Sequential changes in DNA methylation patterns of the rat phosphoenolpyruvate carboxykinase gene during development
(fetus/gene expression/ontogenesis)

Nissim Benvenisty*, David Mencher*, Oded Meyuhas*, Aharon Razin†‡, and Lea Reshef*

*Developmental Biochemistry Research Unit and †Department of Cellular Biochemistry, Institute of Biochemistry, Hebrew University–Hadassah Medical School, Jerusalem 91010, Israel

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ABSTRACT The cytosolic phosphoenolpyruvate carboxykinase [PEPCK; GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] gene was isolated from a rat genomic library, and a map of the methylatable sites C-C-G-G and G-C-G-C has been constructed. The extent of methylation of 18 sites in the PEPCK gene in adult liver, kidney, spleen, and heart muscle and in fetal liver has been analyzed using the 5-methylcytosine sensitive enzymes Hpa II and Hha I. This analysis revealed extensive undermethylation of the PEPCK gene in the adult liver and kidney (PEPCK-expressing tissue), whereas the gene in adult spleen and heart muscle as well as in fetal liver (PEPCK-nonexpressing tissues) was heavily methylated. However, unlike the gene in the adult nonexpressing tissues, a region in the middle of the gene was found to be partially hypomethylated in fetal liver. This hypomethylation correlates with the competence of the fetal liver gene to be expressed. Treatment of fetuses by in utero injection of 5-azacytidine causes a hypomethylation-associated activation of the PEPCK gene. Taken together, the present findings suggest a sequential loss of methyl groups during development. When related to PEPCK gene expression, the sequential loss of methyl groups demonstrates an early stage prior to transcription characterized by hypomethylation of discrete sites and a later developmental hypomethylation of all sites associated with the mature active PEPCK gene around the time of birth.

DNA methylation patterns have been suggested to play an important role in differential gene expression in higher organisms (1–3). One approach that has been used to study the relationship between DNA methylation and gene expression is based on the analysis of the methylation pattern of individual gene sequences in different mature tissues. A large number of genes have been studied using this approach (for review, see ref. 4). Although the data obtained by this analysis suggest hypomethylation of gene sequences during development, it has been difficult to establish a cause and effect relationship with gene activity.

In a few studies, changes in the methylation patterns of specific genes during development have been analyzed (5–10). In some instances [rat liver α-fetoprotein and albumin (5, 6)], the genes are already active in the fetal liver. In others [the globin gene families (7)], various cell lineages may be involved. A recent study of the developmental changes in the methylation pattern of the chicken lens δ-crystallin II gene during early embryogenesis (8) suggests some hypomethylation events concurring with the activation of the gene, but it is still unclear whether this hypomethylation precedes gene activity. We set out to describe developmental changes in the methylation pattern of the rat phosphoenolpyruvate carboxykinase gene. Cytosolic phosphoenolpyruvate carboxykinase (GTP)

[PEPCK; GTP: oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] is a key gluconeogenic enzyme, catalyzing the first committed reaction of this pathway. Potential expression of the PEPCK gene seems to be determined by events occurring during tissue differentiation. It is highly active in the gluconeogenic organs (liver and kidney), active to a lesser extent in adipose tissue (for review, see refs. 11 and 12), and is negligibly expressed in other tissues such as spleen and muscle (13). Likewise, the PEPCK gene is not expressed in the developing fetal liver but is active at birth (14, 15). However, the gene is potentially expressible in the fetus, as indicated by induction of transcription (16–18) and synthesis of PEPCK in utero (19–21).

The relatively late developmental activation of the PEPCK gene provides an excellent opportunity to study the cause and effect relationship between methylation patterns and gene activity. Accordingly, the present report describes features of the developmental course of hypomethylation of the gene. Findings from experiments using the known DNA methylase inhibitor 5-azacytidine (4) provide evidence supporting a cause and effect relationship between hypomethylation and precocious activation of the PEPCK gene.

MATERIALS AND METHODS

Animals. Pregnant rats from overnight mating and 6-week-old male adult Sabra rats (of Wistar origin), provided by the Hebrew University breeding center, were used in all experiments. Individual littermate fetuses (gestational age, 18 days) were injected through the uterine wall as described (15, 18, 21), either with 5-azacytidine (40 μg per 10 μl of saline) or with 10 μl of saline once a day for 2 consecutive days.

Screening of the Genomic Library. The rat genomic library was a gift from James T. Bonner (22). Approximately 500,000 phages were screened for PEPCK genomic sequences using PCK−E5.4 as a probe (an EcoRI fragment of the PEPCK gene generously provided by Richard W. Hanson). Four recombinant phages containing PEPCK sequences differing from one another in the length of the flanking regions were isolated. DNA was extracted from these isolates by the plate lysate method (23) with slight modifications (24).

One of our clones was found by restriction enzyme analysis to be identical with that isolated by Yoo-Warren et al. (25); another one contains an extended 5' region (10.7 kilobase [kb]), and the other two clones have longer 3' ends (4.0 kb).

Genomic DNA Preparation. DNA from rat liver, kidney, spleen, and heart muscle was extracted from isolated nuclei following the technique described by Hewish and Burgoyne (26).

Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; kb, kilobase(s).
†To whom reprint requests should be addressed.
Restriction Enzyme Digestion and Southern Blot Analysis. Digestions were carried out at an enzyme-to-DNA ratio of 2 units/µg or 10 units/µg, as indicated, for 4 hr at 37°C. The resulting fragments were separated by electrophoresis on horizontal 1% agarose gel in a Tris acetate/EDTA buffer (27 and 28) and were transferred onto nitrocellulose filters according to the method of Southern (28). Prehybridization and hybridization were carried out essentially as described by Faliks and Meyuhas (29) for RNA blots, except for the lack of poly(dA) in the hybridization mixture.

RNA Blot Hybridization Analysis. Total RNA from fetal liver was prepared and analyzed as described (18, 30). Autoradiographic exposure was as described by Swanstrom and Shank (31).

Molecular Probes. The molecular probe pPCK-E5.4 is an EcoRI fragment of the PEPCK gene subcloned into the corresponding sites in pBR322. pNoa-2 and pNoa-3 are two cDNA clones constructed as follows: rat liver poly(A)-rich RNA extracted as described (15) from diabetic cyclohexi- mide-treated rats was further enriched for PEPCK sequences (=10-fold) by sucrose gradient centrifugation (10–25% sucrose in 10 mM Hepes, pH 7.6/1 mM EDTA/0.2% NaDodSO4). This enriched fraction was used to construct a cDNA library (24) and to transform Escherichia coli strain 5183. Approximately 2000 tetracycline-resistant transformed colonies were grown on filters and screened for PEPCK sequences by in situ hybridization with a 32P-labeled isolated EcoRI fragment of pPCK-E5.4, according to Grunstein and Hogness (32).

The purified plasmids were 32P-labeled by nick-translation (33) to a specific activity of 1×106 cpm/µg.

RESULTS

As a first step toward elucidating the pattern of methylation of the PEPCK gene in a variety of tissues of the fetal and adult rat, we mapped the recognition sites for Msp I/Hpa II (C-C-G-G) and for Hha I (G-C-G-C) in the isolated gene. Digestion of the isolated gene with Hha I and hybridization with pPCK-E5.4 as a probe revealed four fragments of 4.2, 1.35, 0.9, and 0.25 kb, respectively, and another weak fragment of 0.57 kb. Hybridization with pNoa-2, a PEPCK cDNA clone corresponding to the 3′ region of the gene (Fig. 1), clearly revealed two of the above fragments (0.9 and 0.57 kb) as well as two other fragments (0.8 and 0.2 kb). Another cDNA probe representing the middle of the gene (pNoa-3; Fig. 1) identified the other three bands (4.2, 1.35, and 0.9 kb). Although contained in this region, the 0.25-kb fragment did not hybridize with the pNoa-3 probe, because it resides in an intervening sequence. Double digestion of the gene with Hha I in combination with either EcoRI, BamHI, HindIII, or Sph I determined the final orientation of the Hha I fragments (Fig. 1).

A similar strategy was used to map the Msp I (Hpa II) sites on the PEPCK gene. First, use of pPCK-E5.4 as a probe resulted in the visualization of seven bands that correspond to 10 fragments: 1.5 kb and 1.1 kb (both of which were weakly visible), 3 fragments of 0.8 kb, 1 fragment of 0.75 kb, 2 fragments of 0.6, and one fragment each of 0.55 and 0.25 kb, respectively. The 3′ region cDNA probe (pNoa-2) revealed 2 of these fragments (1.1 and 0.75 kb), and the middle gene probe (pNoa-3) revealed 3 of the fragments (0.8, 0.6, and 0.55 kb). The orientation of the fragments was established by double digestion with Msp I in combination with either EcoRI, BamHI, HindIII, Sph I, Pst I, and Hha I (Fig. 1).

Considering that genomic PEPCK is a unique sequence (25), the detailed map of these CpG sites allowed analysis of the pattern of methylation of the gene in tissues that express or do not express PEPCK. The gene in the liver of adult rats (actively expressing PEPCK) and fetal liver or adult spleen (not expressing PEPCK), when analyzed using pPCK-E5.4 as a probe, exhibited Msp I restriction patterns identical to the isolated gene (Figs. 1 and 2). This indicated that no polymorphism of Msp I sites exists in the genomic PEPCK sequence, permitting us to use the map of the isolated gene as a reference. Digestion of the DNA with Hpa II, an isoschizomer of Msp I that does not cleave the DNA when the inner cytosine residue of C-C-G-G is methylated, yielded a tissue-specific pattern. The adult liver gene showed an extensive undermethylation of C-C-G-G sites, judged by a comparison of digestion patterns with Hpa II and Msp I. Some larger bands were consistently observed (3 and 6.15 kb) that do not seem to result from partial digestion. This conclusion is drawn from experiments in which a 5-fold amount of enzyme yielded the same pattern (Fig. 2). The small fraction of larger bands results from some methylated sites. It is most conceivable that these represent genomic PEPCK deriving from

![Fig. 1. A physical map of the rat cytosolic PEPCK gene region. Black boxes denote exons and white boxes denote introns as reported by Yoo-Warren et al. (25). M1-M11 are the Msp I (Hpa II) sites, and HI-H8 are the Hha I sites. E, Hi, B, S, and P indicate EcoRI, HindIII, BamHI, Sph I, and Pst I sites, respectively. pPCK-E5.4 is an EcoRI fragment of the gene and pNoa-2 and pNoa-3 are two cDNA clones used as molecular probes.](image-url)

![Fig. 2. Southern blot analysis of genomic PEPCK Msp I (Hpa II) sites and a methylation pattern map. 32P-labeled pPCK-E5.4 probe was hybridized to Msp I, Hpa II, or Hpa II and EcoRI digests of 10 µg of rat DNA from adult liver (lanes AL). 18-day gestation fetal liver (lanes FL), and adult spleen (lanes S). Msp I digests represent almost all the repertoire of C-C-G-G sites revealed by this probe in the gene: 0.35–1.1 kb. Bands resulting from partial digests are named according to the region on the map [i.e., total gene (7.75 kb) M1-M11 and the gene digested with EcoRI (5.4 kb) E5′-E3′]. Map below gel shows interpretation of bands with (+) indicating a methylated site, (+/-) indicating a hypomethylated site, and (-) indicating an unmethylated site.](image-url)
non-parenchymatous cells. A similar conclusion was drawn from studies with the albumin gene (5). Analysis of the kidney PEPCK gene (PEPCK-expressing tissue) revealed a pattern of methylation identical to that of the liver (results not shown).

Unlike adult liver PEPCK gene, its counterparts in fetal liver and adult spleen are both heavily methylated yet differ from one another in the pattern of methylation (Fig. 2). The bands corresponding to 4.4 and 1.85 kb are found in the pattern obtained with fetal liver DNA, while bands >8 kb are obtained especially in the pattern with spleen DNA (Fig. 2). Results identical to those of the spleen were found with the PEPCK gene from heart muscle (not shown). The PEPCK gene from spleen and heart muscle is therefore more methylated than its fetal liver counterpart. The difference between the methylation state of the gene in PEPCK non-expressing tissues and fetal liver is even more pronounced, considering that fetal hepatocytes comprise only 34% of liver nuclei on the 18th gestational day (34).

As the pattern of the methylated PEPCK DNA in adult liver (3 and 6.15 kb) resembles that of the spleen rather than the gene from fetal liver (Fig. 2), we suggest that the 1.85- and 4.4-kb fragments found only in fetal liver are unique for this tissue.

To map the hypomethylated Hpa II sites in the PEPCK gene from fetal liver and adult spleen, the 3' region cDNA probe (pN0a-2) was used (results not shown). By this analysis, the 1.85- and 3-kb fragments could be assigned to the 3' regions of the gene. Double digestion of the DNA with Hpa II and EcoRI and hybridization with the genomic DNA probe pPC-5.4 (Fig. 2) eliminated fragments >6.15 kb and a new 5.4-kb fragment appeared (Fig. 2). This result together with the fact that a 5.4-kb fragment is obtained by EcoRI digestion of the PEPCK gene (Fig. 1) indicated that the very large Hpa II bands contain the entire gene. Careful sizing of the bands resulting from the digestion, with reference to the C-C-G-G sites mapped on the gene enabled us to construct a scheme of the methylation pattern of C-C-G-G sites in the PEPCK gene of fetal and adult liver and adult spleen (Fig. 2).

A similar approach has been used to analyze the pattern of methylation of the Hha I sites in the PEPCK gene. Hha I is sensitive to methylation at the internal cytosine of G-C-G-C. As shown for C-C-G-G sites, the G-C-G-C sites in the adult liver gene are essentially not methylated. In contrast, genomic DNA from fetal liver and adult spleen is heavily methylated, as shown by Hha I digest (Fig. 3). Hha I/EcoRI and Hha I/BamHI double digestion further support these conclusions.

Therefore, we conclude that Hha I recognition sites in the adult liver PEPCK gene are essentially unmethylated, while being heavily methylated in the genes from fetal liver and adult spleen. Yet, hypomethylation of some sites of the fetal liver gene is evident (Fig. 3). Sizing of the fragments visualized by the various digests allowed us to construct a scheme describing the pattern of methylated Hha I sites in the PEPCK gene in the three tissues (Fig. 3).

By combining the patterns obtained with Hha I and Hpa II for all tissues analyzed, a statistically significant non-random distribution of 18 5-methylcytosine residues along the gene is identified (Fig. 4). Thus, the 5' flanking regions (5'-f) and a site in the middle of the gene (M7) are partially hypomethylated in both the adult spleen (and heart muscle) and fetal liver. Another partially hypomethylated region is observed only in the fetal liver in the middle of the gene (m-g) containing the consensus hypomethylated site (M7) and one methylated site (M8). Since neither spleen nor fetal liver express PEPCK, this region (m-g) may be specific for differentiated hepatocytes. Partially hypomethylated regions in the fetus become unmethylated during development of the
liver. Moreover, fully methylated regions of the gene in the fetal liver, the 5′ region (5′-g), and the 3′ region (3′-g) become unmethylated in the adult liver. These two regions may be regarded as regions specific for development of the hepatic PEPCK gene.

The detailed time course of the developmental demethylation has been followed and traced to the perinatal period. Thus, unmethylated Hpa II sites were clearly seen in the neonatal liver DNA 7 days after birth, as judged by disappearance of the 4.4-kb fragment from the 5′ region of the gene and appearance of 0.55- to 0.8-kb bands (Fig. 5, lane 3). These small fragments were already detectable as early as the third postnatal day (lane 4). Early events of hypomethylation occurring prior to birth were characterized by appearance of Hpa II fragments (1.2–1.4 kb) in the liver DNA on the 21st day of gestation (lane 6). This state of methylation has been observed through the first postnatal day and disappeared thereafter (lane 5). These results provide evidence that hypomethylation of some sites occurs between the 18th and 21st gestational day. Judged by the increase in liver DNA, the events of the developmental hypomethylation described above occur during one replication cycle prior to birth (between gestational day 18 and 21) and one cycle during the first postpartum week (34, 35). The dormant fetal liver PEPCK gene is still heavily methylated preceding birth, even at gestational day 21, in spite of its undergoing hypomethylation during the last cycle of replication. This period, therefore, was chosen to test whether precocious hypomethylation of the PEPCK gene prior to birth would result in its activation.

Fetuses were treated in utero with the methylase inhibitor 5-azacytidine daily for 2 successive days. This treatment resulted in the accumulation of PEPCK mRNA in the fetal liver (Fig. 6). The low level of PEPCK mRNA observed in livers of the control littermate fetuses is a response to the repeated in utero injections of saline. Concomitant with the appearance of PEPCK mRNA transcripts, the treatment by 5-azacytidine causes hypomethylation of the PEPCK gene, as concluded by the appearance of Hpa II fragments ranging in size from 0.55 to 0.8 kb (results to be published elsewhere). These fragments were not observed in the control littersmates.

**DISCUSSION**

The gene encoding rat cytosolic PEPCK is active in the adult liver and kidney but not in the spleen and heart muscle (11, 13). The results of the methylation pattern analysis of the gene in various tissues, revealing a complete undermethylation of the gene in the mature liver and kidney (Fig. 4), suggest that an extensive hypomethylation of the gene may be an important stage in its activation. We describe here three stages of methylation that correspond to the expressibility of the gene. One stage is represented by the heavily methylated gene in the mature PEPCK non-expressing tissues (spleen and heart muscle); the second stage by the partially hypomethylated (10 of 18 sites) and potentially expressible gene (in response to hormonal treatment in utero) (16–21) in fetal liver. The last stage is represented by the gene in mature liver and kidney, which is active and completely unmethylated in all the 18 sites. The three stages taken together indicate that a sequential process of hypomethylation of the PEPCK gene takes place during development. This process is characterized by a progressive site-specific loss of methyl groups. One group of sites—M7, M9 (Fig. 2), and sites H2-H5 (Fig. 3)—is partially hypomethylated in the fetal liver and methylated in the spleen and heart muscle. Other sites—M3-M6, M8, M10 (Fig. 2), and H6, H7 (Fig. 3)—constitute a group of sites that undergo hypomethylation later in development. These sites are methylated in the fetal liver and are completely unmethylated in the mature liver and kidney. A more rigorous study of the sequential hypomethylation reveals hypomethylation of some sites prior to birth (18 to 21 gestational days) and of others after birth (first week) (Fig. 5). In an attempt to mimic these developmental changes in

**FIG. 5.** Southern blot analysis of liver genomic PEPCK Msp I (Hpa II) sites during development. 32P-labeled pCK-E5.4 probe was hybridized to Msp I digest of 10 μg of adult rat DNA (lane 1) or to Hpa II digests of 5–10 μg of rat DNA from adult (lane 2). Neonates: 7 days old (lane 3), 3 days old (lane 4), and 1 day old (lane 5). Fetuses: 21-day gestational age (lane 6) and 18-day gestational age (lane 7).

**FIG. 6.** Induction of fetal PEPCK mRNA by 5-azacytidine. Total hepatic RNA from 5-azacytidine-treated 21-day gestational age rat fetuses and their saline-treated littersmates was extracted and analyzed by RNA blot analysis. Exp. A: lanes 1 and 2, saline control; lanes 3 and 4, 5-azacytidine-treated for 48 hr. Exp. B: lanes 5 and 6, saline control; lanes 7 and 8, 5-azacytidine-treated for 48 hr. Two amounts of RNA were used [8 μg (odd numbered lanes) and 16 μg (even numbered lanes)].
uterine, we were able to cause premature activation of the gene by 5-azacytidine (Fig. 6), which was accompanied by a variable hypomethylation of the gene sequence.

The partially hypomethylated sites along the fetal liver PEPCk gene constitute two clusters. A concensus cluster flanking the 5' end of the gene (5'-f; Fig. 4), which is found in both the spleen and fetal liver, and a second cluster, in the middle of the gene (m-g; Fig. 4), which is specific for the fetal liver. Such hypomethylated regions may represent conformational changes in the chromatin that facilitate preferential loss of methyl groups.

In several instances a correlation is observed between the degree of methylation at the 5' region of the genes and their expression (36-42). However, hypomethylation of the 5' region of the PEPCk gene, as found in the spleen, is apparently insufficient by itself to promote expression. The additional hypomethylation of the middle of the gene in fetal liver correlates with the competence of the gene to be expressed. The gene at that level of methylation could be activated by glucagon (short term stimulus) or streptozotocin diabetes (long term stimulus). However, no further hypomethylation of the gene could be detected in response to these treatments (data not shown).

The hypomethylation we observed in the fetal liver gene is a solid indication that hypomethylation, which is associated with gene activity, precedes expression. Results that suggest this same idea were reported in a study on the chicken very low density lipoprotein II (10).

However, the vitellogenin gene was shown to undergo hypomethylation at one Hpa II site, ~600 base pairs upstream from the 5' end, following activation of the gene. This hypomethylation was claimed to occur independently of DNA synthesis (43). Although this observation indicates that hypomethylated following expression may occur, it is not clear how this hypomethylated relates to the hypomethylation preceding gene expression that we describe here. This hypomethylation may be a result of another mechanism, especially if it indeed occurs in the absence of replication.

The developmental changes we observe in the methylation pattern of the PEPCk gene may reflect the in vivo mechanism for the maturation of a tissue-specific gene that becomes fully active upon birth (14, 15). The possibility we offer, that hypomethylation proceeds sequentially, has been observed before in a bacterial system (44). It is especially attractive in developmental systems, as it is in accordance with the expected track of a multistep differentiation process.

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