Methylation-dependent Silencing of the Testis-specific Pdha-2 Basal Promoter Occurs through Selective Targeting of an Activating Transcription Factor/cAMP-responsive Element-binding Site*

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Rocco C. Iannello‡§, Jodee A. Gould‡, Julia C. Young‡, Antonietta Giudice‡, Robert Medcalf¶, and Ismail Kola‡¶

From the ³Centre for Functional Genomics and Human Disease, Monash Institute of Reproduction and Development, Monash Medical Centre, 246 Clayton Road, Clayton, Victoria 3168, Australia and the ⁴Department of Medicine, Box Hill Hospital, Nelson Road, Box Hill, Victoria 3128, Australia

In this study, we demonstrate that methylation-dependent repression of the Pdha-2 core promoter is mediated regionally through a consensus activating transcription factor/cAMP-responsive element-binding site located between nucleotides −54 and −62 upstream of the major transcriptional start site. Targeting of the CpG dinucleotide within this cis-element significantly disrupts the ability of this basal promoter to activate gene expression in vitro and completely abolishes promoter activity in vivo. DNase I footprinting experiments indicated that availability of the nuclear factor(s) binding this element is limiting in sexually immature mouse testis, and as such, these factors may play an important role in the coordinate activation of early spermatogenic gene expression. Interestingly, CpG dinucleotides associated with the hypersensitive region flanking the activating transcription factor/cAMP-responsive element-binding site appear to confer some conformational structure on the promoter since mutations at these specific CpG dinucleotides result in elevated basal levels of transcription. This raises the possibility of a potential bifunctional role for CpG dinucleotides in either methylation-dependent or -independent processes. Our data support the notion that hypomethylation and transcription factor recruitment are necessary events that precede gene activation at the early stages of spermatogenesis.

The molecular processes that lead to gene activation at the onset of spermatogenesis are not well defined. Specifically, those involved in (i) the activation of early cell-specific gene expression coincident with the appearance of primary spermatocytes and (ii) the coordination of the expression of these genes during this period are poorly understood. What is becoming clearer, however, is the role of epigenetic influences on gene modulation (1–4). A number of studies have provided strong correlative evidence that suggests that demethylation and chromatin reorganization of the germ cell genome are a prerequisite for gene activation in early spermatocyte differentiation (5–7). Although DNA hypomethylation, in general, appears to be a requirement for transcriptional activation, it alone is not sufficient for the orchestrated activation of spermatocyte-specific genes. This was initially demonstrated by Ariel et al. (5), who showed a 10-day delay in transcriptional activation of Fgk-2 following demethylation of this gene in prospermatogonia. Similarly, we demonstrated that demethylated Pdha-2, a gene that encodes the testis-specific Ela subunit of the pyruvate dehydrogenase complex (8), remains transcriptionally silent in a subpopulation of spermatogonia, but is active in pachytene spermatocytes (9).

Conversely, methylation of Pdha-2 has consistently been associated with transcriptional inactivity in both spermatogonia and somatic tissues. Although the 187-nucleotide Pdha-2 basal promoter can direct transcription in a testis- and temporal-specific manner in vitro, it behaves as a strong constitutive promoter in vitro (9, 10). In a recent study, we demonstrated that this constitutive activity in somatic cells can be significantly reduced following in vitro methylation and showed that an outcome of methylation is the specific ablation of factor binding to a consensus ATF/CRE-binding site within the Pdha-2 core promoter (9). To gain further insight into the mechanisms that lead to the activation of Pdha-2, we sought to look more closely at those processes that are involved in maintaining transcriptional silencing of this gene and specifically how methylation mediates this. In this study, we investigated the relative importance of specific CpG dinucleotides residing within the Pdha-2 core promoter and to what extent the ATF/CRE-binding site and -binding factor(s) may be involved in this process. Our data indicate that methylation-dependent repression is mediated via a CpG dinucleotide within the ATF/CRE-binding site. The contributions of other CpG dinucleotides are negligible, although a pair of CpG dinucleotides flanking this binding site may contribute to a conformational state in a methylation-independent manner. Furthermore, activation of the promoter in vitro is dependent upon the binding of an ATF/CRE-binding factor that appears to be in limiting quantities early in spermatogenesis.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The wild-type Pdha-2 core promoter-CAT construct used in this study has been described elsewhere (10). Briefly, the core promoter construct contains a fragment of the Pdha-2 promoter spanning nucleotide sequences from positions −187 to +22 relative to the transcriptional start site. This promoter cassette was cloned into the CAT reporter-containing vector pCAT-Basic (Promega). In this process, the costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 61-3-9594-7207; Fax: 61-3-9594-7211; E-mail: Rocco.Iannello@med.monash.edu.au.

¶ The abbreviations used are: ATF, activating transcription factor; CRE, cAMP-responsive element; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; CREB, cAMP-responsive element-binding protein; CREM, cAMP-responsive element modulator.
Chain reaction as described by Iannello (13).

The Pdha-2 CRE-binding site is underlined. The CpG dinucleotides at both the ATF/CRE-binding site and flanking hypersensitive sites in the wild-type promoter along with various permutations in the mutant promoters are shown in uppercase letters.

In a previous study, we had established an in vivo correlation between transcriptional activation and demethylation of 5-methylcytosine nucleotides along the length of the Pdha-2 core promoter (9). In the same study, we demonstrated that in vitro methylation of this promoter by SssI methylase significantly reduced its ability to direct reporter gene expression in NIH 3T3 cells. A particularly interesting observation resulting from this earlier work was that CpG methylation specifically inhibited nuclear factor binding to an ATF/CRE-binding site within the promoter. Coinciding with a loss in factor binding was a notable loss of a DNase I-hypersensitive site residing between the ATF/CRE and MPE2-binding sites. Both the ATF/CRE site and DNase I-hypersensitive region contain CpG dinucleotides (Fig. 2). To ascertain the extent to which these changes contribute to the observed methylation-dependent silencing of promoter activity, we generated cassettes in which specific CpG dinucleotides within the Pdha-2 core promoter were mutated (Fig. 1), and the subsequent effects of in vitro CpG methylation were assessed by CAT reporter assays following transient transfection into NIH 3T3 cells (Table I).

FIG. 1. Diagrammatic representation of the nucleotide substitutions generated in the various Pdha-2 core promoter constructs. The Pdha-2 ATF/CRE-binding site is underlined. The CpG dinucleotides at positions −56 and −55 (within the ATF/CRE-binding site) with Cpa (Fig. 1). pQ1.5CAT was generated by mutating the CpG dinucleotide pair at positions −47 to −44 from CCGG to GATC. Finally, to generate pQ2.5CAT, we used the pQΔCRE promoter as template and sequence modified primers such that the final construct contains the CpA substitution within the ATF/CRE-binding site as well as the GATC substitution at nucleotides −47 to −44.

Cell Culture and Transfections—Mouse NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum and 100 units/ml penicillin and streptomycin. Cells were plated at an initial density of 5 × 10^4 cells/ml in 60-mm culture dish and maintained in a 5% CO_2 atmosphere at 37°C. The medium was changed 3 h prior to transfection. Transfection of plasmid DNA was mediated by DNA/calcium phosphate coprecipitation (11) using 15 μg of plasmid DNA/plate. Six hours following transfection, the medium was removed, and cells washed twice with serum-free medium and then incubated with complete medium until ready for harvesting 48 h later.

Generation of Transgenic Mice—The promoter-CAT constructs were linearized and prepared for pronuclear microinjection using standard procedures (12). Fertilized eggs were obtained from superovulated 6-week-old F1(CBA × C57/6J) female mice.

CAT Assays, EMSA, DNase I Footprinting, and Nuclear Extract Preparation—CAT assays and the preparation of cell extracts were performed as described previously (11) using [14C]chloramphenicol. Activity was determined by measuring acetylated and unreacted [14C]chloramphenicol products following ascending thin-layer chromatography and visualization using a Fujix BAS 1000 image analyzer. Electrophoretic mobility shift and DNase I footprinting assays were performed using nuclear extracts prepared from mouse testis and brain as described by Iannello et al. (10). In experiments in which antibodies were used in gel shift assays, antibodies were preincubated with nuclear extracts 10 min prior to the addition of the radiolabeled double-stranded ATF/CRE-binding oligonucleotide. Double-stranded oligonucleotides used in this study that spanned the Pdha-2 ATF/CRE and hypersensitive sites consisted of the wild-type pQ oligonucleotide (5'-CTTGGTGACGTAGC AACCAGG-3'), ΔCRE (5'-CTTGGTGACGTAGC AACCAGG-3'), 1.5 (5'-CTTGGTGACGTAGGACGACAG-3'), 2, and 2.5 (5'-CTTGGTGACGTAGGACGACAG-3'), Pdha-2 core promoter probes used for DNase I footprinting analysis was generated by polymerase chain reaction as described by Iannello (13).

RESULTS

CpG Methylation Abrogates Pdha-2 Promoter Activity Specifically through an ATF/CRE cis-Element—In a previous study, consistent with our previous studies, the wild-type Pdha-2 promoter (pQCAT) showed a significant reduction (68%) in activity following SssI methylase treatment compared with its untreated control (Fig. 3A). As a first step toward examining the relative contribution of specific CpG dinucleotides to this reduction, we generated a promoter construct (pQΔCRECAT) containing a single nucleotide substitution (CpG to CpA) within the ATF/CRE-binding site. A consequence of this substitution is that nuclear factor binding to the ATF/CRE cis-element is ablated as demonstrated by EMSA analysis (Fig. 4).

In this respect, the outcome of this mutation is similar to that observed following methylation of the wild-type promoter, in which binding to the ATF/CRE-binding site is abolished. Results from transient transfection assays indicated that the CpA substitution alone was sufficient to reduce promoter activity by 60% (Fig. 3A). Interestingly, there was no statistical difference between this reduction and that observed following the in vitro methylation of the wild-type promoter, suggesting that the ATF/CRE element may be central to the methylation-dependent silencing of the Pdha-2 gene. Supporting this notion are the results of experiments showing the effect of in vitro methylation on pQΔCRECAT reporter activity. In these studies, only a further 13% reduction in promoter activity was observed following SssI methylase treatment compared with the untreated construct, suggesting that the involvement of the remaining CpG dinucleotides in this mechanism may be marginal.

Additional evidence that methylation-dependent silencing of the Pdha-2 promoter may be mediated predominantly via the ATF/CRE site is derived from the analysis of Pdha-2 promoter cassettes harboring CpG mutations flanking the functional ATF/CRE site (Fig. 1). In vitro methylation of pQ1.5CAT, in which the CpG dinucleotide pair in the hypersensitive site was substituted for GATC, reduced promoter activity by 65% compared with its untreated control (Fig. 3B). The GATC substitution has no direct effect on transcription factor binding to the flanking ATF/CRE site as determined by EMSA (Fig. 4), and the CpG dinucleotide within the ATF/CRE cis-element still remains subject to methylation. Importantly, the percentage reduction in promoter activity following SssI methylase treatment of pQ1.5CAT is similar to that observed following methylation of the wild-type promoter and following perturbation of the ATF/CRE site in pQΔCRECAT. Furthermore, following the introduction of an additional CpG-to-CpA substitution within the ATF/CRE site to generate the mutant construct pQ2.5CAT, SssI methylase treatment did not confer any further inhibitory effect on pQ2.5CAT relative to its untreated control (Fig. 3B),
Regulation of a TATA-less Testis-specific Gene Promoter

A CpG Dinucleotide Pair Confers Functional Constraint on the Pdha-2 Promoter in a Methylation-independent Manner—An analysis of total CAT activities directed by wild-type and CpG mutant promoters (Fig. 5) led to an unexpected finding. Relative to cells transfected with pQCAT (Fig. 5, bar a), those transfected with pQ1.5CAT (bar b) showed 68% higher levels of CAT activity. Although modest, this elevated level of expression was consistent. A possible explanation for this result may be that the CpG dinucleotide pair residing within the hypersensitive site confers some structural or conformational constraint on the promoter. Consistent with this hypothesis is the observation that promoter constructs harboring the GATC substitution (pQ1.5CAT and pQ2.5CAT) retain higher levels of residual or basal activities compared with their respective controls following either in vitro methylation or perturbation of the ATF/CRE-binding site. As shown in Fig. 5, the residual or basal level of activity directed by the promoter cassette in pQ2.5CAT (Fig. 5, bar d) is ~2-fold higher than its respective control, pQ4CRECAT (bar c). SssI methylase treatment of pQ1.5CAT (Fig. 5, bar f) and pQ2.5CAT (bar h) had no effect on their respective residual basal levels of activity compared with their appropriate methylated controls (bars e and g). Our results suggest that the CpG dinucleotide pair flanking the ATF/CRE-binding site may confer some conformational constraint on the Pdha-2 promoter and that this function is distinct and independent of the methylation-induced repression of promoter activity mediated through the ATF/CRE-binding site.

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\begin{align*}
&\text{A} \quad \text{B} \\
&\text{Fig. 3. Histogram showing the relative mean CAT activities of the wild-type and mutant Pdha-2 promoters. A, effects of SssI methylase treatment on the wild-type (pQCAT) and pQ4CRECAT constructs. Meth indicates methylated. Values were taken from Table I, and CAT activity is expressed relative to pQ1.5CAT. B, effects of SssI methylase treatment on pQ1.5CAT and pQ2.5CAT constructs. Values were taken from Table I, and CAT activity is expressed relative to pQ1.5CAT.}
\end{align*}
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\begin{align*}
\text{TABLE I} \\
\text{Effect of in vitro promoter methylation on CAT activities in NIH 3T3 cells transiently transfected with wild-type and mutant Pdha-2 core promoter-CAT constructs} \\
\begin{array}{|c|c|c|}
\hline
\text{Construct} & \text{CAT activity}^a \text{ relative to pQCAT}^b & \text{CAT activity}^a \text{ relative to pQ1.5CAT}^c \\
\hline
\text{pQCAT} & 100 & 100 \\
\text{pQCAT meth} & 32 \pm 9.8 & 32 \pm 9.8 \\
\text{pQ4CRECAT} & 40 \pm 5.9 & 40 \pm 5.9 \\
\text{pQ4CRECAT meth} & 27 \pm 1.0 & 100 \pm 11.9 \\
\text{pQ1.5CAT} & 168 \pm 20.2 & 100 \pm 11.9 \\
\text{pQ1.5CAT meth} & 60 \pm 24.1 & 44 \pm 9.6 \\
\text{pQ2.5CAT} & 88 \pm 11.9 & 52.4 \pm 7.1 \\
\text{pQ2.5CAT meth} & 74 \pm 14.5 & 43.8 \pm 8.5 \\
\hline
\end{array}
\end{align*}
\]

\[^a\text{CAT activity was calculated as the percentage conversion of chloramphenicol% extract, adjusted for background and expressed as percent arbitrary units.} \]

\[^b\text{The relative mean was calculated as the percentage mean relative to pQCAT.} \]

\[^c\text{The relative mean was calculated as the percentage mean relative to pQ1.5CAT.} \]

\[^d\text{Meth designates Pdha-2 promoter constructs methylated in vitro by SssI methylase as described under "Experimental Procedures."} \]
promoter is in a hypomethylated state and transcriptionally active in spermatocytes (9). However, in the same study, we demonstrated that hypomethylation of the \textit{Pdha-2} promoter is initially detectable in spermatogonia, in which this gene is inactive. It has been postulated that during the spermatogonium-spermatocyte transition, a number of events occur that may potentiate transcriptional activity, including the recruitment of transcription factors. To gain some insights as to what mechanism(s) maintain silencing of \textit{Pdha-2} in spermatogonia, we examined the pattern of transcription factor binding on the \textit{Pdha-2} promoter during sexual maturation in mice. This was achieved by performing DNase I footprinting assays using testis nuclear extracts prepared from mice at various stages of sexual maturation. The results shown in Fig. 7 indicate that in testes of 8-day postnatal mice, the transcription factor Sp1 was present in sufficient quantities in our extracts to protect its cognate binding site from DNase I nicking. By day 15, protection was also apparent over the YY1- and MEP2-binding sites. By day 20, all known transcription factor-binding sites residing within the \textit{Pdha-2} core promoter were protected. At this stage, primary spermatocytes are abundant, and \textit{Pdha-2} transcripts are easily detectable by Northern blot analysis (8). The lack of protection of the ATF/CRE-binding site in extracts from 15-day-old mouse testis is, however, not indicative of an absence of factors that bind to this site. Indeed, earlier EMSA studies (10) clearly demonstrated that protein-DNA complexes involving the ATF/CRE site were formed in nuclear extracts prepared from testes of mice as early as postnatal day 12. These data more likely suggest that factors that recognize the ATF/CRE-binding site are present, but less abundant relative to other nuclear factors during the early stages of spermatogenesis.

The identity of the specific factor that recognizes the ATF/CRE site within the \textit{Pdha-2} promoter has not yet been determined. EMSA analysis using adult mouse testis and brain nuclear extracts showed two distinct patterns (Fig. 8). Brain extracts were used in these experiments for comparative anal-

FIG. 4. EMSA analysis showing the effects of nucleotide substitutions within the ATF/CRE and flanking hypersensitive sites on transcription factor binding. Radiolabeled double-stranded oligonucleotides (2 ng) containing both these regions were incubated with 10 μg of NIH 3T3 nuclear extracts. The wild-type \textit{Pdha-2} ATF/CRE and hypersensitive sites are represented by the double-stranded oligonucleotide designated \textit{pq}. The double-stranded oligonucleotide ΔCRE is identical to \textit{pq} with the exception of a CpA substitution of Cpg within the ATF/CRE-binding site. Nucleotide substitutions in the hypersensitive site but containing a functional ATF/CRE site are represented in the double-stranded oligonucleotide 1.5. The oligonucleotide designated 2.5 is identical to 1.5, except that it also contains the Cpa substitution within the ATF/CRE-binding site. Nucleotide substitutions in the hypersensitive site but containing a functional ATF/CRE site are represented by the double-stranded oligonucleotide designated \textit{pQ}.

FIG. 5. Histogram showing the percentage \textit{Pdha-2} promoter-driven CAT activities assayed in NIH 3T3 cells. Bar a, \textit{pQCAT}; bar b, \textit{pQ1.5CAT}; bar c, \textit{pQΔCRECAT}; bar d, \textit{pQ2.5CAT}; bar e, SssI methylase-treated \textit{pQCAT}; bar f, SssI methylase-treated \textit{pQ1.5CAT}; bar g, SssI methylase-treated \textit{pQΔCRECAT}; bar h, SssI methylase-treated \textit{pQ2.5CAT}. Values were taken from Table I, and all CAT activities are presented unbound labeled double-stranded oligonucleotide. The open arrow represents complexes bound to the labeled double-stranded oligonucleotide.

FIG. 6. Representative CAT assay performed on mouse testes obtained from transgenic lines harboring the wild-type promoter (\textit{pQCAT}; A), \textit{pQ1.5CAT} (B), or \textit{pQ2.5CAT} (C). Mouse lines for each of the transgenic constructs are numbered individually. +, CAT-positive control; −, nontransgenic negative control.
The objective of this study was to gain an understanding of the mechanisms responsible for the transcriptional silencing of *Pdha-2*. During spermatogenesis, *Pdha-2* expression coincides with the appearance of early primary spermatocytes (8, 14), whereas in the spermatocyte precursor cells (spermatogonia), transcriptional activity is silenced. Similarly, no detectable levels of *Pdha-2* transcription are observed in somatic tissue. In each case, methylation of the *Pdha-2* promoter has been demonstrated to functionally correlate with the absence of activity (9). Interestingly, prior to transcriptional activation, the *Pdha-2* promoter undergoes demethylation in a subpopulation of spermatogonia, but remains inactive until these cells undergo differentiation into primary spermatocytes. It has been proposed that during this spermatogonium-spermatocyte transition, transcriptional activation is preceded by a number of events that subsequently lead to activation. This period of “potentiation” (6) is believed to involve epigenetic modifications and changes in transcription factor constituency.

Before we can gain insights into this process, we sought to better understand the molecular mechanisms by which methylation-dependent inactivation of *Pdha-2* is mediated. The TATA-less and CAAT-less *Pdha-2* core promoter is composed of ~187 base pairs and contains four major transcription factor-binding sites, three of which are the Sp1, ATF/CRE, and YY1 sites. Although the *Pdha-2* promoter is not a CpG island-containing promoter, several CpG dinucleotides reside within or in close proximity to these transcription factor-binding sites. Therefore, our initial investigations were directed at determining the relative contribution of some of these specific CpG dinucleotides to the modulation of *Pdha-2* promoter activity. Our analysis established that the CpG dinucleotide located within the ATF/CRE-binding element is primarily responsible for mediating methylation-dependent silencing of this promoter in vitro. This conclusion was further strengthened by our observation that this site is critical for functional activation of the promoter in vivo. In contrast, methylation of CpG dinucleotides located within or close to the Sp1- and YY1-binding sites does not appear to contribute to the silencing of the *Pdha-2* promoter, although our data do not exclude a possible contribution that may be masked as a result of a reduced basal activity level following the ablation of ATF/CRE binding. Nevertheless, the data are consistent with previous studies that have demonstrated both transcription factor sensitivity and insensitivity to CpG methylation on several binding sites (15, 16).

The delay in transcriptional activation of hypomethylated genes during the spermatogonium-spermatocyte transition period was originally observed with *Fgk-2* by Ariel et al. (5). Since *Pdha-2* behaves in a similar manner, this suggests that hypomethylation is an early component in the potentiation process and that it precedes other events such as transcription factor recruitment and/or chromatin reorganization. Given the importance of the ATF/CRE-binding site for *Pdha-2* activity, we performed DNase I footprinting assays to determine whether availability of the factor(s) that bind to this site could offer an explanation for the latent activation of the promoter following hypomethylation. Our data clearly show that the factor(s) binding to this site is limited in nuclear extracts prepared from testis coincident with early stages of spermatogenesis. Similar experiments using extracts prepared from purified spermatogonia and spermatocytes will be required to determine unequivocally whether our data correlate with a spermatocyte-specific appearance of this factor. Nevertheless, our results
strengthen the notion that transcription factor recruitment/availability following hypomethylation may, in part, account for the delay in the activation of the \( Pdha-2 \) promoter and are further evidence implicating the ATF/CRE site as central for promoter functionality.

The specific factor that recognizes the ATF/CRE site within the \( Pdha-2 \) promoter has not yet been determined. EMSA analysis using adult mouse testis and brain nuclear extracts showed two distinct patterns. A single complex is formed with brain extracts that can be supershifted by antibodies raised against CREB and that is predominantly abolished by antibodies raised against CREM. However, in testis, multiple complexes are observed, the majority of which remain unaltered by the presence of either the anti-CREB or anti-CREM antibodies. Furthermore, antibodies raised against either the ATF-1 or ATF-2 transcription factor also fail to alter the pattern. CREM may be excluded as a candidate since CREM activator protein is expressed only in haploid germ cells (17). The role of CREB in spermatogenesis is unclear since knockout mice null for CREB show no spermatogenic defects (18). However, given that (i) the integrity of the pyruvate dehydrogenase complex is central for mitochondrial function and (ii) the factor binding to the ATF/CRE site in the \( Pdha-2 \) promoter is essential for activity, the absence of any apparent deleterious spermatogenic phenotype with the CREB-null mice would also suggest that CREB is an unlikely candidate for the factor that binds to the \( Pdha-2 \) promoter during the early stages of spermatogenesis. Identification of this factor may prove to be significant in that it may provide us with some insights into early spermatogenic differentiation akin to the role CREM plays in spermiogenesis.

An interesting observation from our analysis of the \( Pdha-2 \) promoter was the elevation in promoter activity following mutagenesis of a pair of CpG dinucleotides flanking the ATF/CRE-binding site. Although the elevation is modest, it needs to be viewed in the context of a basal promoter-driven activity. It would be interesting to determine if a higher level of activation could be attained if these mutations were present in vivo. Experimentally, this would have to be performed in the context of the highly active full-length promoter since the activity of the core promoter alone in vivo is particularly low (9), making differences between pQCAT and pQ1.5CAT difficult to detect. Indeed, transgenic mouse lines harboring either pQCAT or pQ1.5CAT show no significant differences when comparing CAT activities driven by the transgenic promoters. Random integration, copy number, relatively low basal activities, and the limited number of transgenic lines for each construct \((n = 5)\) make an accurate comparison difficult. Our in vitro data are, however, not entirely surprising. Crystal engineering experiments have revealed a specific role for CpG sequences in DNA-DNA recognition, where they act as anchoring points for DNA self-fitting (19). As such, it has been suggested that CpG dinucleotides may participate in the formation of higher order DNA structures. Therefore, perturbations in gene expression through CpG mutations may occur in at least one of three ways: first, through the disruption of transcription factor-binding sites, thereby inactivating or reducing transcriptional activity; second, by rendering local regions along regulatory DNA sequences incapable of being methylated, thereby maintaining active transcription; and finally, a third process whereby mutations of specific GpG dinucleotides, which do not play a role in regional methylation-mediated modulation, affect the three-dimensional organization of DNA segments and subsequently gene expression. In our case, mutations of the CpG dinucleotide pair in the \( Pdha-2 \) core promoter resulted in an elevated level of transcriptional activity. The observed increased activity is not likely to have occurred as a result of changes in protein-DNA interactions since footprinting studies demonstrated no observable differences in protection of transcription factor-binding sites when comparing pQ1.5CAT and wild-type pQCAT.\(^2\) One mechanism by which mutations in the CpG dinucleotide pair flanking the ATF site may modulate transcriptional expression is through a change in DNA conformation such that it facilitates greater stability in protein-protein interactions. This would be consistent with our in vitro transfection data and footprinting analysis. Given that CpG dinucleotides are under-represented in higher eukaryotic genomes due to the high mutability of methylated cytosine and that this is thought to contribute to genetic disease and cancer (20), our data may point to another model of transcriptional deregulation that may explain some of these disorders.

In conclusion, to better understand the mechanisms that orchestrate the activation of testis-specific genes in early spermatogenic cells, we sought to investigate the processes that precede activation. Using the \( Pdha-2 \) core promoter as a model of study, we conclude from our data that methylation-dependent repression acts via a local rather than global mechanism and that this is mediated through the ATF/CRE-binding site. Availability of the factor(s) that bind to this site may also add another level of modulatory complexity. Since the ATF/CRE-binding site is widespread, ablation of factor binding to this site through methylation may be a common mechanism by which silencing of early spermatogenic genes can be achieved.

REFERENCES

1. Eden, S., Hashimshony, T., Keshet, I., and Cedar, H. (1998) Nature 394, 842
2. Jones, P. L., Jan Vernostra, G. C., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J. and Wolffe, A. P. (1998) Nat. Genet. 19, 187–191
3. Kass, S. U., Pruss, D., and Wolffe, A. P. (1997) Trends Genet. 13, 444–449
4. Siegfried, Z., and Ceder, H. (1997) Curr. Biol. 7, R305–R307
5. Kramer, J. A., McCarron, D. R., Djakiew, D., and Krawetz, S. A. (1998) Development 125, 4749–4755
6. Zhang, P. L., Stroud, J. C., Walker, C. A., Adrian, G. S., and McCarron, J. R. (1998) Biol. Reprod. 59, 284–292
7. Iannello, R. C., and Dahl, H. H.-M. (1992) Biol. Reprod. 47, 48–58
8. Iannello, R. C., Young, J., Summarsono, S., Gould, J., Tymms, M., Dahl, H. H.-M., Hedger, M., and Kola, I. (1997) Mol. Cell. Biol. 17, 612–619
9. Iannello, R. C., Kola, I., and Dahl, H. H.-M. (1993) J. Biol. Chem. 268, 22581–22589
10. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051
11. Hogan, B., Constantini, F., and Lacy, E. (1986) Manipulating the Mouse Embryo, Cold Spring Harbor Press, Cold Spring Harbor, NY
12. Iannello, R. C. (1995) in Methods in Molecular Biology: In Vitro Transcription and Translation Protocols (Tymms, M. J., ed) Vol. 37, 379–391, Humana Press Inc., Totowa, NJ
13. Takakubu, F., and Dahl, H. H.-M. (1992) Exp. Cell Res. 199, 39–49
14. Sugiuchi-Ariga, S. M. M., and Schaffner, W. (1988) Genes Dev. 3, 612–619
15. Tate, P. H., and Bird, A. (1993) Curr. Opin. Genet. Dev. 3, 226–231
16. Steger, K. (1999) Anat. Embryol. 199, 471–487
17. Hummer, K., Cole, T. J., Blindy, J. A., Ganss, R., Aguzzi, A., Schmid, W., Beermann, F., and Schutz, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5647–5651
18. Mayer-Jung, C., Moras, D., and Timsit, Y. (1997) Proc. Natl. Acad. Sci. U. S. A. 91, 5647–5651
19. Jackson-Griseby, L., and Jaenisch, R. (1996) Semin. Cancer Biol. 7, 261–268
20. R. C. Iannello, J. A. Gould, J. C. Young, and Kola, unpublished data.
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