washed. The bound protein was eluted and subjected to SDS-PAGE as described for immunoprecipitations. The 95-kD protein was excised and equilibrated in 0.1M tris-HCl (pH 6.8), 0.1% SDS, 5 mM DTT, and 10% glycerol. The gel slices were inserted into the lanes of a 16% Tricine SDS-polyacrylamide gel, overlaid with 25 μl of Glu-C protease solution (20 μg/ml), and subjected to electrophoresis. The resolved fragments were electroblotted to PVDF membranes, and two fragments of ~6 to 8 kD were excised from the stained blot and sequenced.

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Association of the APC Tumor Suppressor Protein with Catenins

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Mutations of *APC* appear to initiate sporadic and inherited forms of human colorectal cancer. Although these mutations have been well characterized, little is known about the function of the *APC* gene product. Two cellular proteins that associate with APC were identified by nucleotide sequence analysis and peptide mapping as the E-cadherin–associated proteins α- and β-catenin. A 27-residue fragment of APC containing a 15–amino acid repeat was sufficient for the interaction with the catenins. These results suggest an important link between tumor initiation and cell adhesion.

Mutations in the *APC* tumor suppressor gene lead to benign colorectal tumors in about one-third of the population of the Western world (*1*). If not surgically removed, 15 to 20% of these tumors will progress to cancer. Understanding the mechanism by which *APC* mutations lead to tumorigenesis therefore has substantial practical, as well as theoretical, importance.

The *APC* gene was originally identified through positional cloning approaches and encodes a protein of 2843 amino acids (*2*). Somatic mutations in *APC* appear to initiate colorectal tumor development in the general population, whereas germline mutations result in familial adenomatous polyposis (FAP) (*3–5*), an autosomal dominantly inherited disease that predisposes individuals to colorectal cancer. Mice with germline mutations of *APC* manifest a phenotype similar to that of FAP (*6*). To date, more than 120 different germline and somatic mutations of *APC* have been identified, almost all of which result in COOH-terminal truncation of the gene product (*3–5*).

Although APC is known to form homooligomers (*7*), its predicted amino acid sequence provides few clues to its mechanism of action. To help elucidate the function of APC, we used immunoprecipitation to identify cellular proteins that interact with APC in vivo. We studied two human colorectal cancer cell lines—SW480 cells, which express an APC protein that is truncated at codon 1338 (*3*), and HCT116 cells, which express an apparently full-length APC protein (*7*). A monoclonal antibody (mAb) specific for the NH_2-terminus of APC (CF11) immunoprecipitates APC from both SW480 and HCT116 cells, whereas a mAb specific for the COOH-terminus (DB1) immunoprecipitates APC only from HCT116 cells (*7*). Proteins associated with APC should therefore be immunoprecipitated from HCT116 cells by both CF11 and DB1, but should not be immunoprecipitated from SW480 cells by DB1.

We found that two proteins of 95 kD (p95) and 100 kD (p100) were readily detectable in the immunoprecipitates when SW480 cell lysates were treated with CF11. In contrast, neither protein was detectable in the DB1 immunoprecipitates. When HCT116 cell lysates were used, p95 and p100 were immunoprecipitated by both antibodies (Fig. 1A) (*8*). These results suggested that p95 and p100 associate with APC and that the first 1337 amino acids of APC are sufficient for this interaction. Presumably because of its slow turnover rate, APC itself was not detectable in the immunoprecipitates by fluorography, but could be detected by immunoblotting (*7*).

To map the regions of APC that interact with p95 and p100, we examined the ability of different APC fragments to bind to these proteins in vitro. The APC fragments (Fig. 1C) were expressed in *Escherichia coli* as glutathione-S-transferase (GST) fusion proteins and tested for their ability to bind p95 and p100 from metabolically labeled SW480 cells (*9*, *10*). Only GST-APC(B), (G), and (H) bound to p95 and p100, indicating that the binding domain was

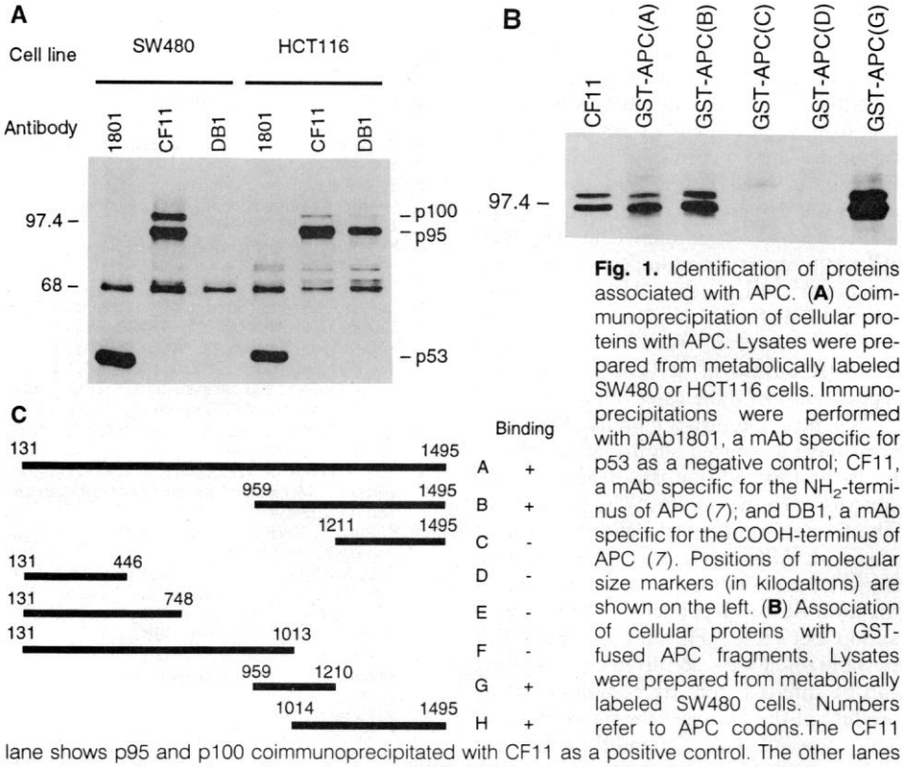

Fig. 1. Identification of proteins associated with APC. (**A**) Coimmunoprecipitation of cellular proteins with APC. Lysates were prepared from metabolically labeled SW480 or HCT116 cells. Immunoprecipitations were performed with pAb1801, a mAb specific for p53 as a negative control; CF11, a mAb specific for the NH_2-terminus of APC (*7*); and DB1, a mAb specific for the COOH-terminus of APC (*7*). Positions of molecular size markers (in kilodaltons) are shown on the left. (**B**) Association of cellular proteins with GST-fused APC fragments. Lysates were prepared from metabolically labeled SW480 cells. Numbers refer to APC codons.The CF11 lane shows p95 and p100 coimmunoprecipitated with CF11 as a positive control. The other lanes show the proteins that bound to the indicated GST-APC fragments. (**C**) Schematic of APC fragments expressed in bacteria as GST fusion proteins and summary of their ability to bind p95 and p100.

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located between amino acids 1014 and 1210 of APC (Fig. 1, B and C).

To obtain cDNA clones for these APC-associated proteins, we screened a cDNA library with radioactively labeled GST-APC fusion proteins (*11*). GST-APC(G) was used as the probe for both primary and secondary screening. The three phage isolates that contained positive clones after the secondary screening were screened again with labeled GST-APC(B) and GST-APC(C) as positive and negative probes, respectively. Positive clones from each of these three phage isolates were plaque-purified and converted to plasmids.

Nucleotide sequence analysis suggested that all three clones represented the same gene and contained the 3′ end, but probably not the 5′ end, of the coding region. Additional cDNA clones covering the entire coding region were then isolated and sequenced (*12*). These cDNAs fell into three classes, probably reflecting alternative splicing within the 3′ untranslated region. This speculation was supported by Northern blot analysis of RNA isolated from SW480 and HCT116 cells, which revealed two transcripts of 3.9 and 4.3 kb (*13*). The cDNA sequences were highly similar to the sequences of mouse and *Xenopus* β-catenin cDNAs and were identical to an unpublished human β-catenin sequence (*14, 15*).

The catenins are a family of cytoplasmic proteins that were originally identified by their association with the cell adhesion molecule E-cadherin (*16*). To confirm that β-catenin was capable of binding to APC, we expressed one of the cDNA clones as a GST fusion protein (GST-CTN) and tested its ability to interact with APC (*17*). GST-CTN bound to APC from SW480 and HCT116 cells, whereas GST alone, GST-APC(G), and a GST fusion protein containing amino acids 536 to 781 of β-catenin (GST-ΔCTN) did not bind APC (Fig. 2A). On the basis of the reported mobilities of the catenins, we hypothesized that APC-associated p95 was β-catenin and that p100 was α-catenin. To test this, we compared the mobilities and one-dimensional peptide maps of these proteins. The p95 and p100 proteins were isolated by immunoprecipitation with antibody CF11 or by binding to GST-APC(G), and the catenins were isolated by immunoprecipitation with HECD-1, a mAb specific for E-cadherin (*18*). Analysis by SDS–polyacrylamide gel electrophoresis (PAGE) showed that p100 comigrated with α-catenin and that p95 comigrated with β-catenin (Fig. 2B). This experiment also showed that E-cadherin was expressed in HCT116, but not SW480, cells (Fig. 2B). The co-identities of p100 and α-catenin and of p95 and β-catenin were confirmed by partial proteolytic analyses with V8 protease (Fig. 2, C and D).

To delineate the catenin-binding domain, we expressed the entire APC coding region in vitro as five overlapping fragments and tested their ability to bind GST-CTN (*5, 19*). Only two fragments, containing part of the overlapping region between GST-APC(H) and GST-APC(G), bound GST-CTN (F2 and F3, Fig. 3A). Accordingly, two additional APC fragments were tested and were also found to be associated with GST-CTN (F2a and F2b, Fig. 3A) (*20*). The minimal overlapping region among the catenin-binding APC fragments was 11 amino acids, suggesting either that these 11 amino acids comprise the catenin-binding domain or that there is more than one catenin-binding domain between amino acids 1014 and 1210 of APC. This region contains three imperfect repeats of 15 amino acids each (Fig. 3B). To test whether these repeats mediated APC interaction with catenins, we expressed APC fragments containing the first repeat alone (R1), the second and the third repeats together (R2), or all three repeats (R3) as GST fusion proteins (Fig. 3A) (*21*). Each of these fragments bound catenins, demonstrating that even a single repeat was sufficient for binding (Fig. 3C).

The association of APC with catenins is consistent with the previous observation that APC is localized in the detergent-insoluble cytoskeleton (*22*). Although both APC and cadherins associate with catenins, these interactions are not identical. The 15–amino acid catenin-binding domain of APC is not present in cadherin. Cadherins appear to associate with similar amounts of α- and β-catenins, as judged by the relative band intensities on fluorographs (for example, see *17, 18*, and Fig. 2B), whereas APC associates preferentially with β-catenin (Fig. 1, A and B). This difference might explain why we isolated cDNAs for β-catenin but not for α-catenin. Alternatively, it is possible that APC does not interact directly with α-catenin but indirectly through binding to β-catenin. The fact that both E-cadherin and APC bind to catenins suggests that cadherins and APC might exist in one complex. However, we have never detected any cadherin coimmunopre-

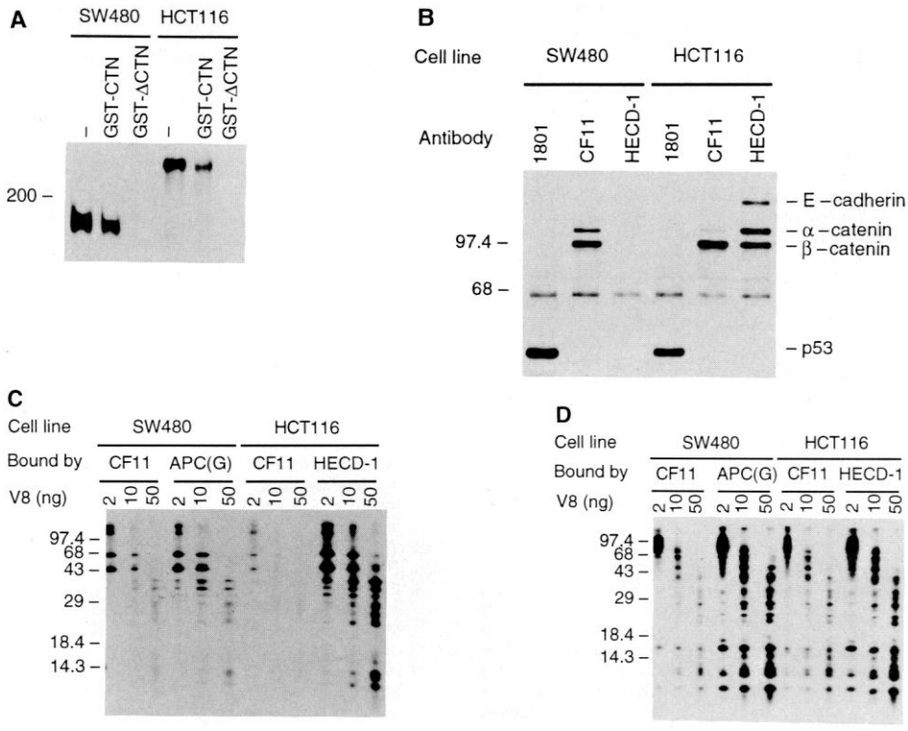

Fig. 2. Identification of APC-associated p100 and p95 as α- and β-catenin, respectively. (**A**) Association of APC with GST-fused β-catenin. Lysates prepared from unlabeled SW480 or HCT116 cells were incubated with GST-CTN (containing β-catenin residues 75 to 781) or GST-ΔCTN (containing β-catenin residues 536 to 781). Bound APC was detected by immunoblotting as in (*22*). Unlabeled lanes contain total cell lysates. (**B**) Comigration of APC-associated p100 and p95 with α- and β-catenin. Lysates were prepared from metabolically labeled SW480 or HCT116 cells and immunoprecipitations were carried out with pAb1801, CF11, or HECD-1, a mAb specific for E-cadherin. Comparative peptide mapping of p100 and α-catenin (**C**) and of p95 and β-catenin (**D**). Proteins were isolated from metabolically labeled SW480 or HCT116 cells by immunoprecipitation with antibody CF11, by binding to GST-APC(G), or by immunoprecipitation with HECD-1. Partial proteolysis experiments were performed with V8 protease (*29*).

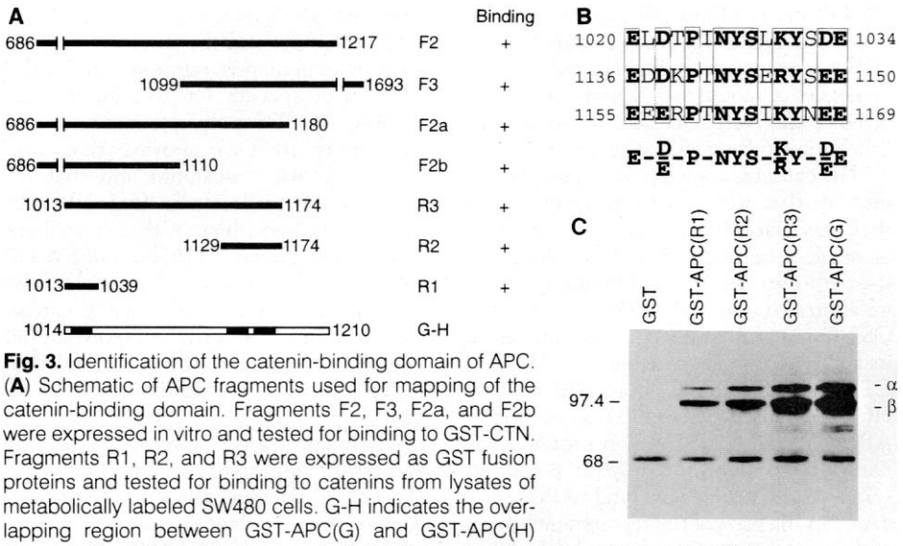

Fig. 3. Identification of the catenin-binding domain of APC. (**A**) Schematic of APC fragments used for mapping of the catenin-binding domain. Fragments F2, F3, F2a, and F2b were expressed in vitro and tested for binding to GST-CTN. Fragments R1, R2, and R3 were expressed as GST fusion proteins and tested for binding to catenins from lysates of metabolically labeled SW480 cells. G-H indicates the overlapping region between GST-APC(G) and GST-APC(H) shown in Fig. 1C. The black boxes in G-H indicate the 15–amino acid repeats. (**B**) Sequence of the three 15–amino acid repeats in APC, with the consensus sequence shown below. Numbers refer to APC codons. Abbreviations for the amino acids are: D, Asp; E, Glu; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; R, Arg; S, Ser; T, Thr; and Y, Tyr. (**C**) Association of APC fragments containing the repeats with the α- and β-catenins.

cipitated with APC or bound to APC fusion proteins (for example, see Fig. 2B). Nor could we detect APC by immunoblotting after immunoprecipitation with an antibody to E-cadherin (*13*).

Cadherins are cell surface molecules that mediate calcium-dependent intercellular interactions and are important for morphogenesis (*23*). Binding to catenins is essential for cadherin function (*24*), and it has been suggested that catenins anchor cadherins to the cytoskeleton (*25*). One model consistent with prior data is that APC modulates the interaction between cadherins and catenins, thereby affecting the pathway through which intercellular interactions control cell growth and differentiation.

A number of other observations have implicated cadherin-catenin complexes in neoplasia. Protein–tyrosine kinase activities, including those associated with Src and the epidermal growth factor receptor, have been localized to cellular junctions, where they can inhibit assembly of cadherin-catenin complexes (*26*). The predicted product of *NF2*, a tumor suppressor gene responsible for neurofibromatosis type 2, resembles ezrin and moesin, two proteins thought to connect membrane proteins to the cytoskeleton (*27*). Finally, loss of cadherin functions through diminished expression of E-cadherin or α-catenin has been associated with increased invasion and metastasis in several human and experimental tumor types (*28*). These observations have suggested that the cadherin-catenin complex is primarily involved in the late stages of neoplasia. The results presented here, however, suggest that interactions involving these proteins may also be critical for the initiation of neoplasia. In combination, the studies indicate that catenin-mediated signals are critical for the control of epithelial cell growth.

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8. Cells in a 25-cm^2 flask were metabolically labeled with 300 μCi [^{35}S]-methionine/cysteine (ICN) in 1.5 ml of Dulbecco's modified Eagle's medium without methionine at 37°C for 4 to 5 hours. Cells were then lysed in MEBC [50 mM tris-HCl (pH 7.5), 100 mM NaCl, 0.5% Nonidet P-40] containing protease inhibitors [0.2% (w/v) 4-(2-aminoethyl)benzenesulfonylfluoride (Calbiochem), and 0.01 mg/ml each of chymostatin, leupeptin, antipain, and pepstatin A (all from Sigma)] and phosphatase inhibitors (0.1 mM Na_3VO_4, 50 mM NaF). Cell lysates were precleared with normal mouse immunoglobulin G and immunoprecipitations were carried out as in (*7*).
9. Expression plasmids were constructed from pGSTag [D. Ron and H. Dressler, *BioTechniques* **13**, 866 (1992)] and a Sca I restriction fragment of APC [nts 391 to 4486 (*2*)]. Fusion proteins were expressed in *E. coli* strain DH10B and purified as in [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)] except that bacteria were sonicated in MEBC containing protease inhibitors. Fusion proteins were purified by binding to glutathione-agarose (Sigma) and washed with MEBC.
10. Lysates were prepared from metabolically labeled SW480 cells, preabsorbed with glutathione-agarose, and then incubated with GST fusion proteins attached to glutathione-agarose for 90 min with rocking at 4°C. The complexes were washed with MEBC, and the proteins detected by SDS-PAGE and fluorography.
11. GST fusion proteins were labeled as in M. A. Blanar and W. J. Rutter [*Science* **256**, 1014 (1992)] with modifications. Fusion proteins bound on glutathione-agarose were washed three times with HMK buffer [20 mM tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 100 mM NaCl, 12 mM $MgCl_2$], then resuspended in 50 μl of HMK buffer containing 1 U/μl of the catalytic subunit of protein kinase A (Sigma). To this 1 μl of [γ-^{32}P]ATP (150 μCi/μl) was added. After a 30-min incubation at 37°C, the reaction was stopped and the complex washed with MDF (MEBC containing 1 mM DTT and 5 mM NaF). The labeled fusion proteins were eluted with MEBC containing 10 mM reduced glutathione and were used to screen a human fetal brain cDNA library that was constructed in lambda ZAPII (Stratagene) and processed as in J. Sambrook, E. F. Fritsch, and T. Maniatis [*Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989)]. The nitrocellulose filters were washed twice with 0.1 M tris-HCl (pH 7.5), 0.9% NaCl, and 0.1% Tween 20 and then blocked for 1 hour at room temperature with cold 10% nonfat dry milk in MDF containing purified GST. After an overnight incubation with labeled fusion protein (100,000 cpm/ml) in MDF containing 5% nonfat dry milk, the filters were washed three times with cold MDF at room temperature for a total of 30 min, air dried, and exposed to film.
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19. Expression of APC fragments in vitro was carried out as in (*5*). The reaction was stopped by adding MEBC containing protease inhibitors (*8*). The mixture was preabsorbed with glutathione-agarose and then incubated with GST-CTN attached to glutathione-agarose. Proteins were separated by SDS-PAGE and APC fragments were detected by fluorography. F2 and F3 are identical to fragments 2 and 3, described in (*5*).
20. The APC fragments were expressed in vitro with templates generated by the polymerase chain reaction (PCR) (*5*). The 5′ PCR primers for F2a and F2b are the same as those for fragment 2 (*5*). The 3′ primers for F2a and F2b are 5′-AACTGAATTCAATAGGCTGATCC-3′ and 5′-CTGAACCATTGGCTCCCCG-3′, respectively.
21. Templates for these APC fragments were generated by PCR with primer pairs 5′-TGGAATTCATATGGATGATAATG-3′ and 5′-TTAAGCTTCCAGAGTTCAACTGC-3′ for R1; 5′-CAGAATTCCTTTGTGTCAAGAAGATGAC-3′ and 5′-ATA-

AGCTTATCCACATGACGTTTC-3′ for R2; and 5′-TGGAATTCATATGGATGATAATG-3′ and 5′-ATAAGCTTATCCACATGACGTTTC-3′ for R3. The PCR products were digested with Eco RI and Hind III, and then inserted in pGSTag.
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Abnormal Chromosome Behavior in *Neurospora* Mutants Defective in DNA Methylation

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The function and regulation of DNA methylation in eukaryotes remain unclear. Genes affecting methylation were identified in the fungus *Neurospora crassa*. A mutation in one gene, *dim-2*, resulted in the loss of all detectable DNA methylation. Abnormal segregation of the methylation defects in crosses led to the discovery that the methylation mutants frequently generate strains with extra chromosomes or chromosomal parts. Starvation for *S*-adenosylmethionine, the presumed methyl group donor for DNA methylation, also produced aneuploidy. These results suggest that DNA methylation plays a role in the normal control of chromosome behavior.

In the DNA of many organisms including mammals, plants, and various fungi, some cytosines are modified by methylation at position 5. DNA methylation in eukaryotes has been correlated with reduced gene activity, and evidence has accumulated that methylation can inhibit gene expression (*1*). In mice, an approximately 70% reduction in DNA methylation, resulting from a mutation in the DNA methyltransferase gene, leads to death during embryogenesis (*2*). Although DNA methylation is required in mammals, it is not yet clear whether its basic function has been identified. The consequences of undermethylation in eukaryotes may be more apparent in organisms that contain methylation but do not require it.

In *Neurospora crassa*, about 1.5% of the cytosines are methylated (*3*), but the bulk of the genome appears devoid of methylation. Only three methylated regions have been characterized in any detail: the tandemly arranged ribosomal RNA genes [ribosomal DNA (rDNA)] on linkage group V (*4*), the zeta-eta (ζ-η) region on linkage group I (*5*–*7*), and the psi-63 (ψ_{63}) region on linkage group IV (*8*, *9*). The latter two regions are relics of RIP (repeat-induced point mutation), a process associated with the sexual phase of the life cycle of *Neurospora*, in which duplicated sequences are riddled with G:C to A:T mutations (*10*). Treatment of *Neurospora* with 5-azacytidine causes substantial reductions in methylation without drastically reducing growth, which suggests that DNA methylation is not essential in this organism (*5*). This observation and the relative paucity of methylation in *N. crassa* prompted us to hunt for *Neurospora* mutants defective in DNA methylation.

To make the mutant hunt as broad as possible, we screened for methylation defects directly by Southern (DNA) hybridization. Reduced methylation in the ψ_{63} region may be recognized by increased digestion of a Bam HI site that is normally blocked by methylation (Fig. 1, A and B) (*11*). A standard laboratory strain, N242, was mutagenized under conditions that induced mutations in a control gene, *mtr*, at a frequency of 1 to 3 out of 1000 survivors (*12*). Approximately 1250 survivors of the mutagenesis were screened, and 25 strains were identified in which methylation at the ψ_{63} Bam HI site had been reduced or eliminated. Thirteen of the putative mutants consistently showed the defects and could be propagated through crosses. Two strains are illustrated (Fig. 1B). Complementation tests performed on pairs of mutants grown as forced heterokaryons revealed that our collection includes mutations in at least three genes important for DNA methylation. An example of complementation between two mutants, *dim-2* and *dim-3* (defective in DNA methylation), is shown in Fig. 1C. The *dim-1* mutant was isolated in a separate mutant screen (*13*). None of the three mutations could always be completely complemented (Fig. 1C) even in control heterokaryons between mutant and wild-type strains, which suggests that the defects are partially dominant (*14*).

No eukaryotic mutants have been described in which DNA methylation is completely abolished. The mouse mutation generated by disruption of the DNA methyltransferase gene (*2*) causes overall reduction, but not elimination, of methylation, whereas a mutation in cultured CHO cells appears to affect only a few sites (*15*). Mutants isolated in *Arabidopsis* show reduced methylation at many, but not all, chromosomal regions (*16*). To further characterize our mutants, we assayed their effects on a second normally methylated region, ζ-η. All of the mutants showed a methylation deficiency in this region. The *dim-3* mutant was typical in showing reduced methylation at both of the diagnostic Bam HI sites in the ζ-η region (Fig. 1, A and D). Another mutant, *dim-2*, appeared devoid of methylation at ψ_{63} and ζ-η (Fig. 1, A, B, and D) and was chosen for further analysis. It showed no methylation at the rDNA region (Fig. 1E) nor at six other chromosomal regions that are methylated in wild-type strains (*17*). The absence of methylation detectable by Southern analysis in a total of nine normally methylated regions suggested that *dim-2* abolishes methylation throughout the genome and distinguishes *dim-2* from other known mutations affecting methylation in eukaryotes.

To test whether the *dim-2* mutation abolishes methylation throughout the genome, we measured the total 5-methylcytosine (5mC) content in DNA from *dim-2* and wild-type strains by high-performance liquid chromatography (HPLC) (Fig. 2). Approximately 1.5% of the cytosines were methylated in the wild-type strain, which is consistent with previous determinations (*3*). No 5mC was detected in DNA from the *dim-2* strain (Fig. 2). Analysis of deoxynucleoside samples from *dim-2* DNA mixed with decreasing amounts of pure 5-methyldeoxycytidine indicated that 5mC amounts corresponding to ≥5% of those found in wild-type *Neurospora* would have been detected. The apparent lack of DNA methylation in *dim-2* strains suggests that DNA methylation is not essential for

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