented their saturation density in culture (*22*). Mutated p53 alleles may confer a growth advantage or a more malignant phenotype to tumor cells without wild-type p53, thereby explaining why mutated p53 alleles are commonly retained in tumor cells. Additional experiments are needed to address this proposal. The idea that mutated p53 has a biological function, and that its function is recessive to that of wild-type p53, is inconsistent with the hypothesis of a dominant negative effect, at least as it applies in natural human tumorigenesis. The dominant transforming properties of mutated murine p53 alleles may be due to the high copy numbers of genes introduced by transfection, and the resulting massive overexpression of mutated p53. Under conditions of equal gene dosage, wild-type p53 is able to override the influence of mutated p53 despite a tenfold molar excess of the latter. These observations may be explained by competition of wild-type and mutated p53 for common cellular targets, for which wild-type p53 is much more avid. In this model, wild-type and mutated p53 would transmit opposite growth signals to these targets, with total absence of p53 perhaps an intermediate signal. Alternatively, mutated p53 may act in an independent pathway to promote selective features of the neoplastic phenotype.

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12. Cloning of wild-type p53 (p53B) cDNA: ~5 μg of fetal brain RNA were mixed with 1.5 μg of oligo(dT) primer and 60 units of avian myeloblastosis virus reverse transcriptase in cDNA buffer (50 mM tris-HCl, pH 8.0, 80 mM KCl, 5 mM $MgCl_2$, 1 mM each deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate, and deoxycytidine triphosphate). The reaction mixture was incubated for 90 min at 42°C. After reaction, RNA was degraded with 0.5 M NaOH, and single-stranded cDNA was precipitated with ethanol. PCR amplification was carried out with one-tenth of the cDNA, 100 ng of each oligonucleotide primer (5′-TGCAAGCTTTCCACGACGGTGACACGCT-3′ and 5′-AGTGCAGGCCAACTTGTTCAGTGGA-3′), and 5 U of Taq polymerase in PCR buffer (50 mM KCl, 10 mM tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, and 0.001% gelatin) for 40 cycles in a programmable heat block (Ericomp, San Diego, CA). Each cycle included denaturation at 93°C for 1 min, annealing at 62°C for 80 s, and primer extention at 72°C for 3 min. PCR products were extracted with phenol and precipitated with ethanol. The precipitate was dissolved in H_2O and digested with restriction enzymes (Hind III and Sma I). The p53 cDNA fragment was subcloned into virus vector to form Vp53B-Neo. Subcloned p53B was sequenced by the dideoxy chain termination method [F. Sanger, S. Nicklen, A. R. Coulsen, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)].
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21. One colorectal carcinoma has been described that contains both mutated ($Asp^{281} \rightarrow Gly$) and wild-type p53 alleles (*14*). Possible explanations for this discrepant case are as follows: (i) an intermediate stage of p53 mutation was coincidentally captured, and p53 had not yet contributed to the neoplastic properties of this tumor; (ii) the "wild-type" p53

allele in this tumor actually carried a functionally important mutation outside of the region sequenced (exons 5 to 9); or (iii) some mutated p53 alleles behave differently than others, or differently depending on cell type.
22. That mutated p53 has a function is also suggested by a study [D. W. Wolf, N. Harris, V. Rotter, *Cell* **38**, 119 (1984)] in which a presumably mutated p53 gene was transfected into Ab-MuLV–transformed murine leukemia cells that lacked endogenous p53 expression. This resulted in cells with more malignant behavior in tumorigenicity assays.
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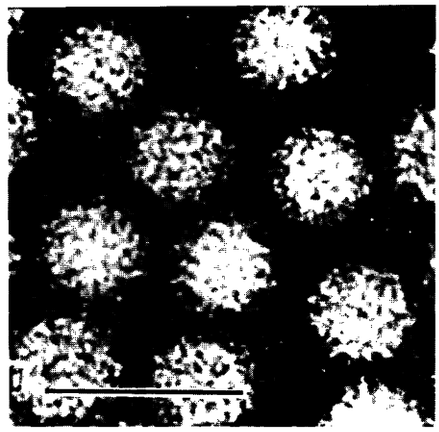

Fig. 1. Electron micrograph of Norwalk virus particles after CsCl gradient purification. Virus was visualized by staining with 1% ammonium molybdate. Scale bar, 50 nm.

Norwalk Virus Genome Cloning and Characterization

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Major epidemic outbreaks of acute gastroenteritis result from infections with Norwalk or Norwalk-like viruses. Virus purified from stool specimens of volunteers experimentally infected with Norwalk virus was used to construct recombinant complementary DNA (cDNA) and derive clones representing most of the viral genome. The specificity of the clones was shown by their hybridization with post- (but not pre-) infection stool samples from volunteers infected with Norwalk virus and with purified Norwalk virus. A correlation was observed between the appearance of hybridization signals in stool samples and clinical symptoms of acute gastroenteritis in volunteers. Hybridization assays between overlapping clones, restriction enzyme analyses, and partial nucleotide sequence information of the clones indicated that Norwalk virus contains a single-stranded RNA genome of positive sense, with a polyadenylated tail at the 3′ end and a size of at least 7.5 kilobases. A consensus amino acid sequence motif typical of viral RNA-dependent RNA polymerases was identified in one of the Norwalk virus clones. The availability of Norwalk-specific cDNA and the new sequence information of the viral genome should permit the development of sensitive diagnostic assays and studies of the molecular biology of the virus.

ACUTE GASTROENTERITIS IS ONE OF the most common illnesses in the United States (*1, 2*), with a large number (up to 42% of outbreaks) of cases estimated to be caused by Norwalk or Norwalk-like viruses (*3*). Both water- and foodborne transmissions of Norwalk virus have been documented, and particularly large epidemic outbreaks of illness have occurred after consumption of contaminated shellfish including clams, oysters, and cockles (*4–10*).

Norwalk virus was discovered in 1973, but knowledge about the virus has remained limited because it has not been possible to propagate the virus in cell culture and suitable animal models have not been found (*2*). Human stool samples obtained from outbreaks and from human volunteer studies have been the only source of virus. Moreover, the concentration of virus in stool is usually so low that virus detection by routine electron microscopy is not possible (*11–13*). Current methods of Norwalk virus detection include immune electron microscopy and other immunologic methods such as radioimmunoassays (RIAs) or biotin-avidin enzyme-linked immunosorbent assays (ELISAs), which use sera from humans in acute and convalescent phases. To date, because of either insufficient quantities or unusual properties of the viral antigen, no hyperimmune serum from animals has been successfully prepared. Preliminary biophysical characterization of virions has indicated that particles contain one polypeptide (*14*), but efforts to characterize the viral genome have failed. Therefore, these viruses have remained unclassified and difficult to study. Molecular cloning was seen as an approach to overcome these problems.

To permit better diagnosis and molecular characterization of Norwalk virus, we constructed a cDNA library derived from nucleic acid extracted from virions purified from stool samples obtained from volunteers. Norwalk virus (8FIIa) was administered orally to adult volunteers (*15, 16*). Stool samples collected after infection were examined for Norwalk virus by either RIAs or direct or immune electron microscopy. Stools from two patients containing the highest amount of Norwalk virus were used as the source of virus for cloning. Norwalk virus was purified by methods used previously for preparation of hepatitis A and rotaviruses from stool, with some modifications (*17, 18*). Basically, stools containing Norwalk virus were treated with Genetron (1,1,2-trichloro-1,2,2-trifluoroethane) to remove lipid and water-insoluble materials. Virus in the aqueous phase was then centrifuged through a 40% sucrose cushion, and the resultant pellets were suspended, sonicated, and loaded in a CsCl gradient for isopycnic centrifugation. Approximately 10^9 physical particles of virus of relatively high purity (Fig. 1) were obtained from 500 g of stool.

Nucleic acids were extracted from these purified viruses by proteinase K treatment of the samples followed by phenol-chloroform extraction and ethanol precipitation (*19*). Because the nature of the viral genome was unknown, the extracted nucleic acids were denatured with methylmercuric hydroxide before cDNA synthesis. Random primed cDNA was synthesized and a small amount of cDNA was obtained (*19*). Direct cloning of this small amount of cDNA was unsuccessful so a step of amplification of the DNA was performed by synthesizing more copies of the DNA with random primers and the Klenow fragment of DNA polymerase I before cloning. This cloning method was developed based on control experiments in which we amplified a known cDNA fragment of hepatitis A virus (*20*). The proce-

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