Interactive report

Site-specific DNA hypomethylation permits expression of the IRBP gene

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Abstract

Interphotoreceptor retinoid binding protein (IRBP), a putative component of the visual cycle, is expressed selectively in the retina and pineal gland. This study examined whether site-specific DNA hypomethylation plays a role in this expression regulation. Southern blotting of HpaII and MspI digests of DNA from various bovine and murine tissues (whole brain, retina, pineal gland, superior colliculus, cortex, thymus, habenular nucleus, cornea, liver, tail, and kidney) revealed that specific Cpg dinucleotides in the IRBP gene promoter are hypomethylated in DNA from retinal photoreceptor cells and pineal gland compared to DNA from other tissues. These sites are methylated in DNA from non-photoreceptor retinal cells. Exogenous methylation of these sites diminished DNA-protein binding in electrophoretic mobility shift assays. HpaII methylation of chloramphenicol acetyltransferase reporter constructs suppressed IRBP but not SV40 promoter activity in transiently transfected primary cultures of embryonic chick retinal cells. These data indicate that specific cytosines in the bovine and murine IRBP promoters are unmethylated in photoreceptive cells but methylated in other tissues. This differential DNA methylation may modulate IRBP gene expression since exogenous methylation of the murine sites suppresses reporter gene transcription, apparently by inhibiting DNA-protein binding events. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Interphotoreceptor retinoid binding protein (IRBP), a large glycoprotein found selectively in photoreceptive tissues including photoreceptors and pinealocytes. It is an extracellular protein found abundantly in the interphotoreceptor space of many vertebrate eyes, binds retinoids and fatty acids, is needed for normal retina morphology and function, and possibly has a role in the visual cycle [12,14,36,47,48,50]. IRBP gene expression is highly regulated. Its mRNA appears at an early and discrete developmental stage, just before the onset of photoreceptor outer segment formation [16,64,68,71], and is limited to the pineal gland and retina [51,69]. IRBP and its mRNA level also are sensitive to lighting [33] and rhythmic cues [52]. As with other genes, this regulation in part derives from the binding of nuclear proteins (trans-acting factors) to DNA targets (cis-acting elements) in the gene and in regions flanking it. Numerous studies have demonstrated cis:trans binding events, especially in the IRBP 5’ flanking region, and functional relevance has been ascribed to them [5,7,18,24].

Gene expression also may be modulated by promoter DNA methylation. For several genes, the amplitude of this methylation is inversely proportional to expression [20,63], and may be associated with stage and tissue specificity [17,32,49,55,59,65]. Most vertebrate DNA is de novo methylated at cytosine residues of CpG dinucleotides [11,20]. As embryogenesis progresses, specific sites are demethylated, permitting transcription [19,23,54]. DNA methylation probably suppresses gene expression by alter-
ing chromatin structure such that the gene promoter becomes inaccessible to transcription factors (for reviews, see [30,43,53]) and possibly by directly hindering the binding of stimulatory trans-acting factors to methylated cis-acting elements [25,26,29,40,41,46,56,58,66,67,74].

If the stage- or tissue-specificity of IRBP transcription is due to hypomethylation, potential IRBP promoter methylation sites might be methylated in non-retinal tissue and unmethylated in the retina. A simple way to analyze CpG dinucleotides for the presence or absence of methylation is to take advantage of the methylation sensitivity of restriction enzymes. For example, HpaII and MspI are iso-schizomers that recognize the sequence CCGG. Methylation of the internal C residue blocks the activity of HpaII but not that of MspI. Genomic digests using the two enzymes should produce identical banding patterns in Southern blots unless sites are methylation-protected. Using this approach, Liou et al. [37] found that at least one HpaII site is specifically hypomethylated in mouse retina beginning at embryonic day 11 (E11) and peaking at postnatal day 4 (P4). Additionally, Albini et al. [1] found that the IRBP promoter of human Y-79 retinoblastoma cells, which produce IRBP, is generally less methylated at one HpaII site than the IRBP promoter from human lymphocytes, which do not produce IRBP mRNA. However, neither group determined whether these methylation differences are functionally relevant; that is, whether DNA methylation regulates gene expression.

Here we show that specific sites in the IRBP 5' flanking region are hypomethylated in DNA from photoreceptors compared to DNA from non-photoreceptor cells and nonretinal tissues. Electrophoretic mobility shift experiments show that DNA methylation suppresses protein binding at two of these sites in the mouse sequence. From these data, we hypothesized that site-specific methylation of the IRBP 5' flanking region should block IRBP promoter activity in photoreceptor cells. Significantly, we find that exogenous methylation of the two sites suppresses mouse IRBP promoter activity in primary cultures of chick embryonic retinal cells. These data establish a cause and effect relationship between tissue-specific DNA methylation patterns and IRBP gene expression.

2. Materials and methods

2.1. Animals

These studies adhered to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. BALB/c and C3H/hen mice were from Jackson Labs (Bar Harbor, ME). B6D2/F1 and FVB/N mice were from Taconic (German-town, NY). The BALB/c and B6D2/F1 mice were used as ‘wild type’ controls. The C3H/hen and FVB/N mice were shown to be rd1°C/rd1°C by PCR analysis and histology. Bovine tissues were obtained at a slaughterhouse (Pel-Freez Biologicals, Rogers, AR).

2.2. Southern blots

Conventional techniques were employed to digest DNAs, run the gels, and blot transfer them [61] (as modified in [9]; see Sambrook et al. [39] for details). HpaII and MspI digests were carried out with 10 µg DNA and 100 U each enzyme in buffer L (Boehringer/Mannheim, Indianapolis, IN) for 10 h at 37°C. 1.5% agarose gels were run in 1X TAE buffer and transferred to Hybond N membranes from Amersham (Arlington Heights, IL). Membrane blots were probed with a DNA fragment of the mouse IRBP gene corresponding to positions −1783 to +101, where the start of transcription is at position +1. For bovine blots, a probe corresponding to positions −4516 to +111 was used. DNA was random-prime labeled with fluorescein-11-dUTP and probe was detected using the ECL Detection System and Hyperfilm-ECL (Amersham). Autoradiograms were scanned with a BioRad GS-700 scanner at 600 by 600 dpi, with a 12 bit A/D converter (4096 shades of gray) and analyzed with “Molecular Imager” software (BioRad, Hercules, CA).

2.3. Promoter activity assays

As previously detailed, primary cultures of embryonic chick retinal cells can be transiently transfected with plasmid vectors containing reporter genes whose expression is regulated by retina-specific and viral promoters [5,6,70]. In a procedure modified from Werner et al. [70], whole retinas from embryonic day 10 (E10) chicks were trypsinized, triturated through a small-bore glass pipet, and plated onto 60 mm plastic culture dishes pretreated with polyornithine at a density of 20×10⁶ cells per dish. Cultures were incubated in Medium 199 supplemented with 10% fetal calf serum for 24 h at 37°C under an atmosphere of 5% CO₂. Cultures then were transfected with plasmid vectors (10 µg DNA) by CaPO₄ precipitation [6,70] containing the gene encoding chloramphenicol acetyltransferase (CAT) and various promoters. After incubation for 16 h, they were fed with fresh medium and incubated for an additional 48 h, fed once more, incubated another 24 h, then harvested by scraping. Cells were centrifuged at 5000×g, medium removed, and the pellet resuspended in 100 µl of 0.1 M Tris–HCl pH 7.8. Suspensions were stored at −80°C until assayed for CAT activity.

To determine CAT activity, cells were sonicated for 2 min on ice using a Vibrancell cup-horn sonicator (Sonic & Materials, Danbury, CT). Sonicates were centrifuged at 15 000×g for 5 min. Supernatants were transferred to fresh 1.5 ml microcentrifuge tubes and heated at 65°C for 15 min. Tritiated Acetyl-CoA (New England Nuclear, Boston, MA) and chloramphenicol (Sigma Chemicals, St.
Louis, MO) were added to the supernatants and this mixture immediately added to an organic scintillation liquid [42]. CAT activity, which was taken as an index of promoter activity, was measured as the accumulation of ³H-acetylatedphamocin in scintillation fluid over time on an LS6500 scintillation counter (Beckman Instruments, Schaumberg, IL).

The promoterless CAT vector pBLCAT3 [38] served as a negative control. The positive control contained the SV40 promoter and enhancer regions (pCAT Control, Promega, Madison, WI) and is referred to as pSV40. The experimental plasmid was the pBLCAT3 vector with an insert comprised of bases −1783 to +101 (relative to transcription start) of the murine IRBP sequence (Genbank Accession AF126968) upstream of the CAT gene. This plasmid is referred to as pIRBP.

To methylate HpaII sites in pSV40 and pIRBP, 200 µg of each plasmid were reacted with 80 U of HpaII methylase (New England Biolabs, Beverly, MA) over 16 h. Fresh S-adenosylmethionine (80 µM F.C.) was added at the start of the reaction and 6 h later. The methylation status of plasmids was determined by HpaII digestion followed by electrophoresis across a 1% agarose TAE gel (data not shown). Promoter data are group means ± SEM and were statistically analyzed by a one-way analysis of variance with Student-Newman-Keuls post hoc analysis.

2.4. Electrophoretic mobility assays (EMSA)

Briefly, mixtures of crude nuclear extracts and a radio-labeled DNA probe were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE). The migration of DNA bound by proteins was retarded compared to free probe. Bound complexes were visualized by autoradiography as slower migrating bands. Assay conditions were optimized for each probe in terms of nonspecific competitor DNA or dye analogs (to suppress nonspecific protein-DNA interactions) and probe concentration [5, 27, 35].

Oligonucleotides were used (sense strand shown): TGTACTAGTGTCCGGACCTTTCATTCATCTT (−735 to −705) and TCCTTCCGGGCTCAAACCAC (−120 to −101). Both strands of a complementary pair were end-labeled with ³²P-γ ATP using polynucleotide T4 DNA kinase (GIBCO-BRL, Grand Island, NY) following the manufacturer’s suggestions, then purified on G-6 BioSpinR columns as per manufacturer’s suggestions (BioRad). Complementary oligonucleotides were annealed by incubation together in 50 mM KCl for 5 min at 90°C in the aluminum block of a dry bath incubator. The block was placed on the bench top until it equilibrated with ambient room temperature over a period of about 2 h. The concentration of the DNA was determined by spectrophotometry and by fluorescence of intercalated Hoechst 33258 (Hoefer Scientific Instruments, San Francisco, CA).

Oligonucleotides were enzymatically methylated (after radiolabeling) as the double-stranded substrate for HpaII methylase. About 10 µg of double-stranded DNA were incubated at 37°C for 4 h with 10 U of HpaII methylase and 80 µM S-adenosyl-methionine or methionine (the latter for unmethylated control) in 50 mM Tris pH 7.5, 10 mM EDTA, 5 mM 2-mercaptoethanol, as per the manufacturer’s suggestions. Complete methylation of probes was confirmed by HpaII digestion followed by PAGE.

Mouse nuclear extracts were prepared immediately before use by the method of Andrews and Faller [2]. When several tissues were used, they all originated from the same mouse to minimize possible experimental variations. Briefly, tissues were homogenized at 4°C in 1.5 ml microcentrifuge tubes using a hand-held homogenizer (Kontes, Vineland, NJ) in 10 mM HEPES-KOH pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF and 200 µg/ml each leupeptin, pepstatin, and chymostatin (all compounds from Sigma Chemicals). Nuclei were allowed to swell on ice for 10 min then vortexed for 10 s. Samples were spun in a microcentrifuge (14 000×g) for 10 s and the supernatant discarded. The pellet was resuspended in 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM PMSF and 200 µg/ml each leupeptin, pepstatin, and chymostatin and incubated on ice for 20 min. The solution was cleared by centrifugation for 2 min at 14 000×g, 4°C. The supernatant was transferred to a fresh 1.5 ml conical tube and protein content determined by BCA assay (Pierce, Rockford, IL).

Several experiments were conducted to optimize the type and concentration of disaggregating agent required to inhibit nonspecific complex formation (poly dIdC [Phar- macia Biotech Inc., Piscataway, NJ) and the protein binding dyes R-478, T-123, and S-119 [Sigma Chemicals, St. Louis, MO [35]]. For the probe containing the promoter activity, was measured as the accumulation of and 80

of bound probe. In determining the effects of methylation on binding, the amount of bound methylated probe was compared to the amount of bound unmethylated probe.

3. Results

3.1. Bovine genomic digests and southern blots

Sequence analysis of the bovine IRBP 5' flanking region and gene (GenBank Accession M32733) shows that there are CCGG sites at positions −4094, −2003, −1870, −1210, −699, and +116 relative to the transcription start site. If these sites exist, digestion of genomic DNA with MspI should result in bands of 2091, 133, 660, 511, and 815 bp in Southern blots (Fig. 1). Excepting one, all bands were detected in DNA from retina, pineal gland, cornea, liver, superior colliculus, cortex, thymus, and habenular nucleus (Fig. 1), indicating and verifying the existence of all of the predicted CCGG sites. As expected, the exception, the 133 bp band, was not seen in any blots as it is too short to be detected under these electrophoresis, transfer, and hybridization conditions (Fig. 1).

Unlike the MspI digestion patterns, the retina and pineal digestion patterns with HpaII differ markedly from that of other tissues, with digestion products of 2884, 2091, 1326, 815, 660, and 511 bp (Fig. 1). These products are not apparent or are comparatively weak with HpaII digests of DNA from other tissues. The retina and pineal bands at 2091, 815, 660, and 511 represent fully digested products and show that the sites at −4094, −2003, −1870, −1210, −699, and +116 are not methylated in DNA from some cells of the retina and the pineal gland. The two prominent bands at 2884 and 1326 bp probably represent mixtures of methylated and unmethylated CCGG sites at −2003, −1870, and −699 bp that may result from the mixed populations of cell types included in the retinal and pineal preparations (e.g., inner retina cells and vascular and connective tissue, which do not express IRBP and hence should be methylated, and photoreceptors or pinealocytes, which do express IRBP and should be hypomethylated).

Alternatively, the 2884 and 1326 bp fragments could result from partial digestions (as may have been the case in with retinal MspI digests, which show a 1326 bp band).

As noted, shorter bands (660, 511, and 815 bp) only appeared in HpaII lanes of retinal or pineal tissue, suggesting that the more proximal CCGG sites are uniformly methylated in other tissues. However, faint HpaII bands can be seen at the higher molecular weight regions in some of the bovine tissues that do not express the IRBP gene. A faint band at 2884 bp in most tissues suggests that the CCGG sites at −4094 and −1210 are sometimes hypomethylated even in tissues that do not express the IRBP gene. These two sites may not play a stringent role in IRBP gene expression.

3.2. Mouse genomic digests and southern blots

Sequence analysis of the mouse IRBP 5' flanking region (see GenBank Accession AF126968) reveals CCGG sites at positions −725, −115, and +153 (Fig. 2). Digestion with MspI and HpaII shows differential patterns of the methylation of two sites in the 5’ flanking region of the mouse IRBP gene. MspI digestion resulted in 1578 and 610 bp fragments for samples from retina, whole brain, tail, liver, and kidney, indicating that CCGG sites exist at −725, −115, and a deduced site at −2303. However, HpaII digests produced 1578 and 610 bp fragments only with retinal DNA (Fig. 2), indicating hypomethylation at these sites compared to nonretinal DNA. (As expected, the predicted 267 bp fragment does not appear probably due to its small size and the assay conditions.)

The presence of faint bands at 2188 and 2455 bp in the retinal HpaII lanes suggests that some DNA in the retina is methylated at the −725 site, producing the 2188 bp band, and at both the −725 and the −115 sites, producing the 2455 bp band. Alternatively, these bands may indicate incomplete digestion at these sites, as suggested by the appearance of a 2188 bp band in some MspI lanes. Photoreceptors are the only retinal cells that contain IRBP.

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The GenBank accession number for the mouse IRBP gene and 5' flanking region is AF126968. That for the bovine sequence is M32733.
mRNA [51,69]. It appears that IRBP DNA from non-photoreceptor retinal cells is methylated at these two sites, hence producing the 2188 and 2455 bp bands in retinal lanes. To test this hypothesis, we compared the methylation status of genomic DNA from the retinas of rd1−/rd1− mice with that of wild type mice. The rd1−/rd1− mutation causes the total degeneration of the photoreceptor cell layer but remarkably leaves the inner cell layers intact (Fig. 3) [15,57]. In Southern blots probing DNA from these retinas, the dominant signals are from non-photoreceptor DNA. While this DNA is not necessarily homogeneous in its methylation pattern for all genes, the rd1−/rd1− retina HpaII lane of Fig. 2 shows a band detectable at 2455 bp but not 1578 bp or 610 bp. This indicates that both the −725 and −115 sites are methylated in non-photoreceptor cell retina nuclei and highlights the specific demethylation of these two sites only in photoreceptor cells.

### 3.3. Protein binding properties of the DNA methylation sites

The Southern blotting data indicate that hypomethylation at the murine −725 and −115 CCGG sites correlates with photoreceptor-specific IRBP expression. If IRBP transcription requires hypomethylation of these sites, it may be that methylation alters the binding of nuclear factors to sequences near or subsuming these sites. To determine whether sequences containing these sites bind nuclear proteins, we tested two radiolabeled DNA fragments containing these sites in EMSAs probing against crude nuclear extracts from retina and other tissues. Both probes bound nuclear complexes (Figs. 4 and 5), suggesting that the sequences might contain cis elements. Figs. 4 and 5 are representative autoradiograms of experiments testing probe −725 and probe −115 sites, respectively. In Fig. 4, two complexes (Bands 1 and 2) are seen in most lanes with unmethylated probe (lanes marked ‘U’), indicating that probe −725 is bound by complexes common to many tissues. The density of Band 1 dropped by about 26% with methylation (lanes marked ‘M’) across tissues. Methylation suppressed the density of Band 2 to background levels. It should be noted that Band 2 was apparent in only two of the four repetitions of this experiment. Densitometry took into account background densities for each lane. The effect of probe methylation on binding with retinal nuclear extracts was tested in several additional experiments. Unmethylated Band 1 optical density averaged 12.4±2.6% of total lane density, whereas methylated Band1 averaged 6.1±2.0%, a statistically significant difference (P=0.0147 by paired, two-tailed t-test, N=9). Thus, methylation of the DNA probe decreased relative binding of a retinal nuclear complex by about 51%.

![Southern blots of murine genomic digests and map of CCGG sites](Image 47x539 to 282x730)

**Fig. 2.** Southern blots of murine genomic digests and map of CCGG sites. Genomic DNA was digested by MspI (M) or HpaII (H) and Southern blotted as outlined in previous figure and text. Blots were probed with a fluorescein-labeled fragment (−1783 to +101) of the murine IRBP gene. The diagram below the blots shows the CCGG sites in a fragment of the murine IRBP gene, which are indicated by vertical marks and numbers below, with numbering relative to transcription start. The CCGG site at −2303 is deduced from fragment sizes. MspI digests (methylation insensitive) indicate that predicted CCGG sites exist in all tissues. HpaII digests (methylation sensitive) show that DNA from wild type retina (far left lane), but not other tissues, is cleaved at the CCGG sites, indicating that this region of the IRBP gene is hypomethylated in retina. The appearance of 2188 bp and 2455 bp bands in HpaII lanes indicates that CCGG sites at −2303 (deduced) and +153 are unmethylated but intervening sites at −725 and −115 are heterogeneously methylated. HpaII digests of DNA from retinas lacking photoreceptors show no bands of 1578 or 610 bp, indicating that the sites at −725 and −115 are methylated in inner retina cells. This suggests that the heterogeneous methylation seen with whole retina DNA is due to differences in photoreceptor vs. inner retina DNA. Data are from four blots, as indicated by separation. Autoradiograms were scaled to align bands.

![Autoradiogram of Southern blot](Image 310x651 to 549x728)

**Fig. 3.** Comparison of age-matched wild type and rd1−/rd1− mouse retina. Left panel: Photomicrograph section of retina from an adult wild type (BALB/c) mouse. The photoreceptors are well-developed, having inner and outer segments and cell bodies with nuclei clearly distinguishable. Right panel: Photomicrograph section of retina from an adult rd1−/rd1− (C3H/HeJ) mouse. The rd1−/rd1− mutation causes selective loss of photoreceptors. There is complete ablation of the outer plexiform and nuclear layers and photoreceptor inner and outer segments. The ganglion cell layer, inner plexiform and nuclear layers, and the retinal pigment epithelium are intact. GCL=ganglion cell layer; IPL=inner plexiform layer; INL=inner nuclear layer; OPL=outer plexiform layer; ONL=outer nuclear layer; IS=photoreceptor inner segments; OS=photoreceptor outer segments; RPE=retinal pigment epithelium.
suggesting that this complex formation was suppressed by methylation.

3.4. Effect of methylation on promoter activity

The above data indicate that the −725 and −115 CCGG sites are hypomethylated in photoreceptors compared to other cell types in vivo and that methylation of these sites inhibits DNA:protein binding. These findings, along with the observation that IRBP is expressed selectively in photoreceptive tissues [51,69], suggest that site-specific DNA methylation suppresses transcription whereas hypo-methylation allows or permits IRBP transcription. A direct test of this hypothesis is to compare the activity of a methylated promoter to an unmethylated one in a functional expression assay. Accordingly, to determine whether methylation at the −725 and −115 sites alters transcription of the mouse IRBP gene, we examined the effect of methylating these sites on IRBP promoter activity in transient transfection assays employing primary cultures of chicken retinal cells. The experiment compared promoter activity from in vitro HpaII methylated and unmethylated pIRBP. Site-specific methylation of the murine IRBP 5′ flanking region by HpaII methylase completely suppressed promoter activity (Fig. 6).

The loss of reporter gene activity could be due to methylation of the CAT gene or other sequences in the vector regions of the plasmid [31]. To exclude this possibility, the effect of methylation on identical CAT plasmids containing either an SV40 promoter (pSV40) or the IRBP promoter (pIRBP) were compared. There are sixteen HpaII sites in pSV40, but none in the SV40 promoter. These same sixteen sites exist in pIRBP in addition to the sites at −725 and −115 of the IRBP promoter insert. Both the unmethylated SV40 promoter and the unmethylated IRBP 5′ flanking region drove CAT activity in primary cultures of chick embryonic retinal cells (Fig. 6). Importantly, enzymatic methylation of plasmid DNA at HpaII sites did not affect pSV40 promoter activity (Fig. 6), showing that methylation of the sixteen HpaII sites in the vector and in the CAT gene had no effect on promoter activity and subsequent transcription of the CAT gene. CAT activity in cells transfected with methylated pIRBP was extinguished (Fig. 6). These data indicate that methylation of the −725 and −115 sites in the IRBP insert suppresses promoter activity, demonstrating that IRBP promoter activity is possible only in the absence of this site-specific methylation, even in the photoreceptor cell.

4. Discussion

Our data support the hypothesis that DNA methylation regulates IRBP gene expression. Exogenous methylation of specific CCGG sites in the murine IRBP 5′ flanking region suppressed promoter activity in transient transfection as-
Fig. 6. Effect of exogenous methylation on promoter activity. Primary cultures of embryonic chick retinal cells were transiently transfected with plasmids containing the CAT gene linked to either an SV40 promoter and enhancer (pSV40), a 1783 to +101 fragment of the murine IRBP gene (pIRBP), or no promoter (None). Both the SV40 and IRBP promoters drove CAT activity. HpaII methylation suppressed IRBP promoter activity but had little effect on SV40 promoter activity. Data are from five experiments, three testing the effects of methylation on pSV40 and two testing pIRBP. Data are means of CAT activities normalized to the response to unmethylated plasmids within each experiment. Error bars represent SEM. Sampling size for each group across experiments is in parentheses above error bar. $P<0.001$ for None vs. methylated or unmethylated pSV40 and pIRBP. $P<0.001$ for methylated vs. unmethylated pIRBP as determined by simple analysis of variance followed by Student–Newman–Keuls post hoc analysis.

saves of cultured retinal cells. The demonstration of this transcription regulation mechanism is important in that Southern blot analysis indicates that these CCGG sites in both bovine and murine IRBP promoters are unmethylated in photoreceptor DNA but methylated in DNA from other cell types in vivo, including non-photoreceptor retinal cells. The photoreceptor- and pinealocyte-specific hypomethylation matches the cell-type-specific transcription of the IRBP gene [51,69]. Additionally, methylation of DNA probes containing either hypomethylation site of the mouse promoter suppressed binding activity in EMSAs. These data suggest that methylation of the IRBP promoter suppresses transcription in non-photoreceptor cells by precluding specific DNA:protein binding events, whereas the lack of methylation in photoreceptors allows transcription. The data also suggest that suppression of IRBP expression in non-photoreceptor cells probably does not result from the binding of silencer proteins at either site, as methylation of probes did not cause additional binding complexes to be detected.

Using Southern analysis, an inverse correlation between DNA methylation and expression has been established for many genes (for reviews, see [20,63,73]), including some specifically expressed in retina. For instance, the 5′ flanking regions of both transducin and IRBP are hypomethylated in expressing cells in comparison to non-expressing cells [1,28]. Liou et al. [37] found a temporal correlation: Specific sites in the 5′ flanking regions of murine opsin and IRBP genes are demethylated several days prior to the appearance of transcripts. These results suggest that demethylation is not a consequence of gene activation, but that activation may require prior promoter demethylation.

The data presented here demonstrate a causal relationship previously suggested but not established by others. Our Southern blotting results are consistent with photoreceptor-specific expression of IRBP. Southern blots with MspI digestion of DNA from multiple tissues show that there are no rearrangements of the IRBP gene in any tissues, irrespective of gene expression, despite differences in HpaII digestions. This suggests that gene rearrangement [3,13,22,62] is not a mechanism for either IRBP gene activation or inactivation. However, our Southern blots show that tissue-specific methylation patterns from photoreceptive cells and non-photoreceptive cells correlate with the tissue-specificity of IRBP gene expression (Figs. 1 and 2). As noted, other researchers also have suggested this correlation [1,37], finding at least one HpaII site is specifically hypomethylated in mouse retina and human Y-79 cells. Expanding their findings, we report that the region of hypomethylation is more extensive than previously thought, up to or exceeding 4 kb in the cow and 2.3 kb in the mouse. Additionally, detection of the phenomenon in three mammalian species (human, bovine, and mouse) reveals that it is generally found in IRBP gene expression.

For both cow and mouse Southern blots, retina lanes show bands whose presence indicates that IRBP DNA is not hypomethylated in every cell of the retina (Figs. 1 and 2). Southern blots of DNA from retinas without photoreceptors (rd1/rd1 mouse retinas) lack bands of 1578 and 610 bp, indicating that DNA from cells remaining after photoreceptor degeneration is methylated at positions −725 and −115. This indicates that photoreceptor cells are the only source of DNA that is hypomethylated at these sites. This is in agreement with northern analyses that indicate that cells of the inner nuclear and ganglion cell layers do not express the IRBP gene [51,69]. The present rd1/rd1 data thus resolve the correlation of IRBP hypomethylation with expression down to specific cell
types of the retina: Photoreceptor cells contain DNA that is hypomethylated and these cells express IRBP, but cells in adjacent inner retinal layers contain DNA that is methylated and these cells do not express IRBP.

The observed correlation between DNA hypomethylation and IRBP tissue-specific expression suggests that methylation may prevent transcription. There are a few proposed mechanisms by which DNA methylation can inhibit transcription. Methylated sequences, whether rich or sparse in CpG dinucleotides, can bind protein complexes that suppress promoter activity [10,11,19,44]. These complexes induce histone deacetylation, resulting in restricted nucleosome mobility and a chromatin structure that makes the gene promoter inaccessible to transcription factors (for reviews, see [30,43,53]). Housekeeping genes with high densities of CpGs (CpG islands) often are regulated in this manner [11]. Silencer complex binding also may occur with specific patterns of low-density methylated CpGs in promoters of tissue-specific genes [44]. Additionally, methylation-induced gene suppression may be more direct. Methylation of a cytosine within a cis-acting element could block the binding of a stimulatory trans-acting factor, as suggested by binding analyses demonstrating methylation-induced suppression of DNA:protein binding for several gene promoters [25,26,29,40,46,56,58,66,67,74].

To determine whether either mechanism could play a role in IRBP expression regulation, we examined the effect of methylation on binding between nuclear extracts and two segments of the IRBP promoter containing the −725 or −115 sites. Our data indicate that both probe sequences bind nuclear complexes from several cell types. HpaII methylation of the probes suppressed binding activity as evidenced by either the complete loss of a band (Band 1, Fig. 5) or diminution of a band (Band 1, Fig. 4), suggesting that both probes contain cis-acting elements whose binding to trans-acting factors is sensitive to methylation. Given that Southern blotting shows that these sites are endogenously methylated only in non-expressing tissue, these data suggest that in vivo, tissue-specific methylation may inhibit the binding of trans-acting factors that are present in many cell types. If these factors are stimulatory, this suppression of binding should inhibit transcription in a tissue-specific manner. It is worth noting that the probes used in the EMSAs contain sequences similar to consensus elements bound by known trans-acting factors. Analysis of the Transfac 3.5 database using MatInspector software (http://www.gsf.de/biodv/matinspector.html) indicates that both probes show sequence homology with the Ets-1 consensus sequence, whose core string includes the CpG dinucleotides. The probe containing the −115 site additionally shows homology with consensus sequence binding elements for nuclear respiratory related factor 2 (Nrf2), RAR-related orphan receptor alpha-1 (RORA-1), Activator Protein 1 (AP-1), and an estrogen receptor (ER), all of whose core strings subsume or immediately flank the pertinent CpG nucleotides.

Because the Southern blotting data revealed a correlation between tissue-specific hypomethylation of promoter DNA and IRBP expression and because the EMSA data indicated that this methylation pattern could result in tissue-specific inhibition of DNA:protein binding, we hypothesized that site-specific DNA methylation underlies the tissue-specificity of IRBP expression. To assess this prediction, we examined the effect of methylating the IRBP 5′ flanking region at −725 and −115 on promoter activity. We measured CAT activity in retinal cells transiently transfected with plasmids containing an SV40 promoter or an insert containing the −1783 to +101 region of the murine IRBP gene. Both the IRBP and SV40 promoters drove CAT activity. Methylation of the plasmids at CCGG sites caused loss of IRBP promoter activity, but not SV40 promoter activity. Though the pSV40 plasmid contains sixteen CCGG sites, none are in the SV40 promoter. This shows that HpaII methylation suppressed IRBP promoter activity due to methylation of the CCGG sites at −725 and −115 and not due to methylation of sites elsewhere in the plasmid or any other effect of methylation of the plasmid.

The lack of methylation-induced suppression of SV40 promoter activity or CAT expression is an important control. Several groups have reported that exogenous indiscriminate methylation of CpG dinucleotides by SssI methylase results in loss of activity for various promoters [44,45,60], leading those researchers to infer a role for methylation in promoter regulation. However, it was also found that methylation of all CpGs in CAT expression vectors suppresses transcription, even with unmethylated promoters [31]. Our data show that methylation of the sixteen CCGG sites in pSV40 had no measurable effect on CAT activity, indicating that our protocol is a viable means for determining the effect of point methylations on IRBP promoter activity.

Methylation is not the sole arbiter of the tissue-specificity of IRBP expression. Previous work in this laboratory showed that transient transfection with unmethylated pIRBP produces CAT activity in embryonic chick retinal cells, but not in cells from whole brain or limb buds [5,6]. Assuming that the plasmid DNA is not being methylated following transfection into the retina cells, this suggests that other mechanisms, even in the absence of methylation at −725 and −115, preclude IRBP expression in nonretinal tissue. Not surprisingly, these data in sum suggest that regulation of IRBP transcription is complex. It may be that proteins that bind at or near the two CCGG sites are themselves bound by adjuncts. These ancillary proteins may be present at different times in different cell types. By this model, the DNA binding protein, its adjunct, and an unmethylated target cis element would all be required for transcription [21]. Alternatively, the pertinent DNA binding protein may appear or bind only following, for example, cell surface receptor activation. As noted above, the methylation sites are subsumed by or immediately adjacent to core consensus elements such as ROA-1, ER,
Ets-1, AP-1, and Nrf-2. It may be that receptor activation stimulates transcription only if the −115 site is hypomethylated, or conversely, that receptor activation itself leads to demethylation [72]. In either scenario (adjunct proteins or activated trans-acting factors), site-specific DNA methylation would prevent improper expression.

Regardless of the complexity of IRBP expression regulation, here we establish that site-specific methylation blocks promoter activity in transient transfection assays and inhibits DNA:protein binding in vitro assays. The importance of these data to the in vivo condition is that such methylation does not exist in expressing tissue. These data indicate that IRBP expression is permitted only in photoreceptors because it is only in this cell type that the IRBP promoter is hypomethylated.

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References


