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Differentiation of Two Mouse Cell Lines Is Associated with Hypomethylation of Their Genomes

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Methyl-accepting assays and a sensitive method for labeling specific CpG sites have been used to show that the DNA of F9 embryonal carcinoma cells decreases in 5-methylcytosine content by ca. 9% during retinoic acid-induced differentiation, whereas the DNA of dimethyl sulfoxide-induced Friend murine erythroleukemia (MEL) cells loses ca. 3.8% of its methyl groups. These values correspond to the demethylation of 2.2×10^6 and 0.9×10^6 5′-CpG-3′ sites per haploid genome in differentiating F9 and MEL cells, respectively. Fluorography of DNA restriction fragments methylated in vitro and displayed on agarose gels showed that demethylation occurred throughout the genome. In uninduced F9 cells, the sequence TCGA tended to be more heavily methylated than did the sequence CCGG, whereas this tendency was reversed in MEL cells. The kinetics of in vitro DNA methylation reactions catalyzed by MEL cell DNA methyltransferase showed that substantial numbers of hemimethylated sites accumulate in the DNA of terminally differentiating F9 and MEL cells, implying that a partial loss of DNA-methylating activity may accompany terminal differentiation in these two cell types.

Changes in methylation patterns occur during cellular differentiation as shown by both tissue-specific differences in 5-methylcytosine (m^5C) content (16, 17, 35) and demethylation at specific sites around active genes, as detected by Southern blotting of DNA fragments produced by cleavage with methylation-sensitive restriction endonucleases (3). Various lines of evidence suggest that m^5C residues (located primarily in the sequence 5′-CpG-3′) are involved in the control of gene expression in the cells of vertebrates (reviewed in reference 13). The 5′ flanking sequences of structural genes tend to have elevated CpG contents (29). In some cases, this tendency is extreme; one example is the human proto-oncogene c-*ras*^{H}, in which the region extending 1.5 kilobases (kb) upstream from the transcription start site has over 180 CpG sites (33). The CpG density of this region is 10 times greater than the genome-wide average.

The haploid mouse genome contains ca. 2.5×10^7 CpG sites, of which 70 to 90% are normally methylated (16, 17). A change of 1% in m^5C content therefore reflects a change in the methylation status of ca. 2.5×10^5 CpG sites per haploid mouse genome. Because analytical methods such as high-pressure liquid chromatography cannot yield statistically significant values for changes in m^5C content of less than several percent, and because redistributions of methylated sites are not discernible by quantitation of the separated bases, significant changes in the methylation status of the genome could occur but remain undetected by standard analytical methods. Some CpG sites can be examined individually for m^5C through the use of methylation-sensitive restriction endonucleases in a Southern blotting protocol, but only if the CpG sites happen to lie within the recognition sequence of one of the small number of suitable enzymes and the appropriate cloned DNA is available for use as a hybridization probe (21, 39). Only a handful of sites, representing a fraction of total CpG sites even in a short stretch of DNA, can be examined in this way. The minute sample size and inherent bias toward CpG sites with defined 3′ and 5′ neighboring nucleotides mean that this method cannot be used to gain information about the methylation status of the genome as a whole. Comparison of the length distributions of bulk cellular DNA cleaved with methylation-sensitive restriction endonucleases is another method which has been used to estimate extent of methylation. This method suffers from a lack of sensitivity and has sometimes given conflicting results when applied to vertebrate DNA (11, 30).

To gain information about the relationship of DNA methylation and cellular differentiation, we have applied sensitive and specific methods to determine the methylation status of the genomes of differentiating F9 murine embryonal carcinoma and Friend murine erythroleukemia (MEL) cells. The F9 cell line was derived from a murine teratocarcinoma and can be induced to differentiate into parietal endoderm by treatment with low concentrations (ca. 10^{-7} M) of retinoic acid (37). The induced cells have a distinctly different morphology and synthesize a number of protein species not present in uninduced cells. MEL cells are virus-transformed erythroid precursor cells which express erythroid characteristics (including hemoglobin synthesis) upon treatment with dimethyl sulfoxide (DMSO) or a number of other agents (15, 38), including drugs which interfere with nucleic acid methylation (12). Work from Christman et al. has implicated DNA hypomethylation in the differentiation of MEL cells (10). We report here that substantial hypomethylation of the genome accompanied the terminal differentiation of both F9 and MEL cells.

MATERIALS AND METHODS

Cell culture. MEL cells were the PC4 subclone of line 745a and were obtained from D. Housman of Massachusetts Institute of Technology. F9 murine embryonal carcinoma cells were obtained from L. Gudas of the Dana-Farber Institute and were propagated as previously described (37). MEL cells were induced to differentiate by incubation in 250 mM DMSO, and F9 cells were induced by incubation in 2 ×

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10^{-7} M retinoic acid (Eastman Kodak Co.). Differentiation of MEL cells was assayed by staining for hemoglobin with benzidine (14); 70% of the cells were positive after 4 days of treatment with DMSO. Differentiation of F9 cells was quantitated by immunofluorescent staining with antibodies against the cytoskeletal protein Endo A (31); antiserum was the gift of R. Oshima, La Jolla Cancer Research Institute. After 4 days of growth in 2×10^{-7} M retinoic acid, 75% of the cells contained cytoplasmic filaments which stained for Endo A. In all cases, cells were harvested after 4 days in the presence of inducer, and DNA was isolated by the method of Blin and Stafford (4). DNA concentrations were determined by UV absorption and confirmed by densitometry of photographic negatives of *Sau*96I-cleaved DNA displayed on agarose gels. Conditions for large-scale culture of MEL cells and for purification of DNA methyltransferase have been described in detail elsewhere (2).

Quantitation of C and m^5C at DNA termini. A modified version of the methods of Christman (9) and Cedar et al. (6) was used to determine the methylation status of *Msp*I (CCGG) and *Taq*I (TCGA) restriction endonuclease recognition sites. Samples (5 μg) of DNA were digested to completion under the conditions recommended by the suppliers (New England Biolabs and Bethesda Research Laboratories, Inc.), recovered by phenol extraction and ethanol precipitation, and labeled at 5′ termini with [γ-^{32}P]ATP and polynucleotide kinase (1). Unincorporated isotope was removed by exclusion chromatography on columns of Sephadex G-50 run in 0.02 M Tris-hydrochloride (pH 7.4)–1 mM EDTA–0.1 M NaCl. DNA in the void volume was precipitated with ethanol and dissolved in 20 μl of 0.05 M sodium acetate (pH 5.2). After the addition of nuclease P_1 (P-L Biochemicals) to 100 μg ml^{-1} and incubation at 50°C for 2 h, the samples were lyophilized, and the residues were dissolved in 10 μl of water and spotted in duplicate on cellulose thin-layer chromatography plates (20 by 20 cm; Brinkmann Instruments Inc.) which were developed in isobutyric acid-NH_4OH-water (66:20:1). The plates were autoradiographed, and the autoradiogram was used to locate radioactive spots. These were scraped from the plates, and radioactivity was measured by liquid scintillation spectrometry. A correction factor for the labeling of random DNA termini was calculated as described previously (9).

Methyl-accepting assays. These were performed essentially as previously described (2), with 1 μg of DNA and 10 U of DNA methyltransferase in a total volume of 100 μl [1 U catalyzes the transfer of 1 pmol of CH_3 to 1 μg of poly(dG-dC) · poly(dG-dC) in 1 h at an *S*-adenosylmethionine concentration of 1.2 μM]. Under the conditions used here, 1 pmol of CH_3 gave 8.9×10^3 cpm. The DNA methyltransferase used here had been purified through the DEAE-Sephacel step and was free of DNA-independent methyltransferase activity. Reactions were stopped by the addition of 2 volumes of ice-cold 1 M NaCl, and trichloroacetic acid-insoluble radioactivity was determined as described previously (2).

The qualitative nature of methyl-accepting activities within the various complexity classes of genomic DNA was evaluated by gel electrophoresis and fluorography of radioactively methylated restriction fragments. Enzymatic methylation was carried out as described above, the DNA was digested to completion with restriction endonuclease, and the fragments were electrophoretically separated on 1.8% agarose gels. Radioactive DNA was visualized by equilibration of the gel with 1.5 M sodium salicylate (7) in methanol, drying under vacuum, and exposure to prefogged Kodak XAR film (26). Liquid scintillation counting of gel slices showed that film darkening was proportional to the amount of radioactivity under the conditions used. This method of fluorography avoids the diffusion of small fragments that can occur during equilibration of gels with aqueous scintillants, and the gels do not crack during drying as do those prepared with diphenyloxazole in methanol (26).

Number average sizes of DNA fragments produced by cleavage of mouse DNA with restriction endonucleases. End-labeled DNA fragments were prepared as described above and electrophoresed through 1.8% agarose gels. An autoradiogram was prepared from the dried gel, and the distribution of radioactivity was determined by densitometry of the film. The midpoint of the distribution was determined and referred to a plot of distance $migrated^{-1}$ versus size for DNA fragments contained in the "kb ladder" DNA size standards (Bethesda Research Laboratories) and run on the same gel as the restriction fragments. By this method, the number average sizes of *Msp*I and *Taq*I fragments of mouse DNA were found to be 1,800 and 1,950 base pairs (bp), respectively.

RESULTS

Demethylation of CCGG and TCGA sites in differentiating F9 and MEL cells. The 5′-terminal nucleotides produced by cleavage of DNA with *Msp*I or *Taq*I are mixtures of C and m^5C, since each enzyme is insensitive to methylation at CpG dinucleotides within their recognition sites (28), although *Msp*I will not cleave the sequence GGCm^5CGG (5, 25). This makes possible an examination of the methylation status of their respective recognition sequences (CCGG and TCGA) by labeling the 5′ ends of restriction fragments with ^{32}P, hydrolyzing the DNA to 5′ dNMP's, and measuring the radioactivity in the dCMP and dm^5CMP fractions after separation by thin-layer chromatography (6, 9). Figure 1 shows the terminal nucleotides produced by cleavage of F9 cell DNA by *Msp*I and its methylation-sensitive isoschi-

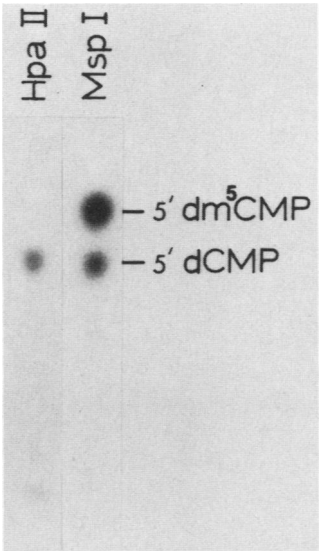

FIG. 1. Terminal deoxynucleotides produced by cleavage of mouse DNA with the restriction endonuclease *Msp*I and its methylation-sensitive isoschizomer *Hpa*II. Endonuclease cleavage, end labeling, hydrolysis to 5′ dNMPs, and thin-layer chromatography were carried out as described in the text. Minor spots represent unincorporated isotope and labeling at random breaks in the DNA; a correction factor was applied to control for labeling at such breakage points as described by Christman (9).

TABLE 1. Change in methylation status of *Msp*I (CCGG) and *Taq*I (TCGA) sites in differentiating F9 and MEL cells[a]

Cell	% of *Msp*I sites methylated	% of *Taq*I sites methylated
F9		
Uninduced	62.7	63.7
Retinoic acid treated	54.5	52.8
MEL		
Uninduced	69.7	52.7
DMSO treated	65.8	49.1

[a] Data were obtained by the end-labeling protocol of Cedar et al. (6) and Christman (9) as described in the text.

zomer *Hpa*II; *Msp*I produced fragments with 5'-terminal dCMP and dm^5CMp, whereas *Hpa*II fragments had only dCMP as 5'-terminal nucleotide.

This method was used to determine the methylation status of the CpG sites in CCGG and TCGA sequences during differentiation of F9 and MEL cells. The DNA of induced F9 cells contained (± standard error) 8.2 ± 1.7% fewer methylated CCGG sites and 10.9 ± 3.0% fewer methylated TCGA sites relative to the DNA of uninduced F9 cells (Table 1). Induced MEL cell DNA contained 3.9 ± 0.9% fewer methylated CCGG sites and 3.6 ± 1.2% fewer methylated TCGA sites than did the DNA of uninduced cells. All differences were significant at $P = 0.05$. Differences in extents of methylation of the two types of sites also existed between uninduced F9 and MEL cells; F9 cells contained 7.0% fewer methylated CCGG sites but 11.0% additional methylated TCGA sites as compared with uninduced MEL cells (Table 1). In the present study, 69.7% of all CCGG sites in uninduced MEL cell DNA were found to be methylated; this agrees very well with the value of 70.8% obtained by Christman (9), who used a very similar method.

Since *Msp*I cleaves mouse DNA to fragments of an average size of 1,800 bp and *Taq*I produces fragments of an average size of 1,950 bp, there must be ca. 1.3×10^6 CCGG sites and 1.2×10^6 TCGA sites per haploid mouse genome, which numbers 2.3×10^9 bp (27). From these figures and the data of Table 1, it can be calculated that ca. 1.1×10^5 CCGG sites and 1.3×10^5 TCGA sites are demethylated during F9 cell differentiation and 5.1×10^4 CCGG sites and 4.3×10^4 TCGA sites are demethylated during MEL cell differentiation (all numbers refer to the haploid mouse genome).

Differentiation of F9 and MEL cells is accompanied by an increase in the methyl-accepting activity of their DNA. Given the apparent lack of dependence on sequence context of CpG sites displayed by mammalian DNA methyltransferase (2, 19), the methyl-accepting activity of a DNA sample should be inversely proportional to the m^5C content of that DNA. An assay based on this concept has been used previously to demonstrate demethylation of DNA during MEL cell differentiation (10).

The time courses for in vitro methylation of F9, MEL, and *Escherichia coli* DNA and poly(dG-dC) · poly(dG-dC) by MEL cell DNA methyltransferase are shown in Fig. 2. It is clear from these data that the DNA of induced F9 and MEL cells had greater methyl-accepting activity than did DNA from the corresponding uninduced cells, indicating that

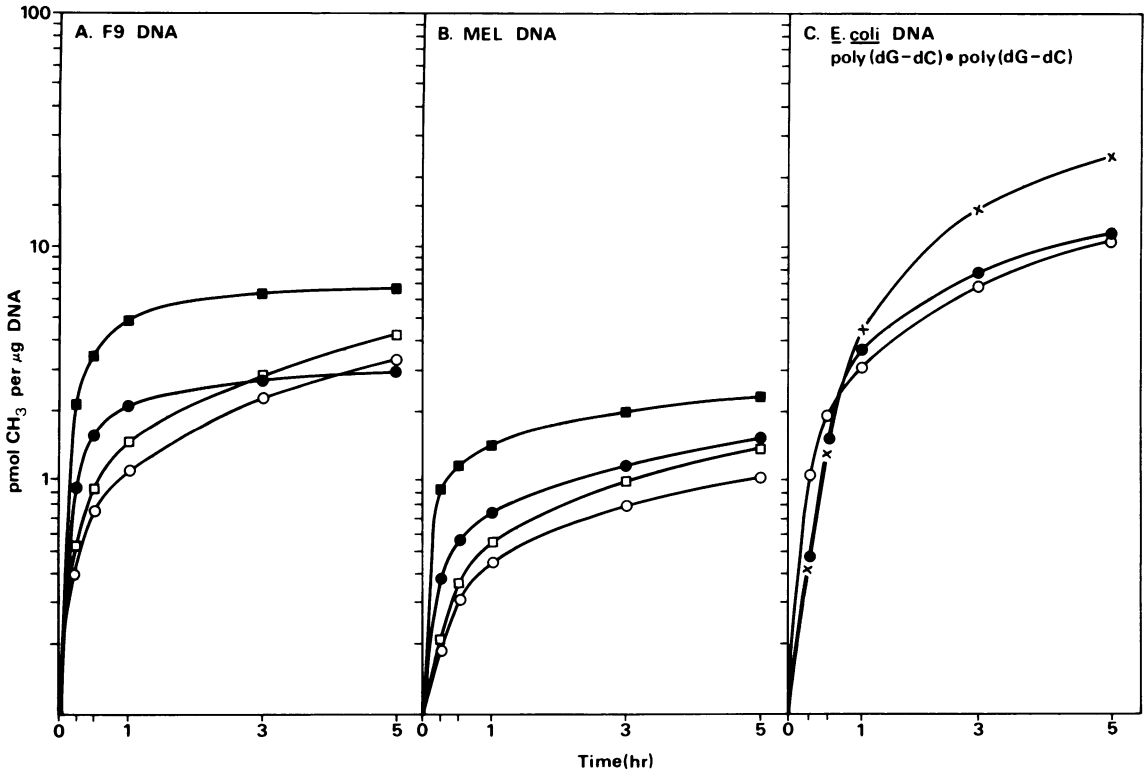

FIG. 2. Time course of enzymatic methylation of DNA from different sources. The scale along the ordinate is logarithmic. In A and B, ■ and □ indicate DNA from induced cells, and ● and ○ represent DNA from uninduced cells. Open symbols indicate DNA that had been heat denatured by incubation at 100°C for 10 min. In C, ● and ○ represent native and denatured *E. coli* DNA, and × represents incorporation into poly(dG-dC) · poly(dG-dC). Notice the very rapid initial rate of methylation of DNA from induced F9 and MEL cells in A and B and the very different kinetics of methylation of native and denatured mouse DNA samples. The points shown are means of duplicate determinations for a typical experiment.

unmethylated CpG sites had accumulated in the DNA of the induced cells. It might be possible to saturate all CpG sites with methyl groups and to use the differences in methyl-accepting capacity of different DNAs as a direct measure of differences in contents of methylated CpG sites, but unfortunately it has recently been demonstrated that substantial incorporation of methyl groups into non-CpG sequences can occur when small amounts of DNA are incubated with large amounts of mammalian DNA methyltransferase for extended periods (18). Under the conditions of the present study, rates of DNA methylation are directly proportional to CpG contents of double-stranded DNA (2). If the differences in methyl-accepting activity at early times (5 h) can be presumed to approximate the differences at saturation, rough quantitative estimates of differences in content of methylated CpG sites can be made. In this way, it has been calculated from the data of Fig. 3 that 10.5 ± 0.98 and 2.2 ± 0.44% of all CpG sites were demethylated during the differentiation of F9 and MEL cells, respectively. These values are likely to be minimum estimates, since continued divergence of the *methyl*-^{3}H incorporation curves would be expected to occur until a maximum difference is reached at saturation. Since high-pressure liquid chromatography measurements of the m^5C content of MEL cell DNA suggest that ca. 81% of all CpG sites in this DNA are methylated (9) and because the *methyl*-^{3}H content of MEL cell DNA after 5 h of enzymatic methylation indicated that ca. 35% of the remaining CpG sites were methylated in vitro (Fig. 2), these quantitative estimates were made from data taken while the reactions were about one-third of the way to saturation.

Qualitatively, measurements of methyl-accepting activity confirmed the more pronounced demethylation of F9 cell DNA during differentiation (as compared with MEL cells) and showed that the uninduced F9 cell genome is hypomethylated relative to that of MEL cells (Fig. 2).

Inspection of the kinetics of enzyme-catalyzed methyl transfer revealed that double-stranded DNA from differentiated F9 or MEL cells accepted methyl groups at an initial rate which was greater than that of poly(dG-dC) · poly(dG-dC), which contained 500 CpG sites per kb; mouse DNA contains only 11, most of which are already methylated. The rate of methylation of double-stranded F9 and MEL DNA abruptly diminished after 30 to 60 min, whereas methylation of the other DNAs and of denatured F9 and MEL DNA continued at a high rate through 7 h (Fig. 2). These data suggest that two kinetic classes of methylation sites exist in double-stranded mouse DNA, whereas single-stranded mouse DNA, *E. coli* DNA, and poly(dG-dC) · poly(dG-dC) contain only one.

The kinetics of methylation of heat-denatured mouse DNA closely resembled those of *E. coli* DNA and poly(dG-dC) · poly(dG-dC); the rapidly methylated sites appeared not to be present in denatured DNA. This can be seen more clearly when the data are presented in the form of first-order plots (Fig. 3). Mammalian DNA methyltransferase has been shown previously to be greatly stimulated by hemimethylated DNA substrates (2, 19), and it is likely that the rapidly methylated sites observed here in double-stranded DNA were hemimethylated sites.

The rates of enzymatic DNA methylation of all DNAs had similar values at reaction times >1 h (Fig. 3). The dotted lines show the extrapolated rate obtained by extending the reaction rate at >1 h to zero time. The content of rapidly methylated (hemimethylated) sites was estimated by computing the difference between actual measured methyl incorporation and predicted methyl incorporation without the rapid initial phase. This quantity was calculated from the *y* intercept of the dotted line in Fig. 3. In this way, the approximate contents of hemimethylated sites were found to be 5.35 ± 0.55 and 11.7 ± 0.2% of all CpG sites in uninduced and induced F9 cells, respectively, and 0.65 ± 0.3 and 3.1 ± 0.1% in uninduced and induced MEL cells. These values must be regarded as rough approximations due to the lack of a method for quantitating hemimethylated sites with confidence.

Satellite DNA is a major methyl-accepting compartment within the mouse genome. The major species of satellite DNA in the mouse genome is a 234-bp repeating unit present in ca.

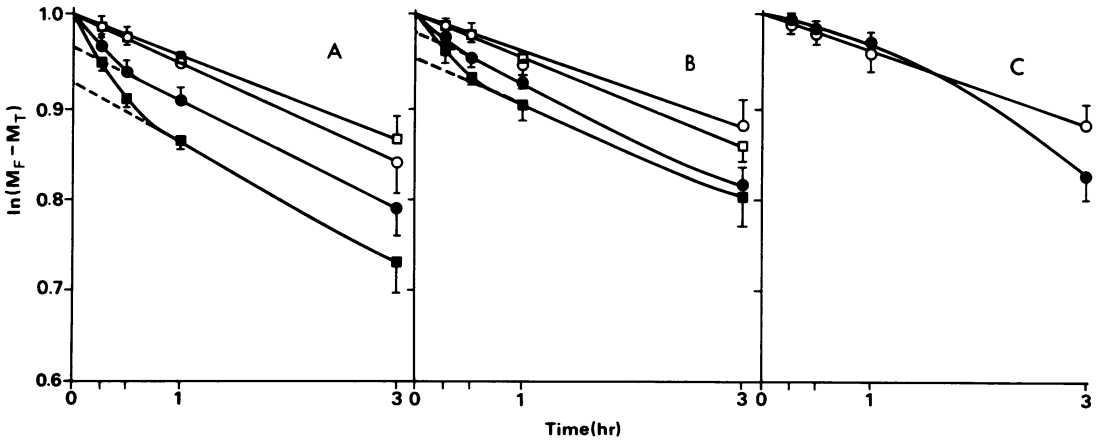

FIG. 3. Analysis of F9 (A), MEL (B), and *E. coli* (C) DNA methylation kinetics by presentation as first-order plots. The ordinate represents the natural logarithm of the differences between *methyl*-^{3}H incorporation at 5 h and incorporation at the times indicated on the abscissa; all data have been normalized with respect to zero time to facilitate comparison of curve shape. Symbols are the same as those in Fig. 2. Notice the distinctly biphasic nature of the curves for native mouse cell DNA in A and B and the straight lines for denatured mouse cell DNA. The dotted lines in A and B are extrapolated segments used to estimate contents of rapidly methylated (hemimethylated) sites as described in the text. In C, a distinct lag period is seen with native *E. coli* DNA; a similar lag was seen when poly(dG-dC) · poly(dG-dC) was used as methyl acceptor. The nature of this lag is not known. The rate of methylation of denatured *E. coli* DNA was very similar to those of denatured F9 and MEL cell DNAs and to native F9 and MEL cell DNAs at times >1 h. Error bars indicate standard errors for four determinations, each with duplicate points.

10^6 copies per cell and constituting 5 to 10% of the total DNA (23). The presence of a centrally located *Sau*96I recognition site in most of the repeating units means that this satellite DNA will form a series of bands on agarose gels which are plainly visible upon staining with ethidium bromide after cleavage of DNA with this enzyme (Fig. 4). The sequence of this DNA is known; there are eight CpG sites per 234-bp repeat unit, giving a CpG density about threefold higher than the genome-wide average (22).

This species of stellite DNA is especially heavily labeled when mouse DNA is incubated with MEL cell DNA methyltransferase and *S*-[*methyl*-^{3}H]adenosylmethionine (Fig. 4). Densitometry of the fluorograms revealed that the four most prominent bands (representing 1, 1.5, 2, and 3 U of the basic repeat) together contain 25 to 35% of the total incorporated radioactivity. Several larger satellite DNA bands were present as a result of sequence degeneracy but could not be distinguished from background with enough accuracy to allow quantitation. As a result, the value of 25 to 35% is a minimum estimate of the fraction of unmethylated CpG sites which reside in mouse satellite DNA.

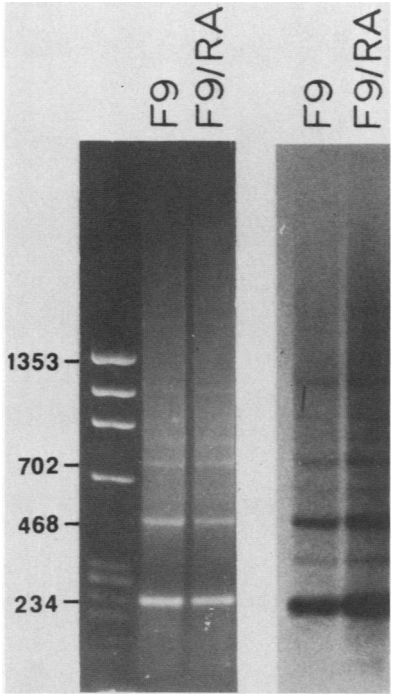

FIG. 4. Distribution of *methyl*-^{3}H among *Sau*96I restriction endonuclease fragments of F9 cell DNA. Samples (5 μg) of intact DNA were incubated for 2 h with 10 μCi of *S*-[*methyl*-^{3}H]adenosylmethionine and 50 U of MEL cell DNA methyltransferase and then cleaved with *Sau*96I, and the fragments were separated by electrophoresis through a 1.8% agarose gel. The left panel is a photograph of the ethidium-stained gel and the right panel is a fluorogram of the same gel. Positions of monomeric, dimeric, and trimeric versions of the 234-bp satellite DNA repeat unit are shown along the left side of the figure. DNA size standards in the leftmost lane are *Hae*III fragments of Φ X174 replicative form DNA. DNA from retinoic acid-induced F9 cells shows elevated levels of *methyl*-^{3}H labeling (relative to DNA from uninduced cells) throughout the distribution of DNA fragments. Notice that the satellite DNA bands have incorporated especially high levels of *methyl*-^{3}H. Similar results were obtained when DNA from induced and uninduced MEL cells was analyzed in the same fashion, with the DNA of induced cells again showing greater methyl-accepting activity (data not shown).

Densitometer sensitivity was adjusted to normalize densitometric scans of autoradiograms with respect to the area under the 234-bp monomer peak. It could then be seen that increased methyl incorporation had occurred throughout the spectrum of fragments prepared by *Sau*96I cleavage of induced F9 cell DNA (Fig. 5). This indicates that demethylation of sites throughout the genome occurred during induced differentiation. When methylated DNA was cleaved with *Eco*RI or *Bam*HI, the distribution of radioactivity was found to parallel the distribution of DNA fragments as assessed by comparison of fluorograms and photographs of the ethidium bromide-stained gels (data not shown.) Neither *Msp*I nor *Taq*I cleaved mouse DNA in such a way as to generate prominent bands when the DNA fragments were displayed on agarose gels, indicating that the end-labeling protocol for m^5C quantitation described above did not preferentially measure the methylation status of satellite DNA sequences.

DISCUSSION

The in vitro differentiation of F9 embryonal carcinoma and MEL cells was found to be associated with demethylation of their DNA, as reflected both by a reduction in the percentage of methylated CCGG and TCGA sequences and by increases in methyl-accepting activity in DNA isolated from differentiated cells. Increased methyl-accepting activity of MEL cell DNA has been reported to accompany DMSO-induced differentiation (10). It has also been reported that embryonal carcinoma cells lines do not show a decrease in m^5C content during in vitro differentiation (36); however, a decrease in m^5C content of less than ca. 10% would not have been detectable by the methods used.

Demethylation seems to occur throughout the genome during cellular differentiation; inspection of the distribution of radioactivity obtained when *methyl*-^{3}H-labeled restriction fragments of DNA were separated on agarose gels revealed that the distribution of label largely paralleled the distribution of DNA, with the major species of mouse satellite DNA (the *Sau*96I repeat) constituting a major methyl-accepting compartment in the DNA of both induced and uninduced cells. The high CpG density of mouse satellite DNA (22) means that it can appear hypermethylated when m^5C contents are measured (16, 34) but can be seen to be hypomethylated when methyl-accepting activity is the criterion.

Hemimethylated sites appear to accumulate in the DNA of differentiating F9 and MEL cells. Analysis of the kinetics of enzyme-catalyzed methyl transfer revealed that native mouse DNA contains a class of rapidly methylated sites which are not present in denatured mouse DNA, *E. coli* DNA, or poly(dG-dC) · poly(dG-dC). Since MEL cell DNA methyltransferase has been shown to be largely unaffected by the sequence context of CpG sites but stimulated 30- to 100-fold by hemimethylated DNA substrates (2, 19), it is probable that the rapidly methylated sites present only in native mouse DNA are hemimethylated sites. Separation of the slow and fast classes of methyl-accepting sites by extrapolation of kinetic data allowed a rough calculation of the content of presumed hemimethylated sites; uninduced F9 and MEL cells had 5.3 and 0.6% of their CpG sites in the hemimethylated state, respectively, whereas the corresponding values for induced cells were 11.7 and 3.1%.

Hemimethylated sites in mammalian DNA have not been described before, but their existence was to be expected since DNA methylation has been previously shown to lag behind DNA replication by 1 to 10 min in mouse L cells (20), in which replication forks move at a rate of ca. 2 kb min^{-1} (24). DNA methylation thus occurs in a region 2 to 20 kb

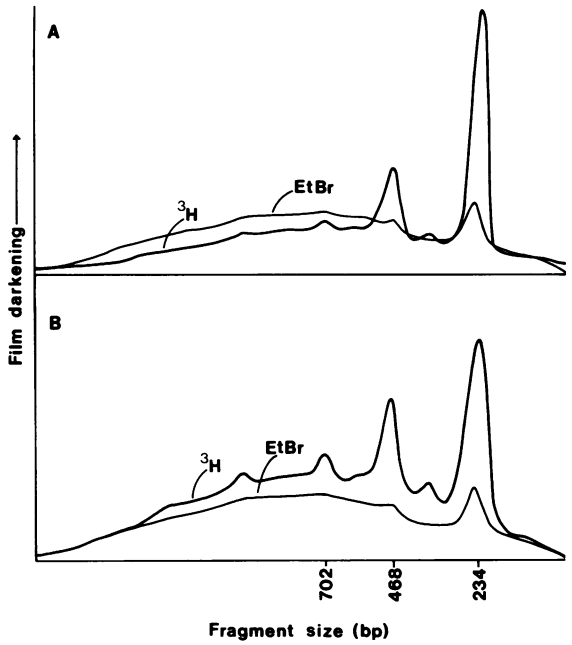

FIG. 5. Densitometer tracings of ethidium negatives and fluorograms. The photographic negatives of the ethidium bromide (EtBr)-stained gels and the fluorograms shown in Fig. 4 were scanned in an Ortec model 4310 densitometer. Weighing of peaks cut from densitometer tracings indicated that 25 to 35% of the incorporated radioactivity in DNA from both induced and uninduced cells comigrated with the satellite DNA bands; for reasons discussed in the text, this is a minimum estimate. Adjustment of densitometer sensitivity was used to normalize the tracings with respect to the area under the 235-bp monomeric satellite repeat unit; increased incorporation of radioactivity can be seen to have occurred among longer, presumably nonsatellite DNA fragments. There was actually twice as much radioactivity in the lane containing DNA from induced F9 cells because of the higher methyl-accepting activity of that DNA. (A) F9; (B) F9/RA.

behind the replication fork. Since this distance is small relative to the size of DNA replicons, at most 5 to 10% of the replicating DNA in an S-phase cell would be expected to exist in the hemimethylated form as a consequence of lagging DNA methylation. The values reported here (5.3 and 11.7% of total sites in uninduced and induced F9 cells, 0.6 and 3.1% in uninduced and induced MEL cells) seem high in view of the calculated upper limit of 5 to 10%. MEL cells accumulate in the G1 phase of the growth cycle upon induction (14), and F9 cells grow only very slowly after 4 days of treatment with retinoic acid (37; unpublished data); for this reason, the presumptive hemimethylated sites probably do not represent newly replicated DNA present when the cells were lysed for DNA isolation. It may be that terminally differentiating cells suffer a partial loss of their ability to methylate DNA during their last cycles of replication or that methylation lags unusually far behind replication in these differentiating cells. Demethylation of DNA during differentiation would not seem to be a random process (as would be expected if demethylation were the result of DNA methyltransferase activity becoming limiting) since tissue-specific methylation patterns are found (35) and because of specific differences in the methylation status of TCGA and CCGG sequences in differentiating F9 and MEL cells (Table 1).

Lastly, the relationship of F9 cells to other lines of EC cells and to cells of primitive ectoderm of the embryo is not clear. F9 cells are known to be capable of forming only derivatives of extraembryonic endoderm, although many lines of EC cells are, like primitive ectoderm, capable of forming many tissue types. Recent work indicates that cell lineage-specific differences in the degree of methylation of *Hpa*II sites in satellite DNA have been established as early as day 4.5 of gestation in mouse embryos, with extraembryonic tissues (trophectoderm and primitive endoderm) being hypomethylated with respect to primitive ectoderm (8). The methyl-accepting assays described in this report showed that F9 cell DNA appeared to be hypomethylated with respect to MEL cell DNA, and *Hpa*II sites within single-copy DNA sequences of F9 cells are hypomethylated with respect to germ cell DNA (32) and presumably to primitive ectoderm as well. Thus, F9 cells display both the biological characteristics and DNA methylation patterns of a committed cell type which has acquired a conditional blockade to differentiation at an early stage in the pathway leading to the formation of extraembryonic endoderm.

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