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Induction of α-Fetoprotein Synthesis in Differentiating F9 Teratocarcinoma Cells Is Accompanied by a Genome-Wide Loss of DNA Methylation

PETER R. YOUNG† AND SHIRLEY M. TILGHMAN*

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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F9 teratocarcinoma cells can be grown as monolayers or aggregates, and upon treatment with retinoic acid they will differentiate into parietal or visceral endoderm, respectively. Visceral endoderm specifically synthesizes α-fetoprotein and albumin mRNAs, which are not found in parietal endoderm. In contrast, both endoderms produce enhanced levels of the major histocompatibility antigen (H2) mRNA compared with F9 cells. F9 cells contain highly methylated DNA as judged by restriction enzyme digestion. However, upon differentiation into visceral endoderm, there is a genome-wide loss of methylation in induced, silent, and constitutively expressed genes. Experiments in which methylation loss is induced via the methyltransferase inhibitor 5-azacytidine result in no induction of α-fetoprotein mRNA and no morphological differentiation, suggesting that methylation loss alone is not sufficient to induce the visceral endoderm phenotype. Likewise, 5-azacytidine treatment of differentiated cells does not result in enhanced expression of α-fetoprotein mRNA. However, the patterns of loss of DNA methylation at all sites examined after differentiation or 5-azacytidine treatment were remarkably similar, suggesting that the two occur by a similar mechanism, the inhibition of DNA methyltransferase activity. These results argue that the specificity for methylation loss at a given site is an inherent property of aggregated F9 cell chromatin. This system provides a model for studying a tissue-specific change in DNA methylation upon differentiation.

The capability of teratocarcinoma cells (EC cells) to differentiate in a controlled manner in culture provides a model system for studying early events of mammalian development. Specifically, the cell line F9, in the presence of retinoic acid, has been shown to differentiate in vitro into cells with the biochemical characteristics of parietal or visceral endoderm, depending upon the culture conditions (24, 28, 55, 56). In this way it mimics one of the early commitment events that occur in the 3- to 5-day mouse blastocyst when the inner cell mass forms these two endodermal cell types (15, 54).

Parietal and visceral endoderm cells can be distinguished in part by their gene products. In particular, α-fetoprotein (AFP), which is the most abundant serum protein of a developing fetus (45), is made only in visceral endoderm cells (13). The F9 cells maintain this specificity upon differentiation in vitro (28) and thus allow us to study the molecular events involved in both induction of the AFP gene and its developmental specificity.

We have previously cloned and characterized the mouse AFP gene (19, 20, 33) and have shown that it is encoded on chromosome 5 of the mouse (11), directly adjacent to the evolutionarily related serum albumin gene (30). In a previous study, we argued that these genes are activated in concert during differentiation of fetal liver but are independently modulated once they have been activated (60). In this paper, we describe their expression upon the differentiation of F9 cells. In addition, we examine the role that DNA methylation plays in their induction. Several studies (12, 16, 27, 42, 51, 53) have suggested that DNA methylation is important in the repression of retroviral gene expression in early embryos and teratocarcinoma cells. It is of interest, therefore, to ask whether cellular genes that are inducible upon differentiation utilize similar control mechanisms.

* Corresponding author.
† Present address: Department of Molecular Genetics, Smith Kline & French Laboratories, Philadelphia, PA 19101.

MATERIALS AND METHODS

Cells and culture conditions. F9 cells were a gift from E. Adamson (La Jolla Cancer Research Foundation, La Jolla, Calif.) and were grown as stem cells in 10% fetal bovine serum (GIBCO Laboratories) and Dulbecco modified Eagle medium (GIBCO). For differentiation to parietal endoderm, freshly seeded F9 cells in the same serum and medium were treated with 10^{-3} M dibutyryl cyclic AMP (Sigma Chemical Co.) and 10^{-7} M all-*trans* retinoic acid (Eastman) (regularly prepared as a 10^{-3} M stock in 95% ethanol and stored at 4 or −20°C) for 3 days (56) and then for 1 or 2 days more with 10^{-7} M retinoic acid. For differentiation to embryoid bodies, F9 cells were seeded at 10^6 cells into 100-mm bacteriological petri dishes and treated with 7.5×10^{-8} M retinoic acid (optimal for AFP mRNA induction) in 10% fetal bovine serum and a 50:50 mixture of Dulbecco modified Eagle medium and Ham F12 medium (GIBCO) (24). The medium was changed on days 3, 5, 6, and usually every day subsequently, with replenishment of retinoic acid unless otherwise stated. 5-Azacytidine (5-azaC; Sigma) was added to cells from a 0.1 mM stock in phosphate-buffered saline stored at −20°C.

Exponentially growing F9 monolayers treated with or without 10^{-7} M retinoic acid were infected with simian virus 40 virus stock prepared from BSC-1 cells at a multiplicity of 3:1 by washing the cells in phosphate-buffered saline, incubating them at 37°C with 2 to 3 ml of virus stock for 2 h, and then adding 2% fetal bovine serum in Dulbecco modified Eagle medium and further incubating at 37°C for 48 to 72 h before harvesting.

Preparation of genomic DNA and RNA. Genomic DNA was isolated from nuclei prepared by the citric acid method

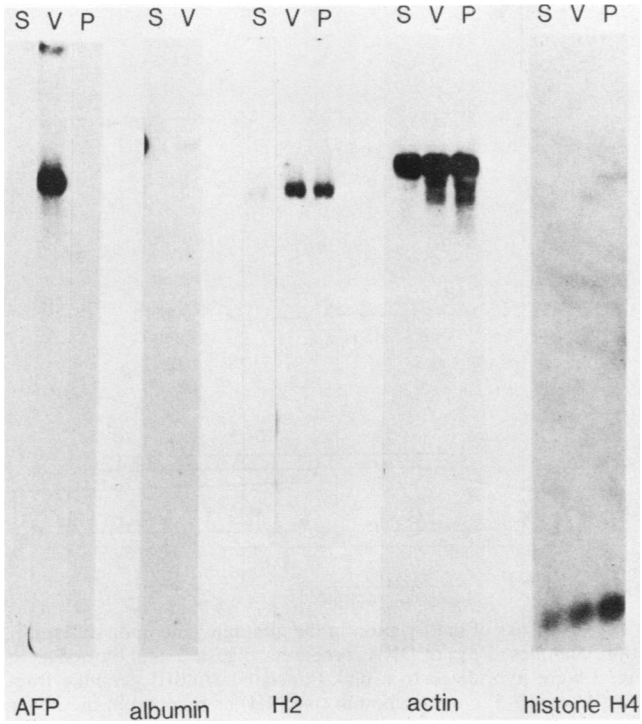

FIG. 1. Northern blot of poly(A)$^+$ RNAs isolated from undifferentiated and differentiated F9 cells. Poly(A)$^+$ RNA was isolated from untreated F9 cells (S, stem), or from cells treated for 5 days in monolayers with 10^{-7} M retinoic acid (P, parietal endoderm), or for 17 days as aggregates with 7.5×10^{-8} M retinoic acid to form embryoid bodies (V, visceral endoderm). RNA (5 to 10 μg) was electrophoresed in 1.5% formaldehyde-agarose gels, blotted onto nitrocellulose, and hybridized with the labeled DNAs listed at the bottom of the figure: the AFP cDNA pmAFP3, the albumin cDNA pmalb 2, the H2 cDNA pMHC-1, the actin cDNA pAM91, and the histone H4 genomic clone pBR-mus-hi-1-H4-*Hin*fI.

(3). Cells or aggregates from 5 to 10 plates (trypsinized if necessary for detachment) were washed in phosphate-buffered saline, spun down, and suspended in 5% citric acid. They were then disrupted in 5% citric acid with a "B" pestle and passed through gauze, and nuclei were purified by centrifugation at 3,500 rpm through a 0.88 M sucrose gradient still in 5% citric acid. Nuclei were then neutralized by gentle suspension in RSB buffer (10 mM NaCl–10 mM Tris-hydrochloride [pH 7.5]–25 mM EDTA) and subsequent centrifugation at 2,000 rpm at least two times until the pH was no longer acidic. The nuclei were then lysed by the addition of sodium dodecyl sulfate to a final concentration of 1%, followed by digestion with 1 mg of proteinase K (Sigma) per ml for at least 1 h. NaCl was then added to 0.5 M, and the solution was extracted twice with phenol-chloroform-isoamyl alcohol (20:20:1), ether extracted, and precipitated with 2 volumes of ethanol. The pellet was suspended overnight in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and digested with 20 μg of boiled RNase A per ml for 1 h. The solution was then reextracted with phenol-chloroform and ether, precipitated with ethanol, and suspended overnight in 10 mM Tris-hydrochloride (pH 7.5)–0.1 mM EDTA. All genomic DNA samples were digested with 2 to 3 U of any restriction enzyme for 2 to 3 h at 37°C, using buffer conditions recommended by the supplier (New England Biolabs). To check for complete digestion with *Hpa*II, picogram quantities of plasmid DNA were sometimes included or enzyme concentrations were doubled with no effect on the hybridization profile. All results were reproducible in different digestions.

RNA was prepared from 5 to 10 plates per time point of washed F9 cells by the hot phenol method (47) and enriched for polyadenylated [poly(A)$^+$] RNA via oligodeoxythymidylate-cellulose chromatography (2). The oligodeoxythymidylate-cellulose was obtained from Collaborative Research, Inc.

Electrophoresis, blotting, and hybridization to labeled probes. RNA samples were denatured by heating for 3 min at 60°C in 60% (vol/vol) formamide, 7% (vol/vol) formaldehyde, 1× MOPS buffer (40 mM morpholinopropanesulfonic acid [pH 7.0]–10 mM sodium acetate–1 mM EDTA) and run in a 1.5% agarose gel containing 6% formaldehyde with 1× MOPS as the running buffer (18). This was blotted onto 0.45-μm nitrocellulose (Millipore Corp.) in 10× SSC by the method of Thomas (59). RNA dot blots (59) were made with a Schleicher & Schuell apparatus and a 0.45-μm nitrocellulose filter (Millipore) wetted in 20× SSC. DNA electrophoresis was in agarose gels, and transfer was by the method of Southern (52). All filters were hybridized to nick-translated DNA fragments (37) purified via electroelution in agarose gels or cloned plasmids labeled with [α-^{32}P]dCTP obtained from New England Nuclear Corp. (38). Prehybridization, overnight hybridization, and subsequent washing (0.1× SSC–0.1% sodium dodecyl sulfate at 52°C) were by the method of Wahl et al. (63).

Autoradiograms of hybridized blots were scanned with an E-C Apparatus Corp. densitometer connected to a Hewlett-Packard 33805 integrator.

DNA probes. The mouse H2 cDNA clone pMHC-1 was obtained from J. Seidman (14); mouse α-actin cDNA pAM91 was from A. Minty (40); mouse genomic histone H4 (pBR-

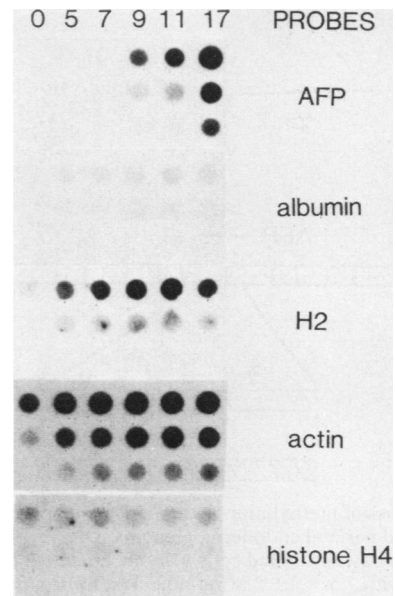

FIG. 2. Time course (days indicated at top) of RNA expression in F9 cells upon differentiation to visceral endoderm. Poly(A)$^+$ RNA was prepared from F9 cells grown in monolayers (day zero) or upon diffentiation with retinoic acid in bacteriological petri dishes for the times shown. Then 1.6 μg of each RNA and threefold dilutions were dotted sequentially onto nitrocellulose (59) and hybridized to nick-translated cDNAs as shown. See the legend to Fig. 1 for details.

mus-hi-1-H4-*Hin*f1) was from A. Seiler-Tuyns (49); mouse dihydrofolate reductase cDNA (DHFR-11) (43) was from J. Schilling and R. Schimke; β-casein cDNA (pCMβ13) was from J. Rosen (26); and pEC_κ, a *Bam*HI-EcoRI cloned genomic fragment from mouse embryonic C_κ region, was from E. Mather and R. Perry (39). All mouse AFP and albumin probes were prepared from previously described genomic clones (20, 30, 33) or subclones derived from them, and cDNA clones (33, 61).

RESULTS

Specificity of induction of mRNAs during differentiation. F9 cells can be grown indefinitely as monolayers, with only a small amount of morphological differentiation at high cell densities (50). Treatment of monolayer cultures with 10^{-7} M retinoic acid and (optionally) 10^{-3} dibutyryl cyclic AMP results in their differentiation into parietal endoderm cells, as judged by both morphological and biochemical criteria such as the expression of plasminogen activator, a key marker of parietal endoderm cells (56). In contrast, if F9 cells are cultured in the presence of retinoic acid in bacteriological petri dishes to which they cannot attach, they form aggregates that consist of EC cells surrounded by an outer layer of visceral endoderm cells, as evidenced by their synthesis of AFP, a characteristic marker of this cell type (28). These are commonly referred to as embryoid bodies because of their resemblance to an early step of mouse embryogenesis (38).

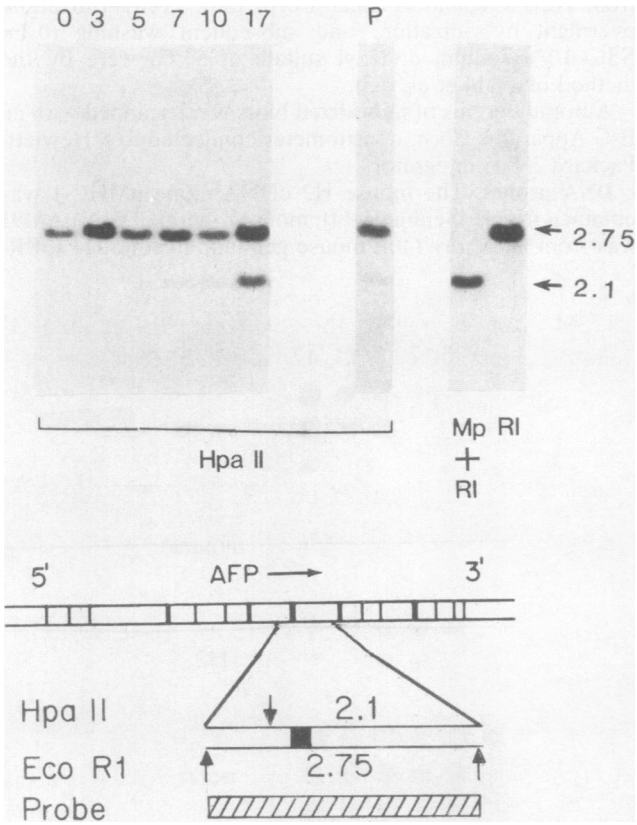

FIG. 3. Loss of methylation in the AFP gene upon differentiation to visceral and parietal endoderm. Genomic DNA was isolated from F9 stem cells (day zero), and cells were incubated in bacteriological petri dishes with 7.5×10^{-8} M retinoic acid for the times shown (in days; lanes 3 to 17) or for 4 days in monolayers with 10^{-7} M retinoic acid and 10^{-3} M dibutyryl cyclic AMP (lane P). Samples were digested with *Hpa*II and *Eco*RI, *Msp*I and *Eco*RI, and *Eco*RI alone as indicated, and 5 μg was electrophoresed in a 0.8% agarose gel, blotted onto nitrocellulose, and hybridized to the nick-translated AFP *Eco*RI genomic fragment, pAFP-C, as mapped in the lower diagram.

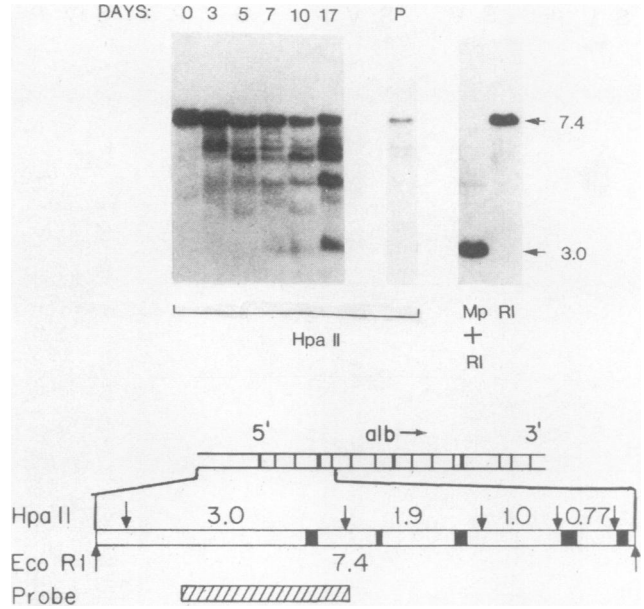

FIG. 4. Loss of methylation in the albumin gene upon differentiation. Samples (5 μg) of DNA, prepared, digested, and blotted as in Fig. 3 were hybridized to a nick-translated *Hin*dIII genomic fragment from the 5′ end of albumin (palbEII) as mapped in the lower diagram.

We wished to ask whether the induction of AFP in visceral endoderm and its absence in parietal endoderm were the consequences of changes at the level of mRNA. Thus, poly(A)$^+$ RNA was prepared from EC cells, and F9 cells differentiated in monolayers or in aggregates. The RNAs were separated on a denaturing gel, blotted onto nitrocellulose, and hybridized to various cDNA clones (Fig. 1). An mRNA that comigrates with authentic AFP mRNA is detected with an AFP cDNA probe only in differentiated aggregates, or visceral endoderm RNA, and is not present in EC cells or parietal endoderm. Apart from confirming the specificity of the differentiation conditions as reported by Hogan et al. (28), these results indicate that the control resides at the level of mRNA production. Recently Grover et al. (23) also observed induction of AFP mRNA upon visceral endoderm formation. Albumin mRNA is also detected only in visceral endoderm RNA, where it is present at very low levels relative to AFP mRNA.

As a control to confirm that differentiation to parietal endoderm was occurring in the monolayers, the major histocompatibility antigen (H2) mRNA levels were measured. Previous studies had shown that H2 antigens and mRNA were not expressed on F9 cells but appeared upon differentiation in monolayers (10, 34, 41). A 2.0-kilobase poly(A)$^+$ mRNA that hybridizes to a cloned H2 cDNA probe is present in both visceral and parietal endoderm in comparable amounts (Fig. 1). However, a low but detectable amount is also present in the EC cells, reflecting a small percentage of spontaneously differentiated cells in the population or a low level of H2 mRNA expression in these cells, or both. As a second criterion for parietal endoderm formation, cells were shown to express simian virus 40 T antigen mRNA

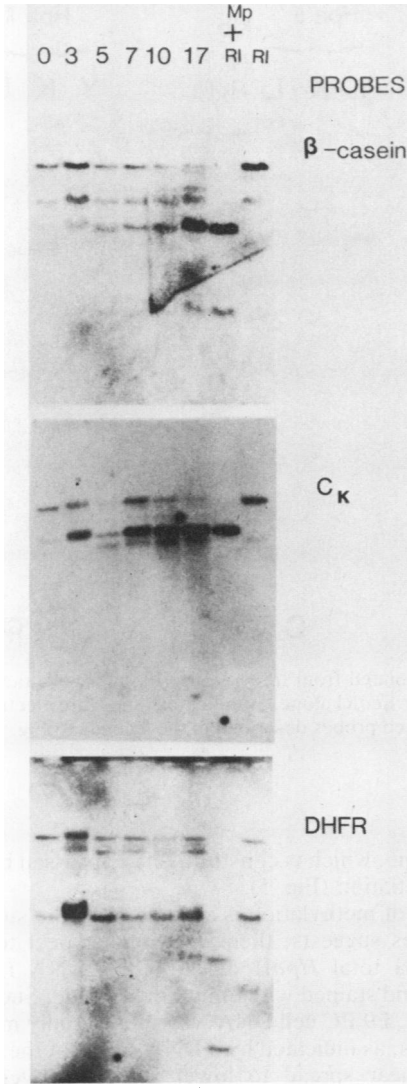

FIG. 5. Loss of methylation in nonregulated genes upon differentiation to visceral endoderm. Samples (5 μg) of DNA, prepared, digested, and blotted as in Fig. 3, were hybridized with the following nick-translated probes: a mouse β-casein cDNA, pCMβ13; a *Bam*HI-*Eco*RI cloned genomic fragment from the mouse germline C_κ gene, pEC_κ; and a mouse dihydrofolate reductase cDNA clone, pDHFR-11. The lowest band in the *Hpa*II-*Eco*RI digests and in the EcoRI digest detected by pEC_κ is a non-reproducible artifact. Numbers at top indicate days of incubation.

after infection with simian virus 40, whereas the EC cells did not, in keeping with the observations of others (36, 48, 57; data not shown).

We also examined the levels of actin mRNA and histone H4 mRNA that represent constitutively expressed genes (Fig. 1) as controls for the RNA preparation and for experiments to be described below. For this purpose, an α-actin cDNA probe homologous in its coding region to the β- and γ-nonmuscle actins was used (40). This probe hybridized mainly to a 2.4-kilobase mRNA, consistent with the size of β- and γ-actin mRNA, that is present in all three RNAs but appears to be induced approximately threefold upon differentiation to either endoderm type (Fig. 2). Histone H4 mRNA is also present in all three poly(A)$^+$ RNAs (Fig. 1), which is presumably the consequence of inefficient separation of poly(A)$^+$ and poly(A)$^-$ RNAs after oligodeoxythymidylate-cellulose chromatography.

Rate of accumulation of specific mRNAs during differentiation to visceral endoderm. When F9 cells are induced to differentiate as aggregates, a ridge separating the inner EC cells from the outer endodermal cells becomes visible between 3 and 5 days after subculturing in petri dishes (24, 28). Is this morphological change correlated with the appearance of AFP mRNA? Total poly(A)$^+$ RNA was prepared from aggregates at different times after exposure to retinoic acid and dotted onto nitrocellulose in a series of dilutions (59). The levels of various RNAs were quantitated after hybridization to cDNA probes by densitometry of the autoradiograms (Fig. 2). AFP mRNA is detectable by dot blot hybridization at day 5, the earliest time examined in this experiment, although it can be first detected at 3 days. It increases again after 7 days, well after the characteristic ridges of the embryoid bodies have formed. In contrast, albumin mRNA is induced by day 5 but remains constant at subsequent time points. At day 7, the levels of AFP and albumin mRNAs, quantitated by densitometry relative to a known standard (60), are comparable: 2 ng/mg of poly(A)$^+$ RNA. However, by day 17, steady-state AFP mRNA increased 100-fold to 200 ng/mg of poly(A)$^+$ RNA.

In contrast to AFP mRNA, H2 and actin mRNAs accumulated in a manner identical to albumin mRNA in that they reached plateaus by day 5 (Fig. 2). Other results with total RNA (not shown) indicate that before day 5 the levels of these mRNAs are increasing to their plateau level. Hence, there is a fundamental difference in the rate of accumulation of AFP mRNA on the one hand and of albumin, H2, and actin mRNAs on the other. The level of histone H4 RNA is constant at all times in culture.

Strickland et al. observed that F9 cells were largely committed to parietal endoderm with only 3 days of treatment with retinoic acid and cAMP (56). To learn whether the time for commitment to differentiate was similarly short for aggregates, the compound was washed out after 3 days of treatment with retinoic acid and aggregates were cultured for 14 days. AFP mRNA was present at levels comparable to those in aggregates that were continuously in the presence of retinoic acid (data not shown), which argues that commitment occurs within the first 3 days. Aggregates formed in the total absence of retinoic acid, on the other hand, exhibited no visceral endoderm ridge and induced only very low levels of AFP mRNA (see Fig. 8).

Loss of DNA methylation upon differentiation. Experiments from several laboratories have implicated DNA methylation as a contributing factor in restricting the ability of the retrovirus Moloney murine leukemia virus to productively infect early embryo cells and teratocarcinoma cells, including F9 (16, 27, 51, 53). We wished to learn whether the hypermethylation of Moloney murine leukemia virus DNA observed in EC cells and the subsequent demethylation after differentiation were peculiar to viral DNA, or whether the induction of endogenous genes such as the AFP gene proceeded by a similar mechanism as well.

For these experiments, the isoschizomers *Hpa*II and *Msp*I were utilized which recognize the sequence CCGG containing the predominant methylation site in higher eucaryotes, CpG. *Hpa*II will cut this sequence only if the internal C is unmodified on both strands, whereas *Msp*I will cut it with few exceptions regardless of its state of methylation (62). The same basic protocol was used in all of the experiments described below. Genomic DNA was prepared from F9 cells before and at various times after differentiation to visceral or

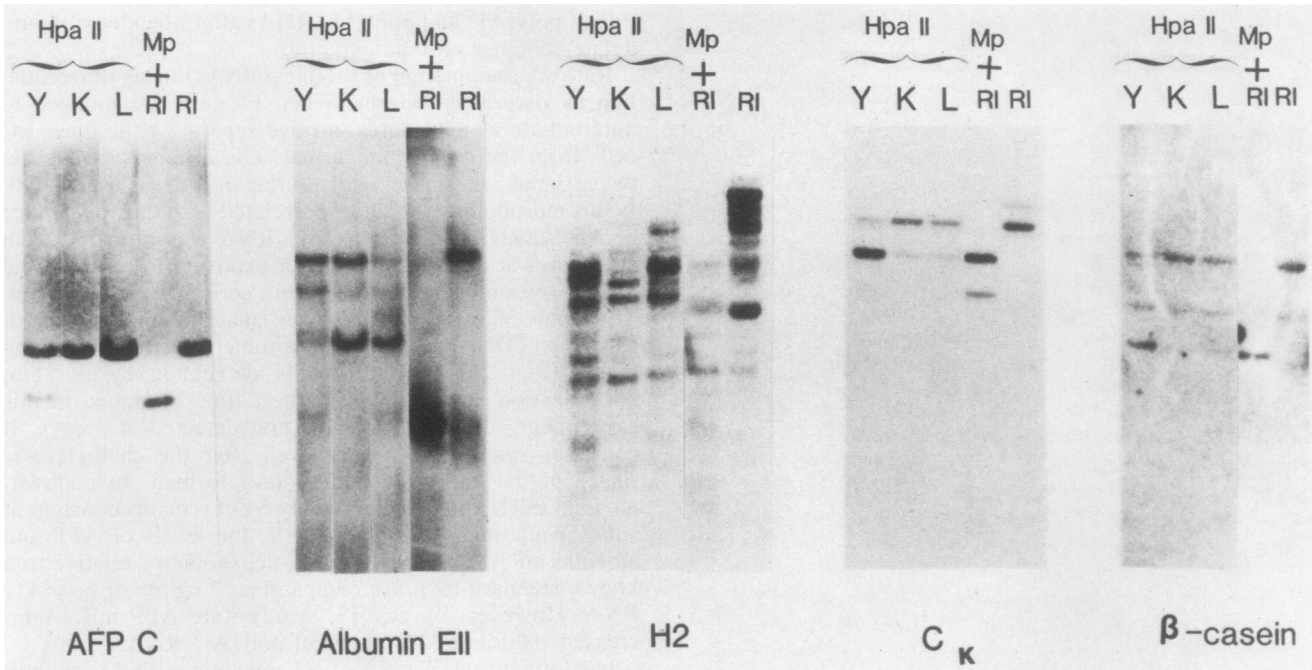

FIG. 6. Tissue specificity of *Hpa*II methylation profiles for several genes. DNA isolated from three mouse tissues, yolk sac (Y), adult kidney (K), and adult liver (L) were digested with *Hpa*II and *Eco*RI, MspI and EcoRI, or EcoRI alone, as indicated, and were electrophoresed through 1% agarose gels, blotted onto nitrocellulose, and hybridized to the nick-translated probes described in the legends to Fig. 2, 3, 4, and 5.

parietal endoderm. The DNA was digested with either *Hpa*II or *Msp*I and then *Eco*RI to obtain reasonably sized bands, separated by agarose gel electrophoresis, blotted, and hybridized to labeled cloned DNA fragments.

Figure 3 shows the results obtained with a probe obtained from the middle of the AFP gene. EC DNA is completely methylated at the single *Hpa*II site in the 2.75-kilobase EcoRI fragment. Upon differentiation to embryoid bodies, there is a significant, though by no means complete, loss of methylation, as shown by the appearance of the 2.1-kilobase band that increases with time in culture. Differentiation to parietal endoderm leads to only a partial loss of methylation at this site, similar to that seen for 7- to 10-day visceral endoderm cells.

The same pattern was observed at two other regions within the AFP gene and at its 5′ end (data not shown). If this loss of methylation is necessary for transcriptional activation, then we might expect that the albumin gene would also lose methylation upon differentiation to embryoid bodies, since Ott et al. (44) have shown a correlation between rat albumin gene expression and undermethylation of the 5′ end of the gene in hepatoma cells. Indeed, this is what is observed at several different locations within and around the gene, as illustrated with a 5′-specific probe in Fig. 4. However, the methylation loss increases with time in culture, whereas albumin gene expression is constant after day 5.

The specificity of the methylation loss was then examined by looking at two genes that are not activated in visceral endoderm. The single-copy immunoglobulin C_κ light-chain and mature β-casein mRNAs cannot be detected in these cells (data not shown), yet their genes undergo substantial methylation losses upon differentiation to embryoid bodies (Fig. 5). A similar result was obtained with the dihydrofolate reductase gene, which is constitutively expressed before and after differentiation (Fig. 5).

If the loss of methylation is as general as the survey with cloned probes suggests, then we would expect to see this reflected in a total *Hpa*II digest of F9 DNA run on an agarose gel and stained with ethidium bromide. Such a result was obtained. F9 EC cell DNA was substantially methylated at *Hpa*II sites, as indicated by a DNA smear at the top of the gel. This smear spread to lower molecular weight upon differentiation to embryoid bodies or parietal cells (data not shown). Additional experiments with the restriction endonuclease *Hha*I, which recognizes the sequence GCGC but will only cut it if the internal CpG is unmethylated, show the same mobility changes, suggesting that a variety of CpG sequences lose their methylation and that the effect is probably not specific for a particular subset of CpG sequences.

Methylation pattern of embryoid body DNA reflects that of mouse yolk sac DNA. The embryoid bodies from which DNA was isolated for the methylation experiments shown in Fig. 3 to 5 are composed of at least two cell types, an outer layer of visceral endoderm that is the AFP-producing cells (24), and the residual EC cells. Thus, one cannot be certain that the undermethylation that is observed is restricted to the visceral endoderm cells, or whether both populations are affected. To ascertain whether the pattern of undermethylation was consistent with visceral endoderm formation, we compared the pattern of DNA methylation in the embryoid bodies with that in mature yolk sac, which is approximately 50% visceral endoderm. The results shown in Fig. 6, compared with those shown in Fig. 3 and 4, illustrate the general finding that the F9-derived endoderm at 17 days closely resembles mature yolk sac endoderm with respect to the degree of methylation of both AFP and albumin genes. The same holds true for the

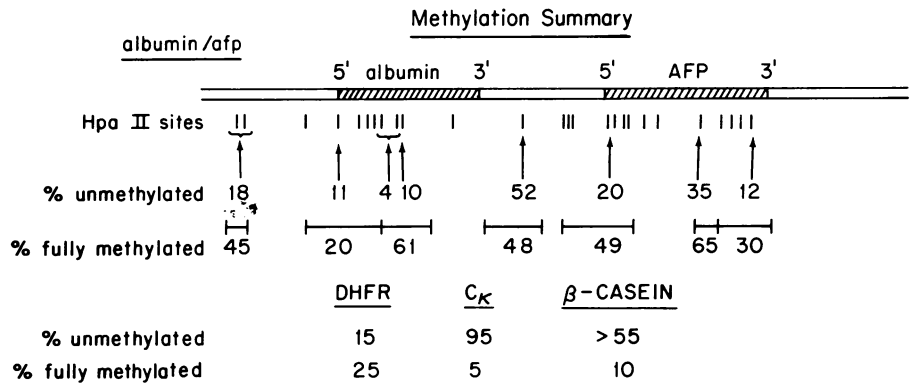

FIG. 7. Quantitative summary of the methylation state of genomic *Hpa*II sites after 17-day visceral endoderm differentiation. Intensities of individual bands on scanned autoradiograms are expressed as a percentage of the total intensity in a lane. Fully methylated and unmethylated percentages refer to fragments corresponding to undigested *Eco*RI fragments or fully digested *Msp*I-*Eco*RI fragments, respectively.

nonexpressed β-casein and Cκ genes as well, in that they are undermethylated in yolk sac DNA (Fig. 5 and 6). Likewise, the pattern for the H2 gene family is similar in 17-day embryoid body and yolk sac DNA (Fig. 6; see Fig. 8B).

Since it could be argued that these genes have similar methylation profiles in many tissues, we also examined *Eco*RI-*Hpa*II digests in adult liver and kidney DNA. There was substantially more methylation for C_κ, β-casein, and several H2 genes in these tissues than in yolk sac (Fig. 6). Thus, 17-day embryoid bodies appeared to mimic the specific pattern of methylation observed in yolk sac, which itself appeared to be a relatively undermethylated tissue. This is consistent with recent observations by Kratzer et al. (35; V. Chapman, personal communication), who showed that satellite DNAs are also relatively undermethylated in yolk sac. These results argue that the undermethylation is occurring in the visceral endoderm cells, but one cannot exclude the possibility that inner cells are affected as well. In fact, the observation that the C_κ gene is 95% unmethylated in 17-day embryoid bodies suggests that at least some sites are undermethylated in all cells of the aggregates (Fig. 5 and 7).

Methylation loss is site specific. The data obtained with the C_κ gene suggested that the extent of methylation loss was not identical at all sites. Additional data in support of this (Fig. 7) were obtained after appropriate exposures of autoradiograms were scanned by densitometry. The percentage of the total intensity in the lane that was present in each band lower than the fully methylated *Eco*RI band was used to estimate the loss of methylation at each site. It must be recognized that these represent an average for the two cell types of the embryoid body. However, comparisons between genes using the same DNA samples are valid indicators of the relative rates of loss of methylation at any given site.

Within the AFP-albumin locus 17 days after induction, the fraction of unmethylated sites varied between 4 and 35%, the one exception being a site between the two genes that was 52% unmethylated. The variability in methylation within the other genes was greater, with the least affected site occurring within the dihydrofolate reductase gene (28% unmethylated), whereas a site within the C_κ gene was almost completely unmethylated. Thus, there was no consistent quantitative pattern of methylation with respect to expressed versus nonexpressed genes. In addition, it was not possible to use the quantitative data to estimate the number of differentiated cells in the aggregates by assuming that the extent of undermethylation equaled the percentage of differentiation, since this varied from site to site. This was particularly well illustrated by the variability in the percentage of totally methylated DNA, which might have been expected to represent the EC cell population. The kinetics of the appearance of unmethylated sites was also variable among the sites examined, arguing that the loss of methylation was not occurring abruptly at the time of commitment but gradually over time.

Effects of 5-azaC-induced loss of DNA methylation. The generalized loss of methylation upon embryoid body formation is not obviously correlated with gene activation, since constitutive and silent genes, as well as inducible genes, are affected. The possibility remains, however, that it is the loss of methylation at a particular subset of sites that is sufficient for induction of visceral endoderm-specific genes.

To explore this, we treated F9 cells with 5-azaC, a drug that inhibits DNA methyltransferase activity, with the idea that if loss of methylation could be induced at these specific sites, the cells would differentiate. Such an effect has been previously observed in mouse embryonic cells (7, 32) and erythroleukemic cells (9). Since long-term treatment of cells with 5-azaC is lethal, we treated F9 cells for 48 h with the drug, either immediately upon subculturing to petri dishes or 3 days later when the aggregates were well formed, in this way hoping to bracket the time of commitment to differentiation. The cells were then allowed to proceed until 7 or 17 days. Within the range of concentrations used (2.5 to 10 μM), no morphological differentiation to embryoid bodies was observed with any of the protocols. Both AFP and H2 mRNAs were made at very low levels, substantially less than those in both untreated aggregates and aggregates treated with retinoic acid (Fig. 8). However, examination of the state of DNA methylation in the AFP and H2 genes showed that the 5-azaC-treated samples were substantially undermethylated relative to the untreated control aggregates (Fig. 8B). It should be noted that the untreated aggregates themselves had undergone some loss of methylation, consistent with the low level of AFP mRNA observed, presumably the result of a low level of spontaneous differentiation.

The foregoing suggested that loss of DNA methylation per se was not sufficient to induce differentiation of the F9 aggregates. However, it was possible that undermethylation was necessary for the selective induction of AFP mRNA shown in Fig. 2. The transient induction of gene expression after 5-azaC treatment has been observed for several genes,

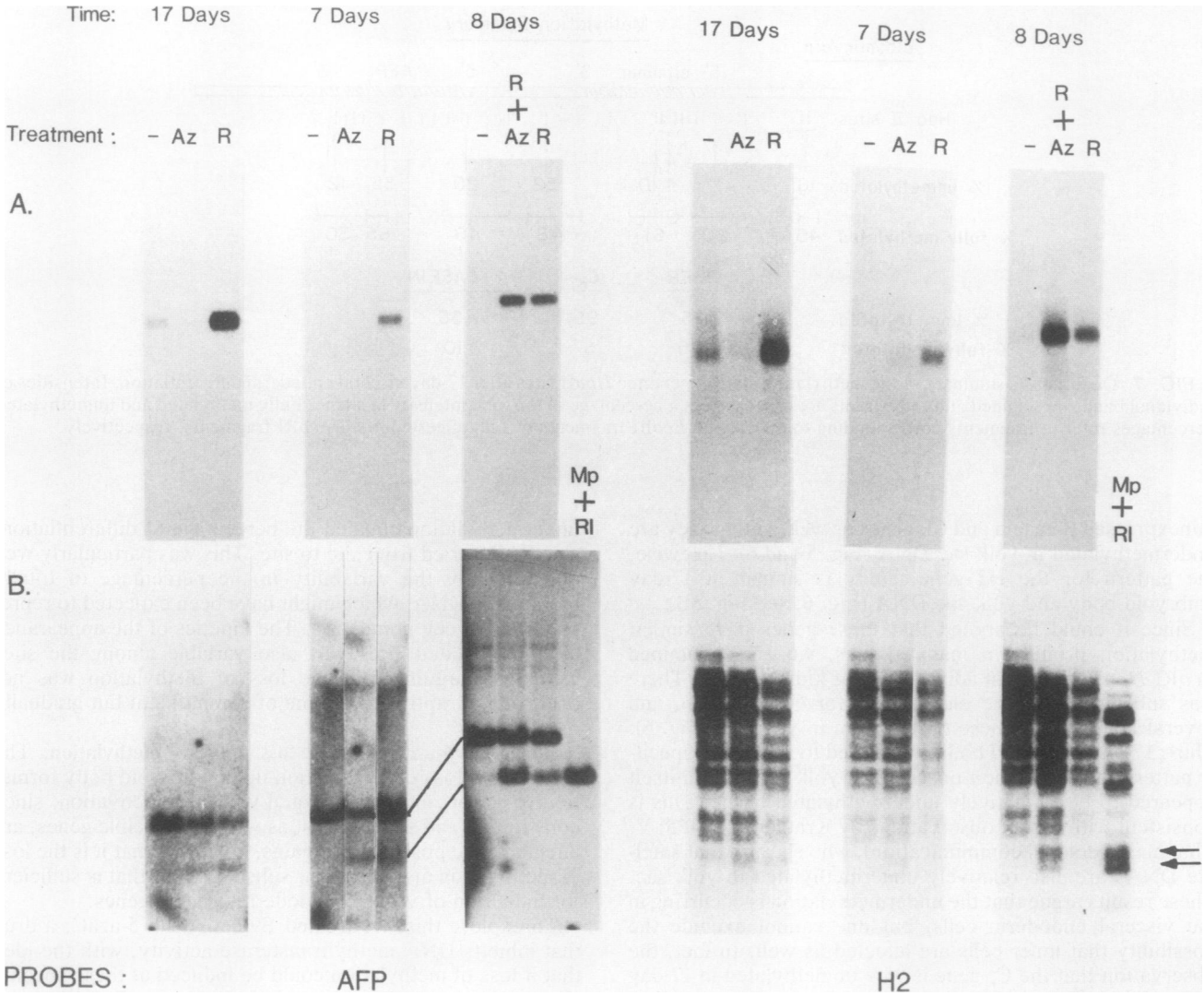

FIG. 8. Effect of 5-azaC treatment upon expression of the AFP and H2 genes. (A) F9 cell aggregates were formed in bacteriological petri dishes and treated on day 3 with 2.5 μM 5-azaC (Az) for 48 h; the 5-azaC was removed and the aggregates were harvested at day 7 or 17. As controls, aggregates were left untreated for 7 or 17 days (−) or maintained in the presence of retinoic acid (R). In a second protocol, F9 cells were incubated in bacteriological petri dishes with 7.5×10^{-8} M retinoic acid and on day 5, 2.5 μM 5-azaC was added for 24 h (R+Az). The cells were harvested on day 8 together with untreated controls (−) and 7.5×10^{-8} M retinoic acid-treated cells (R). Poly(A)$^+$ RNA (3 to 6 μg) from the indicated times was electrophoresed, blotted, and hybridized to nick-translated AFP or H2 cDNAs as described in the legend to Fig. 1. (B) Genomic DNA (5 μg) from the same cells was digested with *Hpa*II and *Eco*RI, or with *Msp*I-*Eco*RI, as shown, and electrophoresed, blotted, and hybridized to nick-translated pAFP-C or H2 cDNA (pMHC-1). The two arrows point to the *Hpa*II-*Eco*RI bands that were diagnostic of the loss of methylation in the H2 genes.

including mouse metallothionien I (6), endogenous avian retrovirus (22), and fetal hemoglobin (5). Therefore, F9 aggregates were treated with retinoic acid for 5 days, 5-azaC was added for 24 h and washed out, and the aggregates were allowed to recover for 2 days. The results (Fig. 8) showed that AFP mRNA levels were not affected by 5-azaC treatment, despite the fact that the *Hpa*II digestion patterns were comparable to those seen in day 17 rather than day 8 embryoid bodies. Interestingly, in contrast, H2 mRNA was substantially induced by 5-azaC (Fig. 8), whereas actin and albumin mRNAs showed little change (data not shown).

In analyzing several genetic loci in the 5-azaC-treated cells, we noticed a striking similarity in the pattern of 5-azaC-induced loss of methylation compared with the pattern after retinoic acid, as reflected in the relative intensities of *Hpa*II-*Eco*RI fragments. A good example of this is shown in Fig. 9, where the 5′ end of the albumin gene was analyzed. Such a similar pattern implies that the demethylation proceeds in the two instances via similar pathways.

DISCUSSION

Expression of AFP mRNA in embryoid bodies. The differentiation of F9 stem cells to visceral endoderm is accompanied by the induction of AFP and albumin mRNAs (24; Fig. 1). This result extends the studies of Hogan et al. (28), who first showed that AFP accumulates in retinoic acid-treated F9 aggregates. Analysis of the time course for induction led to the observation that AFP mRNA increased from 3 to 5 days after retinoic acid addition, remained relatively constant for several days, and then increased again for at least

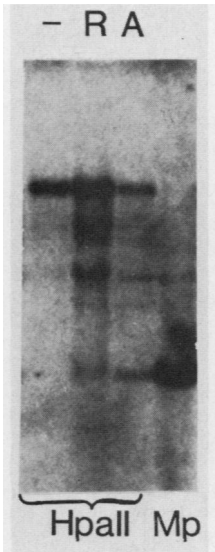

FIG. 9. 5-azaC-induced demethylation is similar to that caused by retinoic acid. Genomic DNA (5 μg) prepared from the 7-day experiment of Fig. 8 was electrophoresed, blotted, and hybridized to nick-translated pALBEII (see Fig. 4). Symbols refer to control aggregates (−), 5-azaC-treated aggregates (A), and retinoic acid-treated aggregates (R).

17 days. Albumin mRNA, as well as H2 and actin mRNAs, increased until day 5 but remained constant thereafter.

It is difficult to rationalize the specific accumulation of AFP mRNA on the basis of continual recruitment of EC cells to visceral endoderm. In that case, all of the mRNAs should have increased in parallel. The fact that the inducer, retinoic acid, could be washed out at day 3 without much effect on AFP mRNA accumulation at day 17 argues, in fact, that commitment occurred very early and was complete by day 3. Instead, these data are consistent with a model whereby differentiation in the first 5 days results in the formation of primary endoderm, the putative precursor to visceral and parietal endoderm (54). Accompanying this is the transcriptional activation of a number of loci, including the albumin and AFP genes. Beyond day 7, when the basement membrane underlying the endoderm has been formed, transcription of the AFP gene or stabilization of its transcript or both are further enhanced, resulting in a differential accumulation of AFP mRNA. Nuclear run-on assays have not been sensitive enough to discriminate clearly between the two alternative mechanisms. The role of the basement membrane in the secondary induction of AFP mRNA is suggested by experiments in which tunicamycin treatment (21) and anti-laminin antibody treatment (23), which prevent basement membrane formation in teratocarcinoma aggregates, prevented accumulation of AFP. However, the former treatment did not inhibit the appearance of cells stained negatively for alkaline phosphatase, a characteristic of primary endoderm cells.

Loss of DNA methylation after differentiation of F9 cells. One consequence of the differentiation of F9 cells into embryoid bodies is an extensive loss of genomic DNA methylation. A much smaller loss seems to be associated with differentiation to parietal endoderm, although this could simply be a function of the fact that parietal endoderm cultures were assayed 7 days earlier than the aggregates. Two lines of evidence argue that the demethylation we observed was occurring primarily in the visceral endoderm cells. First, the methylation pattern for 17-day embryoid bodies was very similar to that from mouse visceral yolk sac. Andrews et al. (1) demonstrated that the unmethylated AFP gene DNA in yolk sac is contributed exclusively by the visceral endoderm layer, whereas the mesoderm layer is fully methylated. Second, aggregation alone fails to result in substantial DNA undermethylation; the presence of retinoic acid is required. Taken together, these data argue that the loss of methylation is occurring at least in part as a consequence of the differentiation of the F9 cells into visceral endoderm.

Given this finding of substantial loss of DNA methylation accompanying differentiation, we are in a unique position to ask whether it has a role in the changes in gene expression that occur. Several results address this point. First, the substantial loss of methylation upon visceral endoderm formation was not specific for visceral endoderm-expressed genes, since all genes examined appeared to be affected. Second, both the commitment to differentiation and the enhanced expression of a number of genes occur before any large changes in their degree of methylation. Third, AFP mRNA accumulation with time is unique in its correlation with demethylation of its gene. Finally, 5-azaC-induced loss of methylation does not lead to differentiation or to enhanced AFP mRNA synthesis after differentiation. Together, these results could be taken as presumptive evidence against a direct causative role for methylation loss in either differentiation or the subsequent enhancement of AFP gene expression, although they do not rule out a secondary or permissive role.

A related conclusion was reached by several groups studying the expression of the Moloney murine leukemia retrovirus, whose expression is restricted in teratocarcinoma cells but becomes activated upon differentiation (53). It has been observed that the time course of Moloney murine leukemia retrovirus restriction in teratocarcinoma cells is substantially faster than the rate of de novo methylation, suggesting that there is a more immediate means than methylation by which EC cells shut down expression (16, 42). In contrast, a more direct test of the effect of methylation on retrovirus gene expression was performed by Simon et al. (51), who showed that retrovirus genomes methylated in vitro by a mammalian methylase were noninfectious, whereas methylation by *Hpa*II methylase, which recognizes the same sequence assayed by *Hpa*II and *Msp*I, had no effect. Clearly, functional tests utilizing gene transfer into recipient cells will be necessary to resolve the participation of methylation at specific sites in gene expression.

In contrast to AFP mRNA, H2 mRNA is induced substantially by 5-azaC treatment at a time when its expression is not changing, suggesting that these genes are sensitive to changes in DNA methylation. The result is consistent with the finding of an increased number of H2 antigen-expressing cells upon 5-azaC treatment of F9 monolayers (8). It may be that 5-azaC treatment results in the loss of methylation of sites not normally affected by differentiation, resulting in increased transcription. On the other hand, the induction of other normally silent H2 gene loci may have occurred.

Mechanism of the loss of DNA methylation. Although the precise involvement, if any, of DNA methylation in the expression of genes during F9 differentiation cannot be unambiguously deduced from the present data, this system does allow one to examine the mechanics of methylation loss during differentiation. The preliminary data suggest that the process is complex in that both the rate and the degree of loss of methylation at different sites are nonuniform.

The decrease in DNA methylation could occur via an inhibition of the well-characterized maintenance methylase(s) through at least two rounds of replication (25, 29, 58, 64), by the inhibition of de novo methylase activity (31), or by a specific demethylase activity for which some preliminary evidence has been obtained (17). A reduction in the maintenance DNA methylase or de novo methylase levels or both upon differentiation could explain the results we observed. Grover et al. (24) have measured a significant increase in cell number in differentiating F9 aggregates, certainly sufficient to account for the rate of demethylation by an inhibition of maintenance methylation alone during replication.

Such a mechanism invites direct comparison with the effects of 5-azaC treatment. 5-azaC is known to inhibit maintenance methylation as the consequence of its low-level incorporation into DNA and subsequent interaction with the methylase, resulting in either covalent adduct formation or modification of the enzyme (46) and reduced levels of the active enzyme (4, 58). It is striking that the *Hpa*II digestion pattern after 5-azaC treatment at several regions of the genome is very similar to that obtained after differentiation with retinoic acid in that partial methylation, rather than complete loss of methylation, is observed and particular partial digest bands seem to have similar relative intensities (Fig. 8B and 9; other data not shown).

One very attractive feature of a model wherein the cellular methylases becomes limiting is that it would account for the apparent sloppiness in the extent of methylation at any one site. It has been observed that the levels of methylation at particular sites can be partial even in monoclonal cell lines (e.g., see reference 39), implying that the efficiency of methylation at particular sites can vary, as would occur if the methylase activity in a cell were limiting.

When during differentiation is the pattern of DNA methylation that will characterize the differentiated cell established? Our observation that the 5-azaC-treated cells and the differentiated cells exhibited remarkably similar *Hpa*II digestion patterns would argue that this pattern is inherent to the chromatin of the aggregated EC cell. Thus, F9 cell differentiation affords an opportunity to examine the means by which one specific pattern of methylation is established during development.

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