Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 1929–1933, March 1992
Medical Sciences

Distinct hypermethylation patterns occur at altered chromosome loci in human lung and colon cancer

(CpG islands/allelic losses/genetic instability)

MICHELE MAKOS*, BARRY D. NELKIN*, MICHAEL I. LERMAN†, FARIDA LATIF†, BERTON ZBAR†, AND STEPHEN B. BAYLIN*‡

*The Oncology Center and ‡Department of Medicine, The Johns Hopkins Medical Institutions, Baltimore, MD 21231; and †The National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD 21701

Communicated by John W. Littlefield, November 25, 1991

ABSTRACT **Regional increases in DNA methylation occur in normally unmethylated cytosine-rich areas in neoplastic cells. These changes could potentially alter chromatin structure to inactivate gene transcription or generate DNA instability. We now show that, in human lung and colon cancer DNA, hypermethylation of such a region consistently occurs on chromosome 17p in an area that is frequently reduced to homozygosity in both tumor types. Over the progression stages of colon neoplasia, this methylation change increases in extent and precedes the allelic losses on 17p that are characteristic of colon carcinomas. We also show on chromosome 3p that regional hypermethylation may nonrandomly accompany chromosome changes in human neoplasia. Increased methylation is consistent in small-cell lung carcinoma DNA at two 3p loci that are constantly reduced to homozygosity in this tumor, but it is not seen in colon cancer DNA, in which these loci are infrequently structurally altered.**

Alterations in DNA cytosine methylation are one of the most consistent, but poorly understood, molecular changes in human cancers (reviewed in refs. 1 and 2). These abnormalities appear early in progression of tumors and reflect an imbalance in the DNA methylation process that includes widespread hypomethylation (3), regional areas of hypermethylation (4), and an increased cellular capacity to methylate DNA (5, 6). The regional hypermethylation may be particularly important. Our laboratory (4) and others (7, 8) have found this change in clusters of CpG dinucleotides near regulatory areas of genes. A normally unmethylated status of these "CpG islands" appears to be essential for active gene transcription (reviewed in ref. 9). Conversely, cytosine methylation within CpG islands can either stabilize or cause alterations in chromatin structure that render chromosome regions transcriptionally inactive (for review, ref. 10) or subject to DNA instability (11).

We now explore whether chromosomal regions that are distinctively altered in human cancers are nonrandomly associated with hypermethylation and how the timing of this abnormality relates to changes in chromosome structure during tumor progression. A CpG-island-rich area on chromosome 17p, consistently reduced to homozygosity in both lung and colon cancers, is extensively hypermethylated in both tumor types. During progression stages for colon neoplasia, this abnormal methylation occurs early, increases in extent, and precedes allelic losses of the involved region. We also find that regions of chromosome 3p that are consistently reduced to homozygosity in lung, but not colon, cancers are hypermethylated only in lung cancer. We suggest that DNA hypermethylation nonrandomly marks chromosome regions altered during the development of specific tumors.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cells and Tissues Studied. All established cell lines of lung and colon carcinomas (4, 12–14) were grown in our laboratory as previously described (4). All fresh tumors and adjacent normal tissues have also been previously studied and were processed as reported (4, 15, 16).

DNA Probes and Hybridization Conditions. All DNA probes have been mapped to chromosome regions and have a known frequency for detecting reductions to allelic homozygosity in the examined tumor types (17–23). Each probe was isolated as an insert from the growth vector and labeled by the random priming procedure (24). Southern hybridizations were performed exactly as in our previous studies (4), using 5–7.5 μg of DNA digested with *Msp* I, *Hpa* II, and *Not* I at high concentrations (15 units/μg or higher) to ensure complete digestion of both normal and tumor DNA.

Pulsed-Field Gel Electrophoresis (PGFE). DNA was prepared in agarose plugs as described (25) and incubated with high amounts of *Not* I (200 units per sample) to ensure complete digestion. The plugs were covered with mineral oil, incubated overnight at 37°C, and then loaded onto 1.0% agarose gels in 0.045 M Tris–borate/0.001 M EDTA, pH 8.0. DNA was separated by pulse times of 70 s for 20 hr and 100 s for 23 hr at 4°C. Separated DNA was transferred to nylon filters and Southern hybridizations were performed exactly as previously reported (4).

RESULTS

Methylation Status of Chromosome 17p CpG Islands in Colon and Lung Cancers. We first examined the CpG island methylation status at the locus 17p13.3, which is consistently reduced to homozygosity in colon cancers (15, 16), lung cancers (26), and breast cancers (27). We employed pYNZ22.1, a probe that frequently detects allelic losses of chromosome 17p in tumors (15, 16, 26, 27). In previous studies with DNA from immortalized lymphocytes of normal individuals (28), probe pYNZ22.1 detected small fragments in digests with *Not* I [4.7 kilobases (kb)] and *Bss*HII (2.4 kb) (Fig. 1*A*). These sizes contrast with the large fragments (>100 kb) usually generated by these restriction enzymes, which recognize 8- and 6-base-pair (bp) sequences, respectively, containing CpG dinucleotides. The enzymes are inhibited by CpG methylation, and clusters of restriction endonuclease sites help define the presence of CpG islands (9, 29). Therefore, the small pYNZ22-complementary fragments

Abbreviations: SCLC, small-cell lung carcinoma; PFGE, pulsed-field gel electrophoresis.

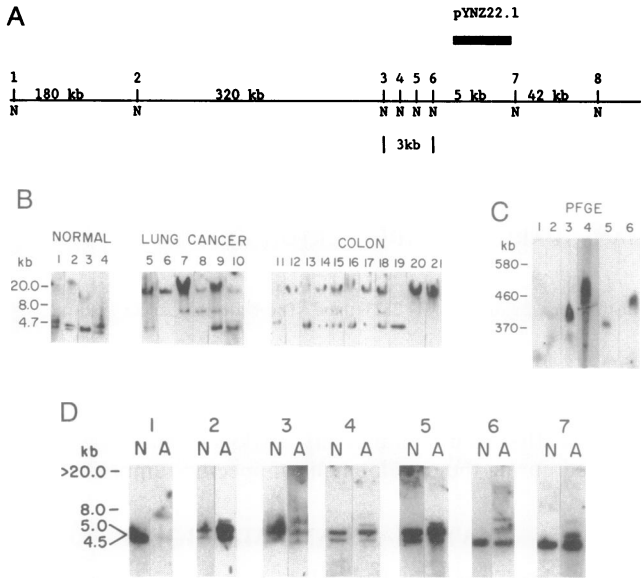

FIG. 1. Methylation patterns for locus 17p13.3 in DNA from normal tissues, lung and colon carcinomas, and colon adenomas. (*A*) *Not* I sites (numbered 1–8) around the recognition site for the pYNZ22.1 probe as discussed in the text. Short bar above the line denotes position of the pYNZ22.1 probe. (*B*) Conventional gels of *Not* I digests (15 units/μg) of DNA samples, probed with pYNZ22.1, including normal lung (lanes 1 and 2), thyroid (lane 3), liver (lane 4), and colonic mucosa (lanes 11, 13, and 16); fresh human small-cell lung carcinoma (SCLC) (lane 5) from the patient whose germ-line DNA is shown in lane 4; established SCLC culture lines (4, 12) NCI-H209, OH-3, NCI-H64, NCI-H60, and OH-1 (lanes 6–10, respectively); fresh colon carcinoma (lanes 12, 14, 15, 17, and 18) from the same patients as in lanes 11, 13, and 16; and three established culture lines (14) of colon carcinoma (lanes 19–21). Lanes 15 and 18 represent longer exposures of samples in lanes 14 and 17, to compensate for the single 17p allelic status of these cancers (see text). (*C*) PFGE of selected DNA samples and hybridization to probe pYNZ22.1. Yeast chromosome marker positions are indicated on the left-hand side. DNA samples are as follows: lane 1, fresh lymphocytes; lane 2, cultured normal fetal fibroblasts (line WI38 from the American Type Culture Collection); lane 3, fresh SCLC; lane 4, NCI-H209 line of SCLC (the same sample as in lane 6 of *B*); lane 5, established line of non-SCLC lung cancer [NCI-H157, large-cell carcinoma (12)]; and lane 6, a line of colon carcinoma. (*D*) Conventional gels of *Not* I digests, probed with pYNZ22.1, of DNA from normal colonic mucosa and colon adenomas from seven patients. Each lane contained approximately 5 μg of DNA. N, normal mucosa; A, adenoma.

produced by *Not* I and *Bss*HII suggest the presence of one or more CpG islands around this region.

We examined the methylation status of five known *Not* I sites clustered in a 8-kb region around pYNZ22 (Fig. 1*A*). In DNA from normal lung, thyroid, liver, and colonic mucosa (Fig. 1*B*, lanes 1–4, 11, 13, and 16) we found these sites to be, as expected, unmethylated. The polymorphic *Not* I fragments between 4 and 5 kb indicate cutting of Not I sites 6 and 7 (Fig. 1*A*). The polymorphisms reflect length differences due to a variable number of tandom repeats within the pYNZ22 region (30). In contrast, 10 of 12 (83%) DNA samples from fresh and cultured cell lines of lung and colon cancers showed abnormal methylation of the *Not* I sites (Fig. 1*B*). This occurred in five of six SCLCs examined, including a non-cultured tumor (lane 5) from a patient whose germ-line DNA does not show this change (lane 4). The abnormal methylation results in partial (lanes 5, 7–10) or complete (lane 6) inability of high amounts of *Not* I enzyme (15 units/μg of DNA) to generate the normal polymorphic 4.0- to 5.0-kb bands (Fig. 1*B*, lanes 5–8). The normal bands are replaced by either a >20-kb band that does not resolve on conventional gels (Fig. 1*B*, lanes 5–9) or a new 8.0-kb band (Fig. 1*B*, lanes 7–9). In one tumor, only some cells show the change, since the normal 5.0-kb bands are present in amounts equal to those of the >20-kb and 8.0-kb bands (Fig. 1*B*, lane 9). The abnormal 8.0-kb band represents methylation of *Not* I sites 4–6 (Fig. 1*A*) and the >20-kb band results from methylation of sites 3–7. The SCLC sample in lane 10 of Fig. 1*B*, gave a normal pattern.

Identical results were obtained in DNA of colon cancer (Fig. 1*B*, lanes 11–21). One of the three fresh colon tumors (lane 12) had virtual loss of the normal 5.0-kb band and presence of only the >20-kb band (Fig. 1*B*; compare lanes 11 and 12). The other two fresh tumors had a decrease of the normal 5.0-kb band and presence of both the abnormal >20-kb and 8.0-kb bands (compare lanes 13–18). Lanes 15 and 18 of Fig. 1*B* are longer exposures for tumor DNA that compensate for the reduced allelic status of these cancers. In two of three established colon cancer culture lines (Fig. 1*B*, lanes 20 and 21) there was complete loss of the normal 5.0-kb band and presence of only the >20-kb band. The third culture line of colon carcinoma gave a normal unmethylated pattern (Fig. 1*B*, lane 19).

We next examined, by PFGE, the distance that the abnormal methylation extended around the pYNZ22 region (Fig. 1*C*). On these gels, the small fragments from normal cultured fibroblasts or fresh lymphocytes were not detected (Fig. 1*C*, lanes 1 and 2). However, in the DNA from lung and colon cancer cultures (Fig. 1*C*, lanes 4–6), and fresh SCLC (lane 3), large fragments are seen that represent resolution of the >20-kb band seen on conventional gels. These indicate that, in tumor DNA, the distance between unmethylated CpG islands around pYNZ22 has been extended from ≈5.0 kb to 300–500 kb, representing methylation of *Not* I sites 1 and/or 2 (Fig. 1*A*) in addition to those clustered directly within the pYNZ22 region (Fig. 1*A*, sites 3–7).

Timing of pYNZ22 Methylation Changes During Colon Tumor Progression. We next explored, in progression stages of colon cancer, the relationships between the pYNZ22 methylation status, allelic losses of 17p, and point mutations in the p53 gene at 17p13.1. These last 2 parameters are the consistent molecular changes associated with chromosome 17p in colon carcinoma DNA (15, 16). We compared the above *Not* I restriction patterns for the pYNZ22 region in DNA from normal colonic mucosa and colon cancers with those for the premalignant adenomas, which are thought to be precursors to colon cancers (for review, see ref. 16).

Table 1. pYNZ22 methylation status and 17p structural changes in colonic neoplasia

Tumor	Type	pYNZ22 methylation	No. 17p alleles	p53 mutation
44	A	Hyper; 8.0	1	Yes
110	A	Hyper; 6.0	2	No
122	A	Hyper; 6.0	2	ND
127	A	Hyper; 6.0	2	No
137	A	Hyper; 6.0	2	No
139	A	Hyper; 6.0–8.0	2	ND
157	A	Hyper; 6.0	2	ND
S15	C	Hyper; >20.0	1	Yes
S93	C	Hyper; >20.0, 8.0	1	Yes
S98	C	Hyper; >20.0, 8.0	1	Yes

A, adenoma; C, carcinoma; Hyper, hypermethylation; ND, not done. Numbers in methylation status column show largest predominant abnormal *Not* I fragments detected; normal fragment size = 4.5–5.0 kb. In Fig. 1*D*, the DNA samples are tumor 44 in lane 1, 110 in lane 2, 122 in lane 3, 127 in lane 4, 137 in lane 5, 139 in lane 6, and 157 in lane 7. In Fig. 1*B*, S15 is in lane 12, 593 in lanes 14 and 15, and S98 in lanes 17 and 18.

In normal colonic mucosa of seven individuals with colon adenomas (Fig. 1*D* and Table 1), probe pYNZ22 detected the normal polymorphic *Not* I fragments between 4.5 and 5.0 kb. By contrast, in seven of seven premalignant colon adenomas from these same individuals (Fig. 1*D*, Table 1), abnormal methylation of the pYNZ22 *Not* I sites was detected. However, the patterns differed between DNA from the adenomas and carcinomas. First, the normal 5.0-kb band is more intense in the adenoma DNA (Fig. 1*D*, each A lane) than in the colon cancer DNA (Fig. 1*B*, lanes 12, 14, 15, 17, and 18). Second, the abnormal bands in adenoma DNA ranged from 5.5 to 8.0 kb (Fig. 1*D*, Table 1) and did not include the >20-kb bands present in the colon cancer DNA (Fig. 1*B*). The bands between 5 and 8 kb represent variable methylation of sites 4–6 (Fig. 1*A*). Thus, while abnormal methylation of the pYNZ22 region exists in the adenomas, the numbers of cells involved and the extent of the abnormal methylation increase in the colon cancers.

We correlated the above methylation data to the timing of 17p allelic losses and point mutations within the p53 gene, which have been previously assessed in most of the samples we studied (15, 16). These structural changes were present in only one of seven adenomas, but each of the three fresh colon carcinomas had both lost one pYNZ22 allele and had mutations in the p53 gene (Table 1). Therefore, abnormal methylation around pYNZ22 precedes the structural changes on 17p that characterize the progression of colon adenomas to carcinomas (16). The abnormal methylation of *Not* I sites is more extensive on the remaining 17p allele in the carcinomas than on the two alleles in the adenomas.

Methylation Patterns of Chromosome 3 in Human Tumors. We next considered whether hypermethylation maps to chromosome loci that are consistently altered in lung cancer and not in colon cancer. We constructed a methylation status map of eight loci along chromosome 3p in DNA from colon cancer and the four major types of lung cancer (SCLC and large cell carcinoma, adenocarcinoma, and squamous cell carcinoma, the latter three referred to as non-SCLC). This entire region exhibits consistent reductions to homozygosity in each of the lung tumor types (17–22), but 3p allelic losses are particularly frequent in SCLC, especially at specific telomeric and centromeric regions (20). Moreover, despite the wide number of chromosome losses that occur in colon cancer, chromosome 3p is not frequently involved (15). We would then predict that the pattern and frequency of hypermethylation at 3p loci might differ between SCLC and other forms of lung tumors and colon cancer.

To study chromosome 3p, we utilized a series of mapped probes that have a known frequency for reductions to homozygosity in human tumors (refs. 17–22 and references therein). For these probes, relationships to CpG islands have not been determined. Therefore, we used the methylation-sensitive enzyme *Hpa* II to examine the methylation status of a short widely distributed sequence, CCGG. *Hpa* II will not cut this site if the internal cytosine is methylated, but its isoschizmer, *Msp* I, cuts regardless of methylation status (31). The *Msp* I cuts recognized by each probe used were identical in normal and tumor DNA. For each probe we also found that the *Hpa* II restriction patterns for normal lung (Fig. 2) and colonic mucosa (data not shown) were similar. None of the probes exclusively detect CpG islands, since some CCGG sites were methylated at each locus examined in normal tissue DNA (Fig. 2*A*, compare lanes 6 and 7 with lanes 4 and 5; Fig. 2*B*, compare lanes 4 and 5 with lane 3; and Fig. 2*C*, compare lanes 4 and 5 with lane 3).

Consistent with the known widespread DNA hypomethylation in cancer cells (1–3), we found a decrease in methylation at six of the eight 3p loci in each of the tumor types examined as compared with DNA from normal tissues (summary in the hatched bars in Fig. 3; an example for a

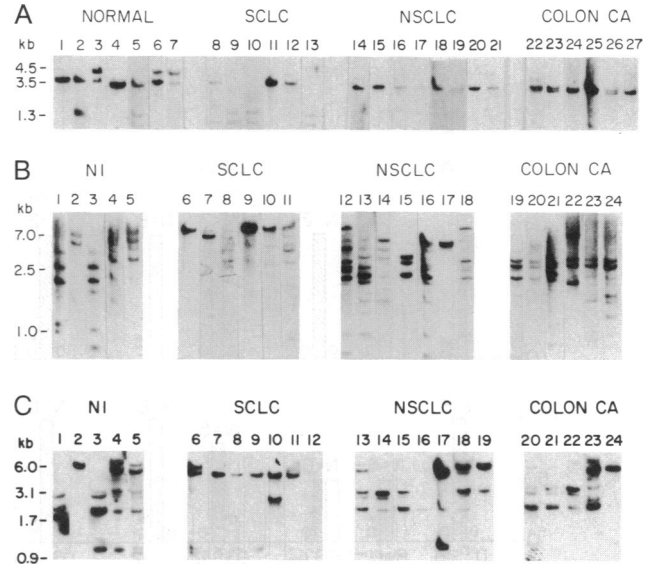

FIG. 2. Methylation status of 3p loci in DNA from normal human lung and established lines of human lung and colon cancers. (*A*) Hybridizations with probe D3S2 located at 3p21. In normal lung DNA, *Msp* I produces a polymorphism with 3.5-kb (lane 1, DNA from a homozygous individual) or 1.3-kb (smaller fragment in lane 2, DNA from a heterozygous individual) fragments. *Msp* I plus *Hin*dIII (lane 3, *Hin*dIII alone) digestion generates either a 3.3-kb fragment (lane 4) or two additional smaller fragments from the smaller *Msp* I allele (lane 5). Normal lung DNA is methylated, since *Hpa* II does not cut the *Hin*dIII fragments (lanes 6 and 7). DNA (cut with *Hin*dIII plus *Hpa* II) from SCLC lines (4, 12) OH-3, NCI-H69, NCI-H64, OH-1, NCI-H60, and NCI-H31 (lanes 8–13, respectively); from non-SCLC (NSCLC) lung cancer lines (13) U1752, NCI-H23, NCI-1355, NCI-H292, NCI-H520, NCI-H322, and NCI-H596 (lanes 14–21, respectively); and from established lines (14) of human colon carcinoma (lanes 22–27) is hypomethylated relative to normal lung DNA, since the 4.5-kb band (lanes 6 and 7) is absent from tumor samples, and, in lanes 8, 9, 10, 13, and 25, *Hpa* II generates fragments from the smaller *Msp* I allele.

(*B*) Hybridization of the above DNA samples to probe D3S18 at 3p25-26 (see Fig. 3). DNA from lungs of normal individuals (NI) was digested with *Msp* I alone (lane 1), *Hin*dIII (lane 2), and *Hin*dIII plus *Msp* I (lane 3). Several *Msp* I sites in lung DNA from two normal individuals are methylated (note higher molecular weight bands, 2.5 to 7.0 kb, in *Hin*dIII plus *Hpa* II digests, lanes 4 and 5). Four of six SCLC DNA samples (lanes 6, 7, 9, and 10), digested with *Hin*dIII plus *Hpa* II, are hypermethylated (note loss of all bands below ≈7.0 kb, as compared to lanes 4 and 5). Only two SCLC samples (lanes 8 and 11) had normal patterns. In contrast, five of seven non-SCLC DNA samples showed hypomethylation (lanes 12, 13, 15, 16, and 18—note accentuation of bands in 2.5-kb region and multiple bands between 1.0 and 2.5 kb). One non-SCLC lung tumor (lane 14, NCI-H292) had a relatively normal pattern and one (lane 17, NCI-H322) showed hypermethylation. All colon cancer samples (lanes 19–24) showed hypomethylation.

(*C*) Hybridization with probe pYNZ86.1 for region 3p13-14 (see Fig. 3). Normal lung DNA was digested with *Msp* I (lane 1), *Hin*dIII (lane 2), and *Hin*dIII plus *Msp* I (lane 3). *Hpa* II plus *Hin*dIII digestion (lanes 4 and 5) produces high molecular weight bands (above 3.1 kb) and diminished hybridization at 1.7 kb (lanes 4 and 5) as compared with *Msp* I and *Hin*dIII digests (lane 3), indicating some methylated CCGG sites in normal lung DNA. A similar pattern was seen in normal colonic mucosa (not shown). Six of seven SCLC DNA samples (lanes 6–11), digested with *Hin*dIII plus *Hpa* II, are hypermethylated (compare to normal in lanes 4 and 5) as evidenced by a marked reduction or loss of bands between 0.9 and 6.0 kb (lanes 6–9 and lane 11) or loss of the 1.7-kb band (lane 10). In contrast, four of seven DNA samples from the non-SCLC lung tumor lines are hypomethylated (note decreased 6.0-kb band and increased bands between 1.7 and 3.1 kb). Two non-SCLC DNA samples (lanes 17 and 18, NCI-H520 and NCI-H320) have a more normal pattern, while line NCI-H596 (lane 19) is hypermethylated. Four of five DNA samples from colon cancer lines (lanes 20–23) show relative hypomethylation and one (lane 24) has a more normal pattern.

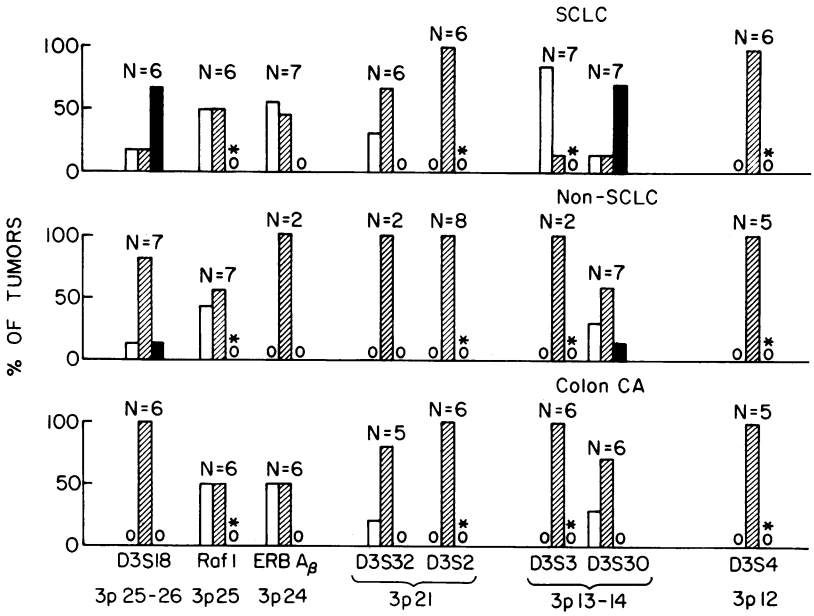

FIG. 3. CCGG methylation status at multiple chromosome 3p sites in the DNA samples shown in Fig. 2. The chromosomal positions of the hybridization probes, from the most telomeric (3p25-26) to the most centromeric (3p12) locations, are shown along the horizontal axis. Precise order of the probes within brackets is not known. No rearrangements for any of the probes have been found in lung cancer DNA (17–22). *Hin*dIII plus *Hpa* II digests were compared for normal vs. tumor DNA samples as shown in Fig. 2. The percentage of DNA samples showing normal methylation (open bars), hypomethylation (hatched bars), or hypermethylation (dark bars) is shown along the vertical axis. A high incidence of hypermethylation or retained normal methylation occurred only in SCLC DNA at loci D3S18 at 3p25-26 and D3S3 and D3S30 at 3p13-14, while these loci, and all remaining regions, were predominantly hypomethylated in the other tumor types tested. *, Maximally methylated in normal lung.

chromosome 3p21 region is shown in Fig. 2*A*—details are given in the figure legend). However, two regions in SCLC DNA, one near the telomere, 3p25-26 (Fig. 2*B*), and one more centromeric, 3p13-14 (Fig. 2*C*), were hypermethylated as compared with the normal lung pattern. Four of seven SCLC DNA samples had these patterns at both 3p regions, and all seven at either 3p25-26 or 3p13-14. In sharp contrast, only one non-SCLC tumor and none of the colon cancers were hypermethylated at either locus (Fig. 2 *B* and *C*; summary in Fig. 3). Moreover, hypomethylation was the predominant pattern in these latter tumor types.

DISCUSSION

We show that abnormal DNA hypermethylation nonrandomly maps to chromosome loci frequently altered in lung and colon tumors. Current data concerning relationships between chromatin structure, regulation of gene expression, and cytosine methylation suggest that such changes, especially in CpG islands, could either mark or cause alterations of chromatin structure associated with DNA instability and gene inactivation. The potential association with DNA instability is stressed by our observation that the abnormal 17p CpG island methylation preceded the structural alterations of this chromosome that characterize colon cancer progression (15, 16). Hypermethylation changes may then be a harbinger of, or directly involved in, allelic losses in cancer. The underlying mechanisms may involve relationships between DNA methylation and DNA replication. Normally unmethylated CpG-rich regions, and transcriptionally active genes, replicate early during the cell cycle (for review, see ref. 32). Increased DNA methylation can cause delayed rates of DNA synthesis (32) which, around fragile sites, could predispose to altered DNA structure (11). This is stressed by recent findings for the fragile X mental retardation syndrome. The appearance of an abnormally methylated CpG island, and an altered length of a series of CGG repeats within this island located adjacent to an X-chromosome gene, coincides with appearance of the disease phenotype (33–35).

If abnormal CpG island methylation is associated with DNA instability, our data would suggest, based on the sensitivity of conventional Southern blots, that at least 10%, or more, of the cells in premalignant colonic adenomas harbor such a tendency on chromosome 17p. This predisposition might explain the high frequency of the genetic changes seen for 17p in the colon cancers that arise from adenomas and in other human neoplasms (15, 16, 26, 27).

In terms of gene inactivation, there is evidence that CpG island hypermethylation can both cause chromatin changes that decrease transcription and mark areas where such chromatin changes are occurring. Transfected genes when methylated are not generally transcribed and assume a closed chromatin transcription configuration (for review, see ref. 10). In adult cells, CpG island methylation occurs only on the inactivated X chromosome in female mammals (for review, see ref. 36). In this setting, the methylation change appears not to precede gene inactivation, but rather to follow and stabilize a chromatin pattern that occurs early in embryogenesis (37).

The potential role of CpG island methylation in neoplasia is well exemplified from findings in immortalized cells in culture. In such cells, CpG island methylation appears to be extensive (7) and may interfere with cell maturation by silencing the expression of differentiation genes, such as the transcription factor myo D (8). In fact, progression from the immortalized to the tumorigenic state is associated with extension of methylation within the myo D gene-associated CpG island (8), as we now report for a 17p region in the progression stages of noncultured colon tumors.

The full implications of the 17p methylation changes we report will await mapping of the methylation status of additional areas of this chromosome. The p53 gene, in which point mutations virtually always coincide with losses of 17p alleles during colon tumor progression (16), is some distance from the pYNZ22 region and is unlikely to be influenced directly by the abnormalities we define. However, hypermethylation can occur over a wide chromosome region, as our current PFGE and previous chromosome 11p data suggest (4). In colon cancers, we have found that, unlike the loss of DNA methylation that is so prevalent in this tumor, the p53 gene retains full methylation of multiple CpG sites (data not shown). Spontaneous deamination of methylated cytosine forms thymidine. There is, in colon cancers (16), a high prevalence of C-to-T mutations in the p53 gene at cytosines methylated in normal tissues (38). The implications of this finding for rates of mutation of p53 during tumor progression remain to be determined.

It is also possible that genes around the pYNZ22 region play a role in tumor progression. Tumor suppressor genes in addition to p53 are thought to reside on 17p somewhere between the region from pYNZ22 to p53 (for example, see

refs. 39 and 40). The frequency with which CpG island sites are found adjacent to genes (9, 29) suggests that multiple genes reside in the pYNZ22 region itself. Microdeletions around the pYNZ22 area cause brain malformations in the rare genetic condition Miller–Dieker syndrome (28). Methylation-associated alterations in expression of such developmentally important genes could play a key role in tumor phenotype.

Finally, our chromosome 3p findings suggest that regional DNA hypermethylation may be specifically associated with chromosomal changes that contribute to individual types of human cancers. In contrast, the widespread loss of DNA methylation in tumor cells seems less specific. Both hypermethylated 3p sites in SCLC involve candidate regions for the initiation or progression of this cancer. First, both the 3p25-26 and 3p13-14 loci are virtually always reduced to homozygosity in SCLC (17–20), less frequently in non-SCLC lung tumors (20), and infrequently in colon cancer (15). Second, the 3p25-26 locus has been linked to the autosomal dominant Von Hippel–Lindau disease, in which renal tumors occur (41). It has been proposed that a tumor suppressor gene(s) for both SCLC and renal tumors resides in this area (41, 42). Finally, regions within 3p13-14 have been found to be homozygously deleted in at least one SCLC cell line (43), and another cell line exhibits reduction to homozygosity due to an interstitial deletion (22). This area also contains the most common fragile site known in the human genome (44).

In summary, we have associated regional DNA hypermethylation with important chromosome changes in human cancer. Studies of the methylation status of defined regions may help elucidate the mechanisms underlying chromosome instability in tumors and help identify chromatin patterns that lead to important losses of gene expression during tumor progression.

We thank Drs. Robert Casero and Andreé de Bustros for reviewing the work. Also, we thank Dr. Bert Vogelstein for providing the majority of the colon samples used in this study and the access to both published and unpublished data concerning 17p loss and p53 mutation. Portions of these studies were funded by Grants CA43318-05 and CA54396-01 from the National Cancer Institute, a grant from the Tobacco Research Foundation, and support from the Clayton Fund. M.M. is a graduate student in a Human Genetics program supported by National Institutes of Health Training Grant GM07814-09.

1. Jones, P. A. & Buckley, J. D. (1990) *Adv. Cancer Res.* **54,** 1–23.
2. Baylin, S. B., Makos, M., Wu, J., Yen, R.-W., de Bustros, A., Vertino, P. & Nelkin, B. D. (1991) *Cancer Cells* **3,** 383–390.
3. Goelz, S. E., Vogelstein, B., Hamilton, S. R. & Feinberg, A. P. (1985) *Science* **228,** 187–190.
4. de Bustros, A., Nelkin, B. D., Silverman, A., Ehrlich, G., Poiesz, B. & Baylin, S. B. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5693–5697.
5. El-Deiry, W. S., Nelkin, B. D., Celano, P., Yen, R.-W. C., Falco, J. P., Hamilton, S. R. & Baylin, S. B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3470–3474.
6. Kautainien, T. L. & Jones, P. A. (1986) *J. Biol. Chem.* **261,** 1594–1598.
7. Antequera, F., Boyes, J. & Bird, A. (1990) *Cell* **62,** 503–514.
8. Jones, P. A., Wolkowicz, M. J., Rideout, W. M., III, Gonzales, F. A., Marziasz, C. M., Coetzee, G. A. & Tapscott, S. J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6117–6121.
9. Bird, A. P. (1987) *Trends Genet.* **3,** 342–348.
10. Cedar, H. (1988) *Cell* **53,** 3–4.
11. Laird, C., Jaffe, E., Karpen, G., Lamb, M. & Nelson, R. (1987) *Trends Genet.* **3,** 274–280.
12. Carney, D. N., Gazdar, A. F., Bepler, G., Guccion, J. G., Marangos, P. J., Moody, T. W., Zweig, M. H. & Minna, J. D. (1985) *Cancer Res.* **45,** 2913–2923.
13. Brower, M., Carney, D. N., Oie, H. K., Gazdar, A. F. & Minna, J. D. (1986) *Cancer Res.* **46,** 798–806.
14. Park, J.-G., Oie, H. K., Sugarbaker, P. H., Henslee, J. G., Chen, T.-R., Johnson, B. E. & Gazdar, A. (1987) *Cancer Res.* **47,** 6710–6718.
15. Vogelstein, B., Fearon, E. R., Kern, S. E., Hamilton, S. R., Preisinger, A. C., Nakamura, Y. & White, R. (1989) *Science* **244,** 207–210.
16. Baker, S. J., Preisinger, A. C., Jessup, M., Paraskeva, C., Markowitz, S., Willson, J. K. V., Hamilton, S. & Vogelstein, B. (1990) *Cancer Res.* **50,** 7717–7722.
17. Brauch, B. H., Johnson, B., Hovis, J., Takahiko, B. A., Gazdar, A., Pettingill, O. S., Graziano, S., Sorenson, G. D., Poiesz, B. J., Minna, J., Linehan, M. & Zbar, B. (1987) *N. Engl. J. Med.* **317,** 1109–1113.
18. Minna, J. D. (1989) *Chest (Suppl.)* **96,** 17S–23S.
19. Naylor, S. L., Johnson, B. E., Minna, J. D. & Sakaguchi, A. Y. (1987) *Nature (London)* **329,** 451–454.
20. Brauch, H., Tory, K., Kotler, F., Gazdar, A. F., Pettengill, O. S., Johnson, B., Graziano, S., Winton, T., Buys, C. H. C. M., Sorenson, G. D., Poiesz, B. J., Minna, J. D. & Zbar, B. (1990) *Genes Chromosomes Cancer* **1,** 240–246.
21. Lerman, M. I., Latif, F., Glenn, G. M., Daniel, B. L., McBride, O. W., Grzeschik, K. H., Takahashi, T., Minna, J., Anglard, P., Linehan, W. M. & Zbar, B. (1991) *Human Genet.* **86,** 567–577.
22. Daly, M. C., Douglas, J. B., Bleehen, N. M., Hastleton, P., Twentyman, P. R., Sundaresan, V., Carritt, B., Bergh, J. & Rabbitts, P. H. (1991) *Genomics* **9,** 113–119.
23. Nakamura, Y., Ballard, L., Leppert, M., O'Connell, P., Lathrop, G. M., Lalouel, J. M. & White, R. (1988) *Nucleic Acids Res.* **16,** 5707.
24. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
25. Kenwrick, S., Patterson, M., Speer, A., Fischbeck, K. & Davies, K. (1987) *Cell* **48,** 351–357.
26. Yokata, J., Wada, M., Shimosato, Y., Terada, M. & Sugimura, T. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9252–9256.
27. Mackay, J., Steel, C. M., Elder, P. A., Forrest, A. P. & Evans, H. J. (1988) *Lancet* **ii,** 1384–1385.
28. Ledbetter, S. A., Wallace, M. R., Collins, F. S. & Ledbetter, D. H. (1990) *Genomics* **7,** 264–269.
29. Lindsay, S. & Bird, A. P. (1987) *Nature (London)* **327,** 336–338.
30. Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumlin, E. & White, R. (1987) *Science* **235,** 1616–1622.
31. van der Ploeg, L. H. T. & Flavell, R. A. (1980) *Cell* **19,** 947–958.
32. Riggs, A. D. (1990) *Philos. Trans. R. Soc. London Ser. B* **326,** 253–265.
33. Oberlé, I., Rousseau, F., Heitz, D., Kretz, C., Devys, D., Hanauer, A., Boué, J., Bertheas, M. F. & Mandel, J. L. (1991) *Science* **252,** 1097–1102.
34. Verkerk, A. J. M. H., Pieretti, M., Sutcliffe, J. S., Fu, Y.-H., Kuhl, D. P. A., Pizzuti, A., Reiner, O., Richards, S., Victoria, M. F., Zhang, F., Eussen, B. E., van Ommen, G.-J. B., Blonden, L. A. J., Riggins, G. J., Chastain, J. L., Kunst, C. B., Galjaard, H., Caskey, C. T., Nelson, D. L., Oostra, B. A. & Warren, S. T. (1991) *Cell* **65,** 905–914.
35. Kremer, E. J., Pritchard, M., Lynch, M., Yu, S., Holman, K., Baker, E., Warren, S. T., Schlessinger, D., Sutherland, G. R. & Richards, R. I. (1991) *Science* **252,** 1711–1714.
36. Migeon, B. R. (1990) *Genet. Res.* **56,** 91–98.
37. Lock, L. F., Takagi, N. & Martin, G. R. (1987) *Cell* **48,** 39–46.
38. Rideout, W. M., III, Coetzee, G. A., Olumi, A. F. & Jones, P. A. (1990) *Science* **249,** 1288–1290.
39. Sato, T., Tanigami, A., Yamakawa, K., Akiyama, F., Kasumi, F., Sakamoto, G. & Nakamura, Y. (1990) *Cancer Res.* **50,** 7184–7189.
40. Coles, C., Thompson, A. M., Elder, P. A., Cohen, B. B., Mackenzie, I. M., Granston, G., Chetty, U., MacKay, J., MacDonald, M., Nakamura, Y., Hoyheim, B. & Steel, C. M. (1990) *Lancet* **336,** 761–763.
41. Seizinger, B. R., Rouleau, G. A., Ozelius, L. J., Lane, A. H., Farmer, G. E., Lamiell, J. M., Haines, J., Yuen, J. W. M., Collins, D., Majoor-Krakauer, D., Bonner, T., Mathew, C., Rubenstein, A., Halperin, J., McConkie-Rosell, A., Green, J. S., Trofatter, J. A., Ponder, B. A., Eierman, L., Bowmer, M. I., Schimke, R., Oostra, B., Aronin, N., Smith, D. I., Drabkin, H., Waziri, M. H., Hobbs, W. J., Matuza, R. L., Conneally, P. M., Hsia, Y. E. & Gusella, J. F. (1988) *Nature (London)* **332,** 268–269.
42. Hosoe, S., Brauch, H., Latif, F., Glenn, G., Daniel, L., Bale, S., Choyke, P., Gorin, M., Oldfield, E., Berman, A., Goodman, J., Orcutt, M., Hampsch, K., Delisio, J., Modi, W., McBride, O. W., Anglard, P., Weiss, G., Walther, M. M., Linehan, W. M., Lerman, M. I. & Zbar, B. (1990) *Genomics* **8,** 634–640.
43. Rabbitts, P., Bergh, J., Douglas, J., Collins, F. & Waters, J. (1990) *Genes Chromosomes Cancer* **2,** 231–238.
44. Smeets, D. F. C. M., Scheres, J. M. J. C. & Hustinx, T. W. J. (1986) *Hum. Genet.* **72,** 215–220.