Tissue-Specific Hypomethylation and Expression of Rat Phosphoenolpyruvate Carboxykinase Gene Induced by in Vivo Treatment of Fetuses and Neonates with 5-Azacytidine†

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ABSTRACT: Rat fetuses of 17–19-day gestation were injected in utero with 5-azacytidine (two to three daily injections of 40 μg/fetus). Neonates were injected with seven daily injections (1 mg/kg). DNA samples were isolated from the fetal and neonatal livers and neonatal spleen and subjected to analysis of their methylation status. Overall methylation was analyzed by the nearest-neighbor analysis (at CpG sites) and the pattern of methylation at CCGG sites by Southern blot analysis using phosphoenolpyruvate carboxykinase (PEPCK) sequences as probes. While DNAs from the liver and spleen undergo hypomethylation to the same extent in response to the 5-azacytidine treatment, the changes in the methylation patterns of the PEPCK gene in the two tissues are strikingly different. The changes observed indicate that a decrease in the methylase activity (inhibition by 5-azacytidine) results in site- and tissue-specific hypomethylation. The tissue-specific changes in the methylation pattern are associated with a tissue-specific expression of the PEPCK gene. Although the gene is hypomethylated by azacytidine in both liver and spleen, it is expressed only in the liver. The expression of already active genes (PEPCK in the kidney and albumin in the liver) is not further enhanced by the drug.

Tissue-specific patterns of methylation have been observed in numerous eukaryotic gene sequences. In these genes, where the methylation pattern has been analyzed in the sperm as well as in somatic tissues, the genes were found to be essentially fully methylated in the sperm and hypomethylated in the expressing tissue, suggesting that methyl groups are lost during the differentiation process (Yisraeli & Szyf, 1984). Analysis of the process by which this loss of methyl groups takes place in the embryo has been a formidable task, considering the small amount of material that is obtainable from early embryos. A model system was therefore required. In recent experiments using teratocarcinoma cells that were induced to differentiate by retinoic acid, massive demethylation has been observed (Razin et al., 1984; Young & Tilghman, 1984). This differentiation is believed to mimic the formation of primitive endoderm. However, this experimental system proved to be unsuitable for demonstrating site- and tissue-specific hypomethylations. We have reported recently a detailed analysis of the methylation pattern of the rat cytosolic phosphoenolpyruvate carboxykinase (PEPCK) gene (Benvenisty et al., 1985). The PEPCK enzyme (EC 4.11.32) plays a key regulatory role in the metabolic pathway of gluconeogenesis, which is vital for the mammal. The PEPCK gene shows tissue-specific expression in the adult as well as in the developing fetus (Tilghman et al., 1976; Benvenisty et al., 1984) and undergoes activation at birth in the rat liver (Ballard & Hanson, 1967; Mencher & Reshef, 1979). It is hypomethylated in the adult expressing tissues (liver and kidney) and heavily methylated in the adult nonexpressing tissues (spleen and heart muscle). A sequential process of hypomethylation takes place in the developing liver. This process is associated with a conversion of the inactive PEPCK gene through a potentially active gene prior to birth to a fully active gene in the adult. Moreover, a cause and effect relationship has been suggested from the course of hypomethylation and activation of the gene (Benvenisty et al., 1985). This is a convenient system that allows a design of experiments aimed at the elucidation of the biochemical mechanism underlying the establishment of a tissue-specific pattern of methylation and gene expression. In the present paper, we demonstrate how the pattern of methylation of the rat PEPCK gene in the liver is changed during development and study the effect of 5-azacytidine (5-aza-C), a known inhibitor of the methylase (Jones, 1984), on the pattern of methylation and expression of the gene in the fetal liver and spleen of the neonate. Treatment of fetuses with high doses of 5-azacytidine has been reported to induce premature appearance of tyrosine aminotransferase activity in fetal liver (Rothrock et al., 1983). Like PEPCK, this enzyme normally appears at birth.

Experimental Procedures

Animals. Pregnant rats from overnight mating, neonates, and 6-week-old male adult Sabra rats (Wistar origin) were all from the Hebrew University breeding center. Individual littermate fetuses were injected through the uterine wall, as previously described (Mencher & Reshef, 1979), with saline (controls) and 5-aza-C (Sigma) (experiment). Similarly, littermate neonates were injected intraperitoneally with either saline or 5-aza-C. In all cases, the volumes injected did not exceed 10 μL.

Genomic DNA Preparation. DNA from rat liver and spleen was extracted by tissue homogenization in 2 mM ethylenediaminetetraacetic acid (EDTA), 0.4 M NaCl, and tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.9, digested by proteinase K (500 μg/mL) followed by four cycles of or-

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ganic solvent extractions, and digested by RNase (50 μg/mL) followed by cycles of organic solvent extractions repeated as described by Hewish & Burgoyne (1973).

Assay of m^5C in CpG-Containing Sequences. The degree of methylation of CpG-containing sequences was determined by an extension of the standard nearest-neighbor analysis (Gruenbaum et al., 1981). DNA samples (1–2 μg) were nicked with DNase I (Boehringer) (0.7 μg/mL) for 15 min at 37 °C. The 3′-ends of the nicks were end labeled with 25 μCi of [α-^{32}P]dGTP (Amersham, 3000 Ci/mmol). *Escherichia coli* DNA polymerase I (9 units, Boehringer) in a 25-μL mixture containing 50 mM Tris-HCl (pH 7.4), 5 mM $CaCl_2$, and 1.4 mM β-mercaptoethanol was used. The reaction was performed at 15 °C for 30 min and terminated by the addition of EDTA to a final concentration of 10 mM. Unreacted labeled dGTP was removed by application to Sephadex G-50 (Pharmacia) minicolumns. The labeled DNA in the effluent was digested to deoxyribonucleoside 3′-monophosphates by treatment with micrococcal nuclease (Sigma, 140 μg/mL) for 1 h at 37 °C in 25 mM Tris-HCl (pH 8.0) and 5 mM $CaCl_2$ followed by a 1-h digestion with spleen phosphodiesterase (Sigma, 7 units/mL) (Knippers et al., 1969). The digest was applied to 10 × 10 cm cellulose-coated thin-layer chromatography sheets (Eastman-Kodak) and chromatographed in two dimensions (Gruenbaum et al., 1981). The chromatograms were autoradiographed, and the spots corresponding to cytosine and 5-methylcytosine were scanned with a quick Helena scan densitometer. To calculate the extent of unmethylated CpG, the areas under the peaks were integrated and corrected for overlap between the major m^5C and minor C peaks. The values obtained represent an average obtained from three experiments in which DNA samples were prepared from three fetuses at least. The values varied in the range of ±2%.

Molecular Probes. The molecular probe pPCK-E5.4 is an *Eco*RI genomic fragment of the PEPCK gene subcloned into the corresponding sites in pBR322 (Yoo-Warren et al., 1983). prAlbI is an albumin cDNA clone (Kioussis et al., 1979). The probes were ^{32}P labeled by nick translation (Rigby et al., 1977) to specific activity of (1–3) × 10^8 cpm/μg of DNA.

Southern Blot Hybridization. DNA was digested by the appropriate restriction enzyme. The digest separated by electrophoresis on horizontal 1% agarose gel as previously described (Benvenisty et al., 1985) was transferred to nitrocellulose filters (Schleicher & Schuell) according to the method of Southern (1975). *Hind*III digest or *Hind*III and *Eco*RI double digest of λ DNA and *Hae*III digest of φX174 DNA were used as size markers. Prehybridization and hybridization were performed according to Wu (1980) in the presence of 10% dextran sulfate during the hybridization.

RNA Extraction and Dot Blot Hybridization. Total RNA from fetal liver and kidney and neonatal spleen was extracted as previously described (Mencher et al., 1984). RNA was dot blotted at increasing concentrations on a 6 × NaCl/citrate-treated nitrocellulose filter using a 96-well minifold TM (SAC 96, Schleicher & Scheull). Prior to dot blotting, the RNA samples were denatured by heating 10 min at 70 °C in the presence of 7.4% formaldehyde, rapidly cooled on ice, and adjusted to 6× NaCl/citrate. RNA fixation, prehybridization, and hybridization were performed as previously described (Mencher et al., 1984).

Quantification of DNA and RNA. Autoradiographic exposures of the Southern blotting and RNA dot blot hybridization were as described by Swanstrom & Shank (1978). Densitometry of the autoradiographic images were performed with the Helena quick-scan R+D densitometer: optical density shows linear relationship with increasing amounts of RNA per dot up to 80 μg.

Results

We have shown in a previous communication that the ultimate pattern of methylation of a tissue-specific gene (PEPCK) is formed during development of the fetus by a process of sequential loss of methyl groups in the gene region. This hypomethylation occurs exclusively in the liver and kidney where the adult gene is expressed (Benvenisty et al., 1985). This developmental system provided us with a tool that allowed a closer insight into the mechanisms by which the sequential loss of methyl groups occurs and its correlation with expression. We have previously hypothesized and shown to be the case in bacteria that the intracellular level of the methylase plays a role in the hypomethylation process (Szyf et al., 1984). We therefore used in the experiments described here 5-aza-C to lower the intracellular methylase level in rat fetuses and neonates by injecting this agent in utero to fetuses and in vivo to neonates. 5-Aza-C is known to be a potent inhibitor of methylase activity. It is believed that 5-aza-C, which is incorporated into the DNA via replication, traps the methylase by irreversible binding of the enzyme to the cytosine analogue (Jones, 1984). It has however been known that azacytidine shows cytotoxic effects (Taylor & Jones, 1982). We have therefore selected conditions for azacytidine treatment that exert minimal cytotoxic effects. At azacytidine concentrations of <40 μg per fetus, no mortality of the fetuses has been observed. Furthermore, no reduction in body or liver and kidney weight or liver and kidney DNA content has been observed as compared with saline-injected littermate controls when the fetuses were injected with two daily injections. A slight reduction in body weight (87% of the controls) was observed after three daily injections (Table I). Since the spleen is undeveloped in the fetus and undergoes rapid growth and development during the first postnatal week (Mackovich & Greengard, 1972), we have chosen to study the effect of 5-aza-C in the spleen of neonates. Injecting neonates with the drug (1 mg/kg of body weight) for the first 7 postnatal days did not affect body and liver growth and had a very slight effect on the growth of the spleen (Table I). These conditions proving not to affect replication and other cell functions were chosen for our experiments.

In order to determine whether 5-aza-C treatment resulted in an overall decrease in the extent of methylation of fetal liver and neonatal spleen and liver DNA, we have used a modification of the nearest-neighbor analysis (Gruenbaum et al., 1981). By this method we could measure the extent of unmethylation of cytosine residues in the dinucleotide sequence CpG, which is essentially the major methylated dinucleotide sequence in eukaryotic DNA (Sinsheimer, 1955). When such an analysis was performed on the DNA of spleen and liver of treated and untreated animals, we found that both tissues underwent a similar degree of hypomethylation (see Figure 1). While 15–23% of the cytosines at the CpG sequences were unmethylated in tissues of untreated animals, 5-aza-C treatment resulted in an about 2-fold increase of the unmethylated CpG fraction to 31–37%. This analysis demonstrated that 5-aza-C has caused a similar decrease in the methylation capacity in spleen and liver as reflected by the level of DNA methylation. It has been previously suggested that a decrease in the methylation capacity of a cell may eventually result in tissue- and site-specific hypomethylations at the gene level (Razin & Szyf, 1984). In order to examine whether this rule holds for the rat PEPCK gene, the 5-aza-C-treated fetal liver

Table I: Effect of 5-Aza-C on Body and Tissue Growth and DNA Content[a]

treatment	dose (μg)	time (days)	age at end of expt	body wt (g)	tissue	tissue wt (mg)	DNA (μg/mg of tissue)
none				2.2 ± 0.23 (4)	fetal liver	110 ± 5.4 (4)	
5-aza-C	40	2	19	2.18 ± 0.08 (8)		102 ± 5.4 (4)	
none				3.79 ± 0.16 (6)		258 ± 24 (6)	4.3 ± 0.51 (4)
5-aza-C	20–40	2	20–21	3.63 ± 0.16 (8)		246 ± 10 (12)	4.2 ± 0.28 (4)
5-aza-C	10–30	3	20–21	3.28 ± 0.08 (4)			
none				4.72 ± 0.04 (4)	fetal kidney	23 ± 0.48 (4)	2.4 ± 0.18 (4)
5-aza-C	30	3	21.5	4.46 ± 0.10 (4)		21 ± 0.53 (4)	2.2 ± 0.01 (4)
none				17.20 ± 0.80 (4)	neonatal liver	556 ± 29 (4)	3.06 (1)
5-aza-C	5	7	7	8.83 ± 0.45 (6)		289 ± 17 (6)	3.23 (1)
none				19.40 ± 0.26 (5)		659 ± 22 (5)	
5-aza-C	1	7	7	18.50 ± 0.48 (4)		609 ± 44 (4)	
none				19.40 ± 0.26 (5)	neonatal spleen	87 ± 16 (3)	1.08 (1)
5-aza-C	1	7	7	18.50 ± 0.48 (4)		70 ± 2 (3)	1.24 (1)

[a] Littermate fetuses (17 or 19 gestational age) were injected daily in vivo with 5-aza-C (experiment) or saline (control). Dose (μg) per fetus and treatment times were as specified. Littermate neonates were injected daily ip for the first 7 postnatal days with 5 μg/g of body weight or 1 μg/g of body weight 5-aza-C as specified (experiment) or saline (control). Body and tissue weights and tissue DNA content were determined at the end of the experiment and are given as the means ± SEM of the number of animals indicated in parentheses.

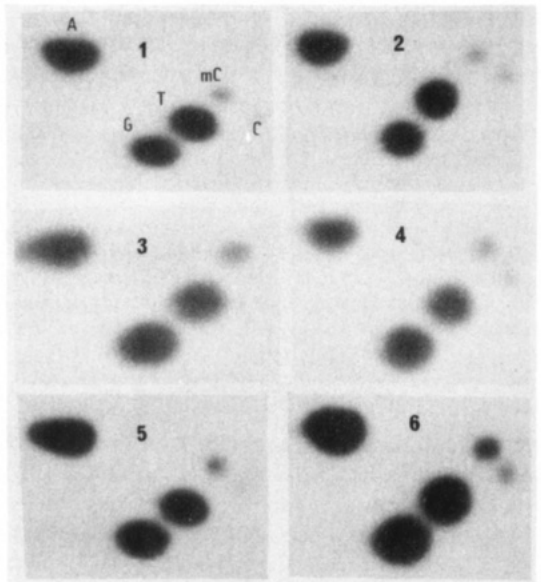

FIGURE 1: Extent of DNA hypomethylation in fetal and neonatal liver and spleen treated with 5-aza-C. DNA was purified from normal and 5-azacytidine-treated fetal liver and neonatal spleen and liver. The extent of hypomethylation at CpG sequences was determined as described under Experimental Procedures. The values calculated from a densitometric scan of the autoradiograms were as follows: (1) 21-day fetal liver (23%), (2) 21-day fetal liver treated for 2 days with 5-aza-C (37%); (3) neonatal liver (15%); (4) neonatal liver post 7-day 5-aza-C treatment (31%); (5) neonatal spleen (20%); (6) neonate spleen post 7-day 5-aza-C treatment (32%).

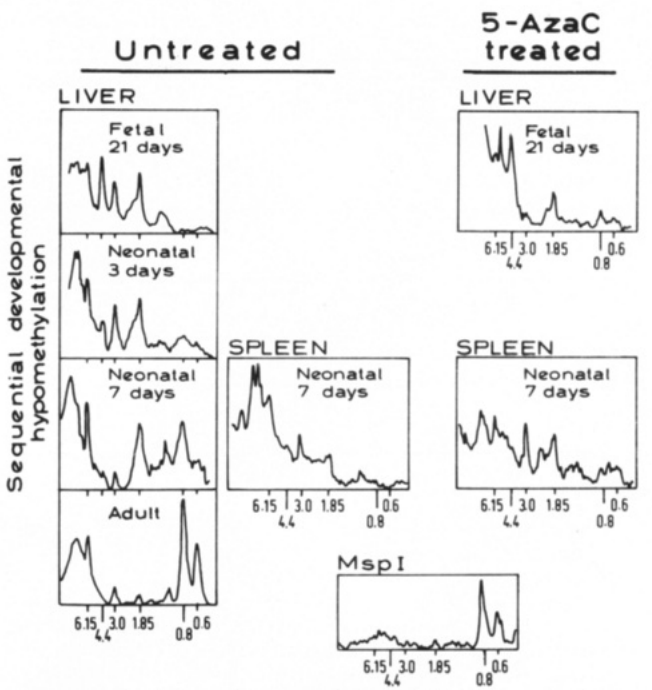

FIGURE 2: *Hpa*II digestion patterns of the PEPCK gene in the developing fetal rat liver and 5-aza-C-treated fetal liver and neonatal spleen. DNA was isolated, digested with *Hpa*II, and size fractionated by gel electrophoresis. The fractionated DNA fragments were transferred to nitrocellulose sheets and hybridized to ^{32}P-labeled probe pPCK-E5.4 and autoradiographed as described under Experimental Procedures. The autoradiograms were scanned with the Helena quick-scan densitometer.

and neonatal spleen DNA was digested with *Hpa*II and analyzed by Southern blot hybridization with the genomic DNA fragment probe as described before (Benvenisty et al., 1985).

The *Hpa*II digestion patterns of the PEPCK gene have been autoradiographed and scanned by densitometry (Figure 2). The changes in the patterns during development are characterized by a gradual decrease in the 4.4-kb band characteristic of the fetal liver PEPCK gene (Benvenisty et al., 1985), a decrease in the 3-kb band, an increase in the small fragments ranging in size between 0.55–0.8 kb occurring between the 3rd and 7th day of the neonate and a decrease of the 1.85-kb band in the adult (Figure 3). These changes in the pattern reflect events of hypomethylation that occur in vivo during normal development. To examine our hypothesis that suggests that the intracellular level of the methylase may determine the pattern of methylation, it was necessary to establish whether similar hypomethylation will occur when the intracellular methylase level is decreased by a methylase inhibitor. two daily injections of 19 gestation day fetuses with 40 μg per fetus of 5-aza-C resulted in an increase of the 4.4-kb band, a virtual disappearance of the 3-kb band, a considerable decrease of the 1.85-kb band, and the appearance of the small fragments (0.55–0.8 kb). The hypomethylation of the gene observed in the spleen in response to 5-aza-C is quite different from that described above for the liver. Although the small fragments (0.55–0.8 kb) appear, there is either an increase (1.85 kb) or no change (3 kb) in the large-sized bands as well as the appearance of additional bands (Figure 2 and 3). As a result, two different tissue-specific patterns of methylation are obtained (Figure 2 and 3) in spite of the fact that a very close overall hypomethylation was observed in the spleen and liver in response to 5-aza-C treatment (Figure 1). The PEPCK gene has been shown to be heavily methylated in the fetal liver and spleen (PEPCK nonexpressing tissues), hypomethylated

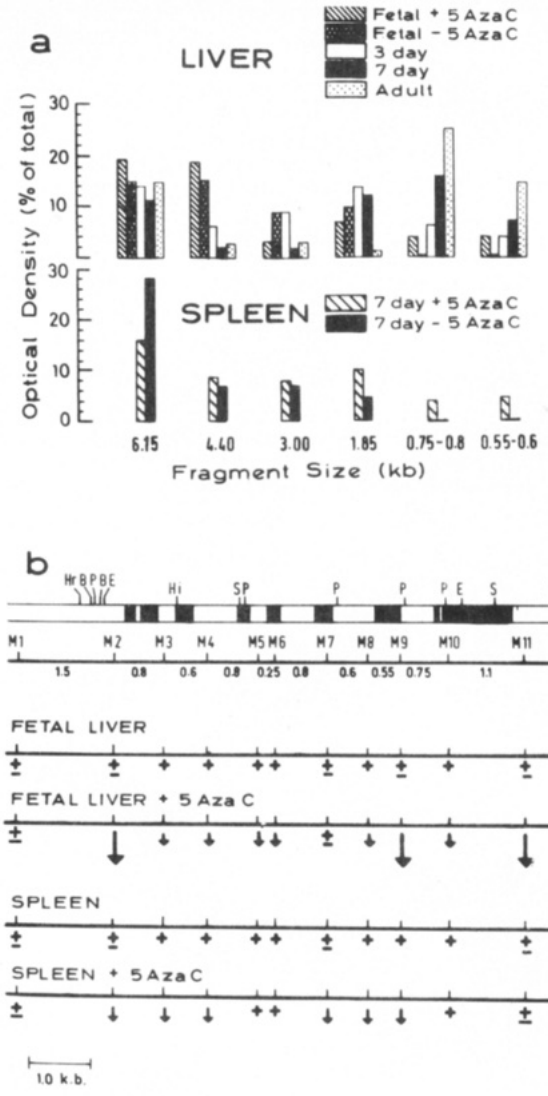

FIGURE 3: Quantitation of the changes in the *Hpa*II digestion patterns described in Figure 2. (a) To visualize the changes in the abundance of definitive *Hpa*II bands in the Southern blots, the area under the peaks corresponding to 6.15-, 4.40-, 3.00-, 1.85-, 0.8–0.75-, and 0.60–0.55-kb fragments was computed and expressed as percent of the total area under the corresponding densitograms. (b) Scheme of hypomethylation events. Our interpretation of the hypomethylation events following 5-azacytidine treatment is represented. The first line describes a physical map of the PEPCK gene. Black boxes denote exones and white boxes introns, as reported by Yoo-Warren et al. (1983). M1–M11 are the *Msp*I (*Hpa*II) sites. E, Hi, B, S, and P indicate *Eco*RI, *Hind*III, *Bam*HI, *Sph*I, and *Pst*I sites, respectively. The second line is map of the *Msp*I sites. The sites corresponding to the visualized bands have been previously mapped (Benvenisty et al. 1985): 6.15, M2–M11; 4.40, M2–M9; 3.00, M2–M7 and M7–M11; 1.85, M9–M11; 0.75–0.8, M2–M3, M4–M5, M6–M7, and M9–M10; 0.55–0.6, M3–M4, M7–M8, and M8–M9. The next two pairs of lines describe the methylation state in fetal liver DNA and 5-aza-C-treated fetal liver and neonatal spleen and 5-aza-C-treated spleen. The second map in each pair describes the hypomethylation events after 5-azacytidine treatment. Short arrows indicate minor events and long arrows major hypomethylation events. Major events occurred in the fetal liver in sites 2, 9, and 11, giving rise to a 6.15-kb band (M2–M11) and a 4.4-kb band (M2–M9). Minor events in sites M3–M6, M8, and M10 account for the virtual disappearance of the 3-kb band (M2–M7 and M7–M11) and the decrease in the 1.85-kb band (first two maps). 5-Azacytidine treatment induced minor hypomethylation events in most sites of spleen DNA, except M5, M6, and M10, which account for the decrease in the 6.15-kb band (M2–M11), the rise in the 3-kb band (M2–M7 and M7–M11), the 1.85-kb band (M9–M11), and the appearance of two new bands (1.4 kb and 2.4 kb) (see Figure 2).

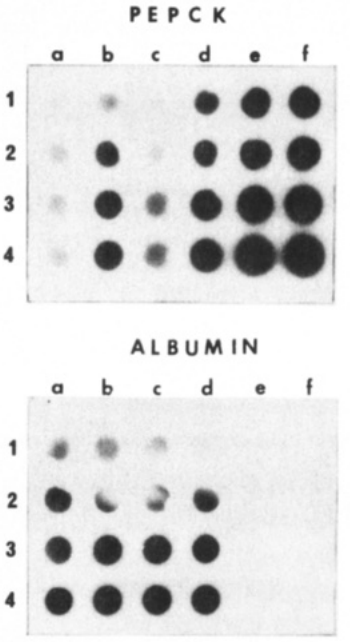

FIGURE 4: Dot blot analysis of PEPCK and albumin RNA in non-treated and 5-aza-C-treated fetal rat liver and kidney. Total RNA was extracted from either control (saline injected) (a, c, and e) or 5-aza-C-treated (b, d, and f) littermate fetuses from three separate litters. RNA pooled from three to six littermate fetal liver (a–d) or kidney (e and f) was applied to nitrocellulose filters in the following increasing amounts: (1) 5 μg; (2) 10 μg; (3) 20 μg; (4) 30 μg. The nitrocellulose filters were hybridized with ^{32}P-labeled pPCK-E5.4 (PEPCK genomic fragment) or pAlb-1 (albumin cDNA).

in the adult liver and kidney (PEPCK expressing tissues), and undergoing sequential hypomethylation in the liver in the perinatal period while acquiring its expression capacity (Benvenisty et al., 1985). Dot blot hybridization analysis (Figure 4) shows indeed a premature appearance of PEPCK mRNA sequences in the fetal liver after 2 days of 5-aza-C treatment. Shorter periods of treatment (2–24 h) were ineffective (results not shown). The appearance of PEPCK mRNA in fetal liver was accompanied by comparable appearance of PEPCK catalytic activity (results not shown), substantiating the notion that under our experimental conditions minimal toxic effects were exerted by 5-aza-C on other systems (transcription, protein synthesis, etc.). We did not observe any PEPCK mRNA sequences in the spleen 7 days after 5-aza-C treatment, in spite of the substantial hypomethylation observed in the gene during this period. Similarly, no albumin mRNA sequences were observed in the fetal kidney, an albumin nonexpressing tissue (Figure 4). It should be kept in mind that the hypomethylation of the gene in the spleen was different from that obtained in the liver. If this difference in the final pattern of methylation does not account for the lack of expression in the spleen, it should be argued that hypomethylation in itself is insufficient. Some additional tissue-specific conditions are necessary for gene activation. To examine whether 5-aza-C treatment affected genes that already express in the fetus, we have determined PEPCK mRNA in the fetal kidney and albumin mRNA in the fetal liver, which are expressed in these tissues prior to birth. As shown in Figure 4, these mRNAs are observed in the appropriate fetal tissues, but their abundance is not further increased by 5-aza-C treatment.

Discussion

Association between the process of cell differentiation and tissue-specific hypomethylation (Razin & Szyf, 1984) demands

clarification of several questions: (i) What are the characteristics of a tissue-specific methylation pattern? (ii) How are the tissue-specific methylation patterns formed? (iii) What is the cause and effect relationship between the methylation pattern of gene sequences and their activity? As part of an attempt to answer these questions, we have studied the in vivo effect of 5-aza-C on the overall extent of DNA methylation of various tissues in the rat fetus and neonate. In addition, we studied the detailed methylation pattern of the PEPCK gene and its expression in the various tissues in response to the 5-aza-C treatment. Several features of the PEPCK gene make this system an ideal tool to examine the above-mentioned questions: (1) The PEPCK gene is tissue specific, being active in the liver and kidney and inactive in other tissues (Tilghman et al., 1976; Benvenisty et al., 1984). (2) The PEPCK gene undergoes terminal differentiation just before birth, which results in its activation upon birth (Ballard & Hanson, 1967; Mencher & Reshef, 1979). (3) The process of activation of the PEPCK gene is accompanied with a sequential hypomethylation (Benvenisty et al., 1985; Figure 2).

Three major observations have been made in the present study: (1) 5-Aza-C causes similar hypomethylation levels of the liver and spleen DNA while tissue-specific hypomethylation is observed in the PEPCK gene sequence. (2) Hypomethylation of the fetal liver PEPCK gene is associated with its expression. (3) Although the PEPCK gene undergoes substantial hypomethylation in the spleen, the methylation pattern obtained is not sufficient for gene expression. These observations indicate that activation of the gene depends either on a very precise process of hypomethylation of the gene or that additional factors may be needed for gene activation. The mechanism by which an overall hypomethylation (as a result of inhibition of the methylase by 5-aza-C) can result in tissue-specific patterns of methylation is still obscure. It is however clear, by examination of the patterns of the hypomethylated PEPCK gene in the liver and spleen, that the loss of methyl groups is not random; given sites are readily hypomethylated while other sites are more resistant to the loss of methyl groups. Superimposed on this site specificity is the tissue-specific susceptibility of the various sites to hypomethylation. A readily hypomethylatable site is more resistant to hypomethylation in a nonexpressing tissue. The results described here can be interpreted as follows: A decrease in methylase activity (5-aza-C) results in a nonrandom hypomethylation, which is determined by the different affinities of the enzyme to the various methylatable sites (Razin & Szyf, 1984) as it was unequivocally shown in *E. coli* (Szyf et al., 1984). In addition, a tissue-specific conformation of the DNA and/or chromatin at the gene region determines the final tissue-specific pattern of methylation. The 5-aza-C-induced pattern of methylation that we observe in the fetal liver PEPCK gene suggests that even the normal sequential hypomethylation that occurs in the fetal liver during development is restrained. In the tissue where the gene has to be expressed, specific conditions have to prevail to allow the formation of a methylation pattern favoring gene activity.

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