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High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer

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ABSTRACT **DNA methylation abnormalities occur consistently in human neoplasia including widespread hypomethylation and more recently recognized local increases in DNA methylation that hold potential for gene inactivation events. To study this imbalance further, we have cloned and localized to chromosome 19 a portion of the human DNA methyltransferase gene that codes for the enzyme catalyzing DNA methylation. Expression of this gene is low in normal human cells, significantly increased (30- to 50-fold by PCR analysis) in virally transformed cells, and strikingly elevated in human cancer cells (several hundredfold). In comparison to colon mucosa from patients without neoplasia, median levels of DNA methyltransferase transcripts are 15-fold increased in histologically normal mucosa from patients with cancers or the benign polyps that can precede cancers, 60-fold increased in the premalignant polyps, and >200-fold increased in the cancers. Thus, increases in DNA methyltransferase gene expression precede development of colonic neoplasia and continue during progression of colonic neoplasms. These increases may play a role in the genetic instability of cancer and mark early events in cell transformation.**

Tumor progression involves interactions between multiple adverse genetic events, including chromosomal translocations, deletions, amplifications, and point mutations (1–5). The etiology of these progressive changes in transformed cells is unknown. One candidate mechanism is the abnormal pattern of DNA methylation that occurs consistently in human tumors and includes, simultaneously, widespread genomic hypomethylation (6–10) and regional areas of abnormally increased methylation (11–14). Both changes appear early in tumor progression, such as in the premalignant polyps that precede colon cancer (15, 16), and could contribute to genetic instability through alterations of DNA conformation (17) and/or DNA–protein binding patterns (18, 19) and increased fragile-site instability (20). Furthermore, local increases in DNA methylation in cancer cells can be strategically placed in C+G-rich areas, termed "CpG islands," including regions on the short arm of chromosome 11 (21) where tumor suppressor genes are known to reside (22–24), on chromosome 16 in HeLa cells (18), and on multiple areas in immortalized cells (25), including the muscle-differentiating gene *MyoD* (26). These islands are important for maintenance of "open" transcriptional conformation around genes, are normally unmethylated in adult cells except on the inactivated X chromosome (14, 25, 27), and are in a "closed" chromatin pattern when abnormally methylated in cancer (21) or immortalized cells (25). Antequera *et al.* (25) suggest that silencing of differentiation genes by such abnormal CpG island methylation may prevent differentiation in immortalized cells.

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One mechanism for local increases in DNA methylation in neoplastic cells may be increased activity of DNA methyltransferase (DNA MTase), which catalyzes methylation of cytosines at CpG sites. Despite widespread DNA hypomethylation, DNA MTase activity is actually increased in rodent and human tumor cells (28). The mechanism underlying such an increase, including how DNA MTase activity is regulated with respect to the time of onset of tumorigenesis and/or tumor progression has not been elucidated. We have now cloned a region of the human DNA MTase gene, shown its chromosome location, and demonstrated that expression of this gene is incremental over the progression stages of human cancer.

MATERIALS AND METHODS

Cell Lines. WI-38 fetal lung fibroblasts and WI-38 VA13 simian virus 40-transformed fibroblasts were obtained from American Type Culture Collection and BALB/MK keratinocytes (29) were from Stuart Aaronson and William Taylor (National Institutes of Health). All other established cell lines have been described (30–32).

RNA and DNA Hybridizations. Poly(A)$^+$ RNA and DNA were isolated and analyzed by Northern and Southern blot analyses, respectively, as described (11, 13, 16, 21, 33). A mouse cDNA for DNA MTase was a gift from Timothy Bestor (34), a v-fos probe (35) was obtained from Oncor (Gaithersburg, MD), and a human β-actin probe was a gift from Don Cleveland (Johns Hopkins).

PCR Methodology. We used a modification of PCR procedures (36, 37) to clone a portion of the human DNA MTase gene and compare its expression with that of β-actin. Oligonucleotides for rodent DNA MTase gene sequence (34) (synthesized by the core facility of the School of Public Health, The Johns Hopkins University School of Medicine) were used to amplify the human DNA MTase gene and included: for the sense 5′ DNA MTase primer in the Lys-Gly repeat region (see Fig. 1*A*), 5′-GGGAAGGGGAAAGGGAAGGG-3′ and, for the 3′ antisense MTase primer (Fig. 1*A*), 5′-GCGGGCACATTCCCG-3′. Oligonucleotides used to detect transcripts for the human β-actin gene spanned a 1.0-kilobase (kb) region in the far 5′ region of the gene (38) and included: for the 5′ primer (base pairs 25–44), 5′-GTGGGCCGCTCTAGGCACCAA-3′ and, for the 3′ antisense primer (base pairs 938–957), 5′-GATGGAGCCACCGATCCACA-3′. To clone and detect expression of the human DNA MTase gene, 0.1–1.0 μg of total RNA was incubated for 1 hr at 37°C with the 3′ antisense oligonucleotide for DNA MTase plus 100 units of Moloney murine leukemia virus reverse transcriptase in 10 μl of 50 mM

Abbreviations: DNA MTase, DNA methyltransferase; RT, reverse transcription; SCLC, small cell lung carcinoma.

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Tris·HCl, pH 8.3/75 mM KCl/10 mM dithiothreitol/3 mM $MgCl_2$/5 units of RNasin containing all four DNA nucleotides (0.5 mM). For the PCR, the final reaction mixture was 25 μl of the 5′ and 3′ oligoprimers (each at 1.0 μM)/25 mM Tris·HCl, pH 8.3/1–3 mM $MgCl_2$/60 mM KCl/0.5–5 units of *Taq* DNA polymerase (Amplitaq, Cetus). PCR was performed in a Techne Laboratories (Vineland, NJ) programmable Dri-Block (PHC-1) using cycle stages of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min. Samples (5–15 μl) were electrophoresed on 2% agarose gels and gels were stained with ethidium bromide, transferred to nylon filters, and hybridized as described (11, 13, 16, 21) with 1×10^7 cpm of oligolabeled probes. In all studies, omission of RNA or reverse transcriptase resulted in no subsequent amplification signals. By using RNA from the human small cell lung carcinoma (SCLC) cell line HUT249 (30, 31), a 1.3-kb fragment of the DNA MTase gene was amplified (see Fig. 1*A*), cloned in the *Eco*RI site of λ ZAPII (Stratagene), and recovered in pBluescript phagemid with helper virus as described by the manufacturer.

Chromosomal Localization of the Human DNA MTase Gene. A panel of human–hamster somatic cell hybrid DNAs digested with *Hin*dIII was obtained from Bios (New Haven, CT) and probed with the above DNA MTase cDNA.

RESULTS

Comparison of Rodent and Human DNA MTase Genes. A 1.3-kb cDNA for the human DNA MTase gene and a 2.0-kb mouse DNA MTase cDNA (Fig. 1*A*) detected the same restriction fragments in human DNA (Fig. 1*B*, lanes 1–6) and the same 5.2-kb mRNA transcript in human cells (Fig. 1*C*, lanes 1 and 8–12) and mouse keratinocytes (Fig. 1*C*, lanes 2 and 3) that was reported in murine tumor cells (34). The human cDNA was, by dideoxynucleotide sequencing, ≈85% homologous to mouse DNA MTase (data not shown).

Chromosomal Localization of the Human DNA MTase Gene. Hamster–human somatic cell hybrid DNA was probed for the human DNA MTase gene. Of 25 hybrids examined, 7 were positive and all contained human chromosome 19, which was absent in the 18 negative hybrids. All other chromosomes showed discordancies ranging from 12 to 52%.

Steady-State Expression of the Human DNA MTase Gene in Normal and Neoplastic Cells. By Northern blot analysis, the rodent and human DNA MTase probes readily detected a 5.2-kb transcript (Fig. 1*C*, lanes 2–5) in immortalized but nontumorigenic mouse keratinocytes (29). Interestingly, transcripts were not detected, even with prolonged exposures, in mRNAs from normal or virally transformed cultured human fibroblasts (Fig. 1*C*, lane 6) or from fresh samples of human lung or liver (data not shown) or total RNA from human cancer cells (Fig. 1*C*, lane 7). However, a 5.2-kb transcript was detectable (Fig. 1*C*, lanes 8–12) with mouse or human cDNA probes in poly(A)$^+$ RNA from multiple lines of cultured human cancer cells. No gross alterations in the DNA MTase gene were found in DNA from these or other human tumors examined (Fig. 1*B*, lanes 1–12).

DNA MTase Expression During Cell Growth–PCR Analysis of DNA MTase Expression in Rodent and Human Cells. The reverse transcription (RT)–PCR technique was used for sensitive detection of DNA MTase mRNA. For semiquantitation, we compared, as have others (37, 39), amplification of DNA MTase transcripts to a reference gene, β-actin. The transcripts were first compared in total RNA of quiescent and growth factor-stimulated mouse keratinocytes (29) by Northern blot and RT–PCR analyses with excellent correlation (Fig. 2). DNA MTase transcripts were low in quiescent rodent keratinocytes but rose dramatically, as compared to actin transcripts, before and during DNA synthesis. The increase in MTase transcripts occurred well after that for the early-response gene c-*fos* (Fig. 2*A*). Densitometric analysis

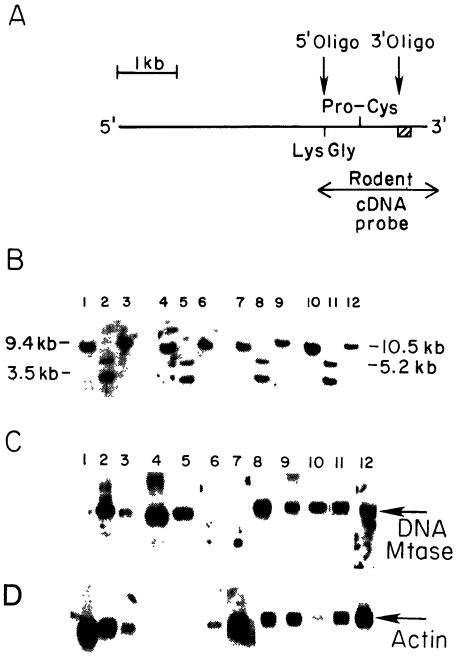


FIG. 1. Genomic structure and expression of DNA MTase. (*A*) Mouse DNA MTase gene including areas conserved with prokaryotic CpG DNA MTases (34). Lys-Gly, Lys-Gly repeat area; hatched box, the highly conserved region; Pro-Cys, conserved Pro-Cys dipeptide essential for enzyme activity; ⟷, 2.0-kb mouse cDNA (34); vertical arrows, regions used to amplify a human cDNA. (*B*) Southern blots of human DNA (7.5 μg) digested with *Bgl* II (lanes 1, 4, 7, and 10), *Hin*cII (lanes 2, 5, 8, and 11), or *Hin*dIII (lanes 3, 6, 9, and 12) at 3–4 units/μg of DNA. Lanes: 1–3, normal lung probed with the mouse DNA MTase cDNA (exposure time, 72 hr); 4–6, same lanes, after probe removal, probed with the human cDNA (exposure time, 24 hr); 7–9, SCLC probed with human cDNA; 10–12, medullary thyroid carcinoma probed with the human cDNA. All final washes were in 0.1× standard saline citrate at 65°C. (*C*) Northern blot hybridizations were exposed for 24–96 hr except where noted. Lanes: 1, 10 μg of poly(A)$^+$ RNA from a squamous cell carcinoma cell line derived from human lung; 2, 10 μg of poly(A)$^+$ RNA from cultured mouse keratinocytes; 3, 20 μg of total RNA from the same mouse keratinocytes. Lanes 1–3 were probed with the mouse MTase cDNA. Lanes: 4 and 5, same as lanes 1 and 2, after removal of the mouse cDNA probe and hybridization with the human cDNA; 6, 6 μg of poly(A)$^+$ RNA from simian virus 40-transformed human lung fibroblasts (12-day exposure); 7, 40 μg of total RNA from the human SCLC cell line shown in lane 8 (12-day exposure); 8–11, 10 μg of poly(A)$^+$ RNA from human SCLC cell lines; 12, 10 μg of RNA from a human medullary thyroid carcinoma cell line. Lanes 4–12 were probed with the human cDNA. (*D*) The blots in *C* were rehybridized with human β-actin probe.

revealed a roughly 10-fold increase in MTase/actin mRNA ratios in the stimulated cells by Northern blot analysis and a 30-fold (12 hr) to 50-fold (24 hr) higher ratio by RT–PCR (Fig. 2*B*). The data, which validate our RT–PCR technique, fit well with an observed rapid increase in DNA MTase activity during DNA synthesis (40) and suggest that this regulation occurs, primarily, at the transcript level.

In human cells, RT–PCR of total RNA (Fig. 3 *A–D*) revealed, as a function of multiple cycle number and amount of RNA added, that the DNA MTase/actin transcript ratio in logarithmic-phase cultured lung cancer cells was several hundredfold greater than that for growing normal human fibroblasts (Fig. 3*E*). DNA MTase mRNA levels were also increased to a lesser level (>30- to 50-fold) in the same fibroblasts transformed with simian virus 40 (Fig. 3), although levels were still not detectable by Northern blot analysis of poly(A)$^+$ RNA (see Fig. 1, lanes 6).

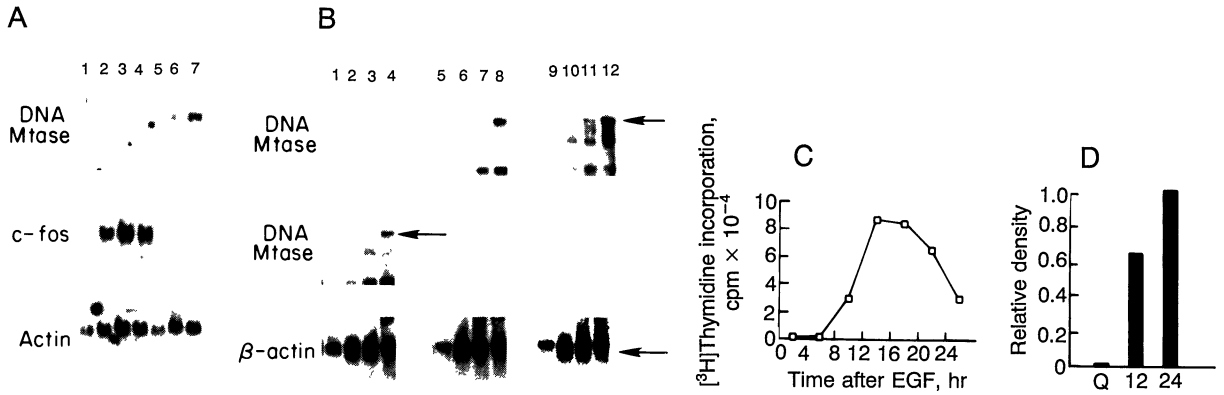


FIG. 2. DNA MTase and β-actin expression in BALB/MK mouse keratinocytes (29). After 2 days in low-calcium minimal essential medium plus 10% (vol/vol) dialyzed fetal calf serum, cells were stimulated with epidermal growth factor (EGF) (4 μg/ml). (*A*) Northern blots (20 μg of total RNA) were probed sequentially with cDNAs for mouse DNA MTase, human c-*fos*, and human β-actin genes. (*B*) RT–PCR analysis of same RNA (1 μg). PCR products were electrophoresed and hybridized with the above probes. Exposure times, 2 hr, plus a 24-hr exposure for DNA MTase in quiescent cells (second panel from top). Arrows, predicted amplification products. Lanes: 1–4, quiescent cells; 5–8, cells stimulated with EGF for 12 hr; 9–12, cells stimulated with EGF for 24 hr. Cycle numbers are as follows. Lanes: 1, 5, and 9, cycle 15; 2, 6, and 10, cycle 20; 3, 7, and 11, cycle 25; 4, 8, and 12, cycle 30. Cycle numbers beyond the linear range produced unidentified smaller products. (*C*) DNA synthesis rate, 4 hr per confluent well, was measured by [^{3}H]thymidine uptake in a 24-well plate. (*D*) Within linear regions (see below, Fig. 3), densitometry was used to compute DNA MTase/β-actin transcript ratios. Bars indicate time after EGF stimulation. Bars: Q, quiescent cells; 12, 12 hr of stimulation; 24, 24 hr of stimulation.

DNA MTase Gene Expression Increases over Progression of Human Colon Cancer. To understand more fully potential relationships between increased DNA MTase gene expression and transformed cells, we investigated the progression of human colon cancer. In this model, molecular events can be studied in accessible stages of transition from premalignant lesions (benign polyps) to advancing stages of malignancy (41–44). Further, in such tissues, abnormalities in DNA methylation, consisting simultaneously of hypomethylation (15) and regional hypermethylation (16) in premalignant polyps, are the earliest consistent molecular changes.

We observed a distinct correlation between increasing DNA MTase gene expression and colon cancer evolution (Figs. 4 and 5). DNA MTase/actin transcript ratios in RNA from eight of nine colonic adenocarcinomas (CA; Figs. 4*A* and 5*C*) were higher than the highest ratio found in 13 normal mucosal samples from patients without neoplasia (Nl/Nl; Figs. 4 *A* and *B* and 5*A*). The values in cancer tissue ranged up to 4000-fold higher than the median value for the samples from patients with benign disorders [median increase (Fig. 5), >200-fold; $P < 0.001$]. Similarly, the MTase/actin ratios were higher in six benign villous adenomatous polyps (VA; Figs. 4*A* and 5*B*) than in 12 of the 13 normal mucosa samples from patients without neoplasia (median value, >60-fold higher; $P < 0.001$). An important finding regarding tumor progression is that values in the carcinomas are, as a population, significantly higher than those in the benign polyps (median value, 4-fold increased; $P < 0.05$—see VA and CA, Fig. 5 *B* and *C*), which may precede colon cancer in many patients (45–48). Furthermore, analysis of paired normal and cancer samples from individual patients always showed higher levels in the neoplastic lesions (Fig. 4*A* and see dotted lines in Fig. 5*C*).

A critical finding is that, as a population of values, DNA MTase/actin RNA ratios were much higher in histologically normal colonic mucosa distant from either polyps or cancers as compared to colonic mucosa from patients with nonneoplastic disorders (Figs. 4 *A* and *B*; also compare lanes Nl/Nl to Nl/VA, to Nl/CA, Fig. 5 *A–C*). As shown in Fig. 5, 9 of 17 histologically normal samples from patients with either polyps (Nl/VA) or carcinomas (Nl/CA) had values higher than did 12 of the 13 mucosal samples from patients with nonneoplastic disorders (Nl/Nl) (median values, 15-fold higher; $P < 0.001$).

The increased DNA MTase gene expression in histologically normal colonic mucosa must be related to defined increases in DNA synthesis and an accompanying block to

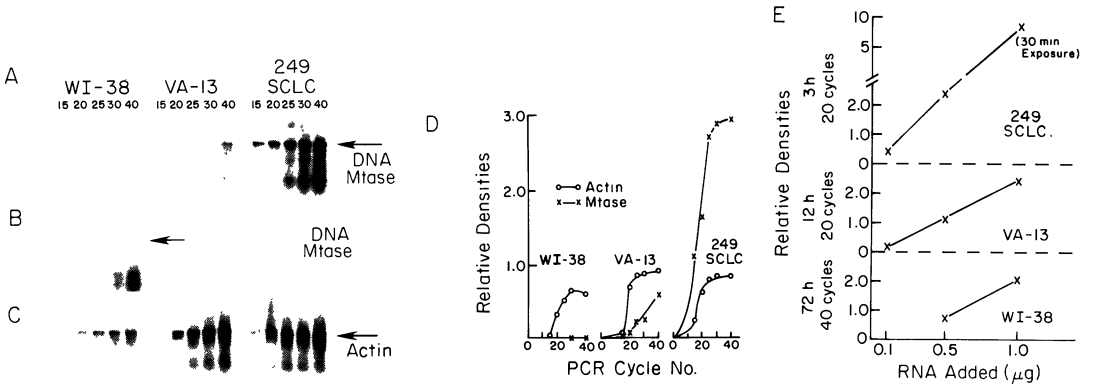


FIG. 3. RT–PCR analysis of DNA MTase and β-actin RNA transcripts from RNA of WI-38 normal human fetal lung fibroblasts, a simian virus 40-transformed line (VA-13) of these same fibroblasts, and a human SCLC cell line (NCI-H249). (*A*) Hybridization signal (5-hr exposure) to human DNA MTase cDNA (arrow, predicted 1.3-kb product), for multiple PCR cycles (indicated as lane labels). (*B*) Exposure (24 hr) of blot in *A* for normal fibroblasts only. (*C*) Actin hybridization signal (5-hr exposure). (*D*) Densitometric analysis of both transcripts. (*E*) Densitometric analysis of DNA MTase transcripts in RNA concentration (*x* axis) curves showing that the relative amplitude differences (shown by exposure times and cycle numbers on *y* axis) between the cell types are maintained throughout. h, hr.

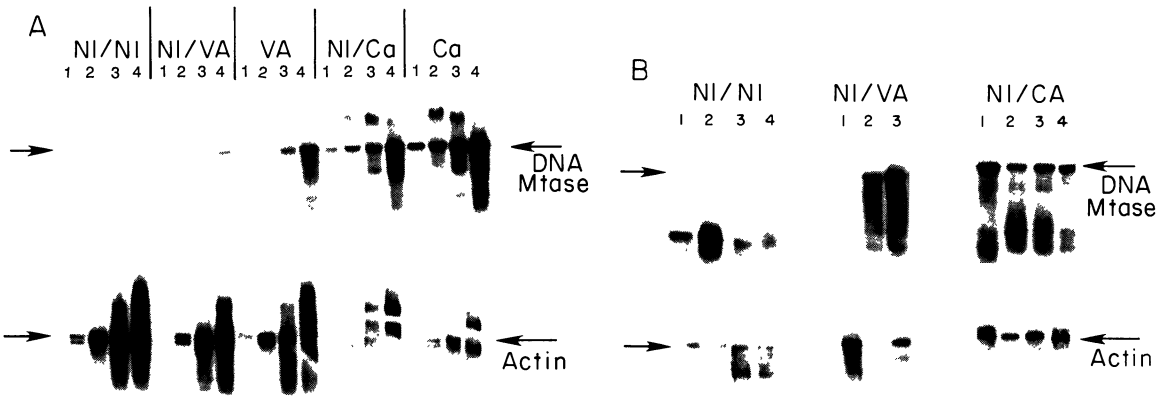


FIG. 4. Examples of RT–PCR analysis of DNA MTase and β-actin transcripts in RNA from human colonic mucosa and colon neoplasms. Exposure times were 2–3 hr. The high cycle numbers necessary to visualize the lowest levels of DNA MTase transcripts lead to variable bands smaller than the predicted product (arrows) and a marked overexposure of β-actin. (*A*) Histologically normal colonic mucosa from a patient with diverticular disease and no evidence of neoplasia (NI/NI), normal mucosa of a patient with a benign villous adenoma polyp (NI/VA), the polyp in the same patient (VA), normal mucosa in a patient with colon adenocarcinoma (NI/CA), and the carcinoma itself (CA). As in previous studies (16, 41–44), the normal colonic mucosa samples from patients with neoplasms were taken several centimeters distal to tumors and judged free of tumor cells by multiple microscopic examinations. Cycle numbers are as follows: Lanes: 1, cycle 15; 2, cycle 20; 3, cycle 25; 4, cycle 35. (*B*) DNA MTase (30 cycles) and β-actin (20 cycles) transcripts in histologically normal colonic mucosa from four additional patients without neoplasia (NI/NI; lanes 1–4), three patients with villous adenomas (NI/VA; lanes 1–3), and four patients with colon cancer (NI/CA; lanes 1–4).

normal intestinal cell maturation that involve a broad region of colonic mucosa distant from actual tumors (45–48). This so-called "field effect" may reflect the initial changes in which polyps and cancer subsequently develop. We have determined, by staining the cell-cycle-specific antigen Ki-67 (49), that maximum increases in colonic mucosa of patients with cancer are 2-fold greater than values in patients without cancer (data not shown). This difference is identical to those reported (45–48) and much less than the 15-fold increase we find in DNA MTase transcripts. Furthermore, in the colon cancers we have studied (Fig. 5*C*), a maximum increase of 2-fold was found for mitotic indices between cancers spanning a 400-fold difference for DNA MTase expression. Finally, the average mitotic index for these cancers, 1.4%, was only 3-fold higher than that present in normal colonic mucosa from patients without cancer (45–48) as compared to the

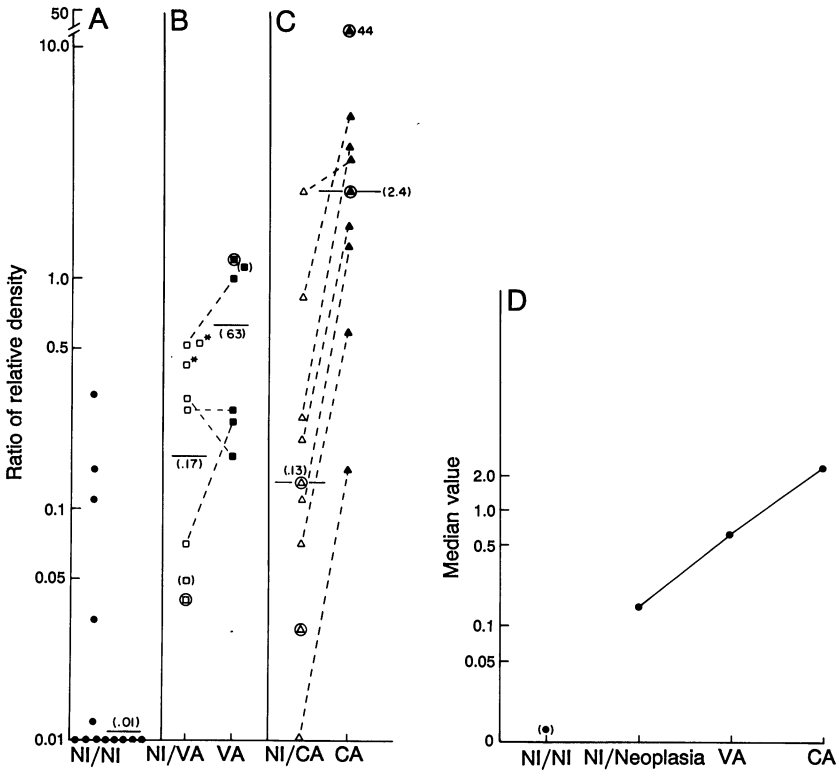


FIG. 5. (*A–C*) RT–PCR analyses of 45 samples from 32 patients. The types of data in Fig. 4 were analyzed by densitometry, in linear regions, to compute ratios of DNA MTase to β-actin transcripts and expressed as the relative density for the DNA MTase/β-actin ratio. Samples from 23 of the 44 patients were analyzed in two or more RT–PCRs and the others were analyzed in one assay, with an interassay variability of 2-fold at the lowest range and 6-fold at the highest values. MTase and β-actin signals were always analyzed in the same RT–PCR assay. Results were statistically analyzed by the rank sum test. Age and sex distribution were similar in each category including 18–81 years for 13 patients (four males and nine females) with no evidence of neoplasia, NI/NI (*A*), 48–83 years for 6 patients (four males and two females) with premalignant polyps and no history of familial disease and 23 and 39 years for two patients with the genetic disorder familial adenomatous polyposis, NI/VA (normal mucosa/villous adenoma) (*B*), and 39–83 years for 10 patients (five males and five females) with adenocarcinomas, NI/CA (normal mucosa/cancer) (*C*). No correlation of results to age or sex was found. Of the 13 patients without neoplasia, 7 had diverticular disease, 4 had inflammatory disease of the small bowel, 1 had radiation proctitis, and 1 had volvulus. Samples were taken from ascending and descending colon and the results for normal mucosa and tumors showed no correlation to site. Dotted lines (*B* and *C*) link paired normal and tumor samples from a given patient, as does the circle or parentheses in *B*. The values in *B* marked with an asterisk are mucosal samples from two patients with the autosomal dominant genetic disease familial adenomatous polyposis. In *C*, circled values indicate normal or cancer samples for which paired specimens were unavailable. Numbers in parentheses are median values. (In *B*, the 2 patients with familial polyposis were excluded because no other patients in this study had a documented family history of colonic neoplasia.) (*D*) MTase/β-actin transcripts as a function of colon neoplasia progression. *x* axis, constant arithmetic time frame to progress from the "field" changes to cancer (42); *y* axis, logarithmic plot of the median value of DNA MTase/β-actin for each category. NI/NI from A, NI/neoplasia from *B* and *C*; VA, benign adenomas from *B*; CA, cancer from *C*.

>200-fold average increase we found for DNA MTase expression. Thus, in patients with neoplasia, the striking increases in DNA MTase transcripts do not bear a simple relationship to the increased cell proliferation in the "field defect" or tumors.

DISCUSSION

In this study, high steady-state DNA MTase transcript expression is found to be characteristic of all human cancer cell lines examined. This result, although surprising, considering the widespread hypomethylation in cancer cell DNA, fits with the high DNA MTase activity detected in neoplastic cell lines (28). The data emphasize that increased DNA methylation capacity may be a mechanism for inducing the "hot spots" of increased DNA methylation now being recognized in cancer cells (18, 21, 25, 26) and being associated with altered chromatin conformation (25) and gene expression (26).

In patients with colon neoplasms, increases in DNA MTase gene expression appear to precede the local increases in DNA methylation observed in the premalignant polyps (16) and to continue increasing during the progression of neoplasms. In fact, if plotted over a predicted arithmetic time period for colon cancer evolution (45, 46), median values for DNA MTase gene expression actually increase exponentially (Fig. 5*D*). Increases in DNA MTase expression may then accompany changes preceding polyps and carcinoma and the remarkable accrual of genetic instability events that ensue once lesions have developed (41–44).

"Field changes" in colonic mucosa are most pronounced (45–48) in patients with autosomal dominant diseases that predispose to colonic carcinomas (50, 51). Notably, two of the three highest DNA MTase transcript values in histologically normal mucosa come from the only two patients we have studied with such genetic disorders (Fig. 5*B*). A genetic predisposition may also underly the common forms of colon carcinoma with a possible gene frequency in the population of 19% (52, 53). In this regard, 3 of the 13 values (23%) for colonic mucosa from patients without neoplasia are 3- to 10-fold greater than the highest value for the other 10 samples (Fig. 5*A*). Increased, DNA MTase gene expression may then help mark the predisposition state for colonic neoplasms as well as having potential functional significance for abnormalities of DNA evolving during tumor progression.

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