Activation of a Methylated Promoter Mediated by a Sequence-specific DNA-binding Protein, RFX*

Received for publication, April 27, 2005, and in revised form, August 19, 2005 Published, JBC Papers in Press, September 15, 2005, DOI 10.1074/jbc.M504633200

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The roles of eukaryotic DNA methylation in the repression of mRNA transcription and in the formation of heterochromatin have been extensively elucidated over the past several years. However, the role of DNA methylation in transcriptional activation remains a mystery. In particular, it is not known whether the transcriptional activation of methylated DNA is promoter-specific, depends directly on sequence-specific DNA-binding proteins, or is facilitated by the methylation. Here we report that the sequence-specific DNA-binding protein, RFX, previously shown to mediate the transition from an inactive to an active chromatin structure, activates a methylated promoter. RFX is capable of mediating enhancerosome formation on a methylated promoter, thereby mediating a transition from a methylated-dependent repression of the promoter to a methylated-dependent activation of the promoter. These results indicate novel roles for DNA methylation and sequence-specific DNA-binding proteins in transcriptional activation.

Methylation of DNA cytosine residues is strongly associated with higher order chromatin formation, heterochromatin, and repression of RNA transcription. Several molecular mechanisms leading to transcriptional repression, as a result of DNA methylation, have been elucidated. For example, MeCP2, one of a family of proteins with a methyl-DNA binding domain (MBD), binds to 5-methylcytosines recruit a histone deacetylase, which in turn leads to nucleosome stabilization, recruitment of proteins that mediate chromatin condensation, and the repression of transcription (1–8).

In several rare cases, DNA methylation has been shown to enhance transcription. DNA methylation of the far upstream region of the INTERLEUKIN-8 gene and methylation of the EARLY GROWTH RESPONSE-2 intron are associated with increased promoter activity (9, 10). The mechanism of these effects is unknown, and no DNA-binding proteins involved in these processes have been identified. Other studies have indicated that methylated DNA can be transcribed, presumably when there is an absence of proteins that directly mediate transcriptional repression (11). However, none of these studies involve a transition from repressed to activated methylated DNA. Recently, Lembo et al. (12) identified a protein that facilitates a transition from MBD-mediated repression to MBD-mediated activation. However, the molecular mechanism that specifies a transition from repressed methylated DNA to methylated DNA that becomes or remains transcriptionally active, for a given promoter, remains unknown.

Here we report that the sequence-specific DNA-binding protein, RFX, can mediate the transcriptional activation of a methylated major histocompatibility (MHC) gene promoter that was repressed by the methylation. These results indicate that DNA demethylation is not necessary for promoter de-repression, i.e. for a loss of proteins that block the binding of positive transcriptional regulatory proteins; and that DNA demethylation is not necessary for promoter activation, defined as an initial promoter-protein interaction that facilitates the interaction of the promoter with positive transcriptional regulatory proteins.

MATERIALS AND METHODS

Azacytidine Treatments and Agarose Gel PCR—Suspension B-cell lines (Raji, 6.1.6., and BLS1) were seeded at 10^5 cells per ml in a 20-ml volume of media. Cells were treated with 5 μM azacytidine for 72 h. Total RNA was prepared as described (13), and 5 μg of RNA were used in a standard reverse transcriptase-PCR for HLA-DRA mRNA and for γ-actin mRNA, exactly as described in Ref. 14. PCR was performed for 40 cycles and the PCR products were detected by 2.2% agarose gel electrophoresis.

Methylation of HLA-DRA Promoter-Luciferase Construct and Transfections—The HLA-DRA promoter-luciferase construct, pDNA derived from pGL3 (14), was methylated using SssI methylase (20 μg of pDNA, 5 mM S-adenosylmethionine, 8 units of SssI, 10 × New England BioLabs buffer number 2, H20 to a total volume of 100 μl). Mock methylated pDNA was prepared in the same way but without SssI methylase in the reaction. Methylated pDNA, or mock methylated DNA, was extracted with phenol:chloroform, ethanol precipitated, resuspended in water, and quantified by absorbance at 260 nm and agarose gel electrophoresis. Methylation was confirmed by the treatment of the methylated and mock methylated pDNA with a methylation-sensitive restriction enzyme. Fifty nanograms of methylated pDNA were added to each of six wells containing 5 × 10^5 5637 cells (ATCC). DNA transfection was performed using the TransIT Reagent according to the vendor’s instructions. IFN-γ was added to 400 units/ml following the transfection, and luciferase assays were performed 24 h following the transfection. Each bar graph represents the average and standard deviations for six transfections.

Co-transfections of RFX Expression Vectors and the HLA-DRA Promoter-Luciferase Construct—Fifty ng of methylated pDNA (Me-pDNA) was co-transfected with 50 ng each of cytomegalovirus-based expression vectors for RFX5, RFXAP, and RFXB (also termed RFXANK or Tvl-1), representing the RFX trimer, or with 150 ng of empty expression vector, into 5 × 10^4 5637 cells, as described in the legend to Fig. 1.
Following the transfection, cells were treated with IFN-γ. Luciferase averages and p values were obtained by using six wells for each transfection. Quantification of the luciferase assay is indicated in the left of the bar graphs and the p value is indicated in boxes.

**Methylation Sensitive Real-time PCR**—Twenty-four hours following transfection of the indicated plasmids, total cellular DNA was isolated. Cells from a 100-mm plate were washed with phosphate-buffered saline, lysed by passage of the cell lysate through a 20-gauge syringe exactly 10 times. The cell lysate was incubated with proteinase K and RNase, and DNA was extracted using phenol/chloroform using standard procedures for isolating cellular DNA. The DNA was ethanol precipitated, resuspended in water, and quantified. Equal amounts of DNA (about 1% of the DNA recovered from a 100-mm plate) were digested with the indicated methylation sensitive restriction enzymes and were amplified by real-time PCR using a Bio-Rad iCycler and Bio-Rad iQ SYBR Green Supermix, according to the vendor’s instructions. Primer positions a–f are indicated in Fig. 4D; primer a, CTTTATTTTTGGCCTCCTTCA; b, CTAGCAAATA-AGGTGCTCCTCC; c, TACACGAAATTGCTTCTGGTGCCG; d, CCA-GATCCACAACTTCTTGCTCTCA; e, ACTGTCATGCACTCCGTAGATGC; f, CTCACACAGCGTGAAGATCTTCTA. Primers e and f were used to determine the relative amounts of transfected plasmid present for each of the indicated transfections, as these primers amplify a segment of pDRA that does not include a site for either Hgal or Aval. For the MS-PCR of the endogenous T5–1, 6.1.6, and SJ2 HLA-DRA DNA of Fig. 5A, the primers were: (i) forward, aagagtctgtcctgcatgaac; and (ii) reverse, ctgtcgctgtcctgcatgaac.

**Bisulfite Sequence Analysis**—Total cellular DNA was isolated using standard techniques and assayed by bisulfite treatment, PCR amplification, and DNA sequencing using the CpGenome kit (Chemicon). The primers for the PCR amplification were: forward, TTGTTTGTGTTTGTGTTTAAGAATTTTATT; reverse, AAATCTCCACTATAACCAATTTCTCTCA.

**Chromatin Immunoprecipitation Assay**—Chromatin immunoprecipitation assay was performed (Ref. 15 and references therein). Briefly, cells were cross-linked with 1% formaldehyde for 20 min at room temperature; the cells were harvested and lysates were prepared. The His tag (27E8) monoclonal antibody (Cell Signaling Technology) was used for immunoprecipitation. Immunoprecipitations were analyzed for the presence His-tagged RFX on the HLA-DRA promoter. Rabbit antimouse secondary antibody was used as the control for all reactions. The PCR were then performed using 2.5 μl of DNA from the immunoprecipitation reactions or 1 μl of DNA from the input reaction as template. PCR cycling conditions were as follows: 94 °C for 2 min; then 35 cycles at 94 °C for 30 s, 43 °C for 30 s, and 68 °C for 30 s; followed by 68 °C for 2 min. The sequences of the primers used in the PCR were HLA-DRA promoterprimers described as primers a and b above (see also Fig. 4D).

**RESULTS**

**DNA Methylation Represses Transcriptional Activity of the HLA-DRA Promoter**—RFX was first identified as a protein defective in bare lymphocyte syndrome, an immunodeficiency disease due to the lack of transcription of the MHC class II genes, which encode the class II antigen presenting molecules (16). RFX has two apparent functions in the testis (30, 31). RFX also mediates the establishment of an MHC class II DNase I hypersensitive site (16), thus facilitating a transition from an inactive, condensed form of chromatin to a form of promoter chromatin that permits enhancedase assembly and transcriptional activation. RFX5, one of the three subunits of RFX, is a member of a family of methyl-DNA-binding proteins (24–28), some of which also have a connection with the regulation of transcription of DNA that is in a condensed form. For example, *Drosophila* RFX functions in sperm (29), and RFX2 and RFX4 function in the testis (30, 31).

B-cells specifically lacking RFX, but possessing the other required transactivators, do not have a transcriptionally active HLA-DRA gene, the human MHC class II gene prototype for the study of MHC class II gene regulation. RFX-defective B-cells have a condensed, inaccessible HLA-DRA promoter chromatin conformation. To determine whether DNA methylation plays a role in preventing transcription of HLA-DRA in RFX-negative cells, we treated two RFX-negative cell lines with azacytidine (AzO) treatments as indicated. Panel B, luciferase activity of methylated and mock methylated pDRA plasmids as indicated.
Methylated Promoter Activation

FIGURE 2. RFX activation of methylated pDRA. Panel A, co-transfection of methylated pDRA (Me-pDRA) and RFX expression vectors, or empty vector, as indicated, followed by treatment of cells with IFN-γ and assay for luciferase activity. The methylated or mock methylated promoter-luciferase constructs were transfected into cells as indicated under "Materials and Methods." p values are indicated in boxes. Panel B, methylated pGL3-Control (Me-pGL3-Control) co-transfected with the RFX expression vectors, or with an equivalent amount of empty vector, as described in panel A. Relative luciferase activity is indicated to the right. 1.0, the amount of luciferase activity for the unmethylated pGL3-Control during a simultaneous transfection. Thus, results indicated essentially no increase in activity of Me-pGL3-Control because of RFX expression.

these results are consistent with the possibility that RFX-negative cells have a methylated HLA-DRA gene (see also Fig. 5).

To determine whether an HLA-DRA promoter-reporter construct could be repressed by methylation, we methylated a previously described (14) pDRA (luciferase) construct with Sssl methylase, which methylates all plasmid CpG dinucleotides. The methylated pDRA was transfected into 5637 cells, which require IFN-γ treatment for the induction of CIITA and the activation of the pDRA promoter. The Sssl methylation significantly reduced the IFN-γ activation of the pDRA (Fig. 1B).

RFX Facilitates Activation of a Methylated HLA-DRA Promoter-Reporter Construct—Methylated pDRA was co-transfected into 5637 cells with equal amounts of either (a) empty vector or (b) the RFXAP, RFXB, and RFX5 expression vectors, representing the three subunits of RFX. Cells were treated with IFN-γ and assayed for luciferase activity 24 h following the transfection. The methylated pDRA co-transfected with RFX showed a 10-fold increase (Fig. 2A) in luciferase activity compared with the control samples lacking the exogenously expressed RFX.

To be certain that the increase in transcriptional activity was caused by RFX binding to the HLA-DRA promoter sequences of pDRA, we assessed the effect of RFX on pGL3 promoter-reporter constructs lacking any known RFX sites or lacking any apparent sites for the other sequence-specific DNA-binding proteins of the MHC class II enhancerosome. We methylated the pGL3-Basic and pGL3-Control luciferase constructs and transfected them into cells. pGL3-Basic, lacking any known promoter elements, had very little activity whether methylated or unmethylated (data not shown). Methylation of the pGL3-Control luciferase construct, which contains the SV40 enhancer, reduced its promoter activity (data not shown). The methylated pGL3-Control luciferase construct was then co-transfected into cells with the RFXAP, RFXB, and RFX5 expression vectors or with empty vector. No increase in transcriptional activity was observed for the pGL3-Control luciferase construct (Fig. 2B), consistent with the conclusion that RFX activates methylated pDRA by binding to its cognate site in the HLA-DRA promoter (Fig. 4E). Methylated pDRA co-transfected with the RFX expression vectors represented a positive control for the RFX effect (data not shown).

RFX is Required for the CIITA Activation of Methylated pDRA—In general, the sequence-specific DNA-binding proteins can occupy the HLA-DRA promoter without the advent of HLA-DRA transcription. Transcriptional activation commences when the formation of the enhanceosome, including RFX, is completed by availability and the enhanceosome binding of CIITA, which in turn leads to the recruitment of histone acetyltransferase activity (32, 33). CIITA synthesis is induced by IFN-γ, as a result of the IFN-γ induction of activated STAT1 and IRF-1 and the binding of these two transactivators to the CIITA promoter (34, 35).

To be certain that RFX facilitated the IFN-γ activation of the methylated pDRA promoter by cooperating with CIITA, rather than by any unappreciated indirect effect, we co-transfected the methylated pDRA with a CIITA expression vector and with either the RFX expression vectors or with control empty vector. RFX expression strongly enhanced the CIITA activation of the methylated pDRA (Fig. 3).

RFX Does Not Facilitate the Activation of Nonmethylated pDRA and Does Not Facilitate Demethylation of pDRA—Whereas RFX is capable of low affinity binding to nonmethylated HLA-DRA promoter DNA and
of participating in enhanceosome formation in vitro in the absence of DNA methylation, it is not known whether RFX is important in the activation of nonmethylated HLA-DRA promoter DNA. We co-transfected either the RFX expression vectors or empty vector with nonmethylated pDRA (Fig. 4, A–C), which led to a decrease in CIITA-dependent, HLA-DRA promoter activity. This result may indicate that another protein, rather than RFX, mediates the function of the X1 HLA-DRA promoter element, which includes the RFX binding site on nonmethylated DNA (Fig. 4E) (36). Alternatively, the 5637 cells may not support a post-translational modification of RFX required for activation of an unmethylated promoter or may represent some other cell-specific effect that interferes with the activation of the nonmethylated HLA-DRA promoter-reporter construct.

To rule out the possibility that RFX facilitated demethylation of the pDRA, we recovered total DNA from cells co-transfected with either the RFX expression vectors or the empty vector in combination with either methylated pDRA or nonmethylated pDRA. The recovered DNA was digested with either Hgal, the site that overlaps the CG dinucleotide, or with other methylation sensitive restriction enzymes. The results for pDRA and other Hgal digestion sites are shown in panels F–I, which were not normalized and, in the case of panel I, include a “no-transfection” control. The y values for panels G and I bar graphs represent relative cycle numbers for each transfection using the results obtained for each transfection, were subtracted from the number of cycles obtained for the RFX expression vectors or the empty vector in combination with either methylated or nonmethylated pDRA. The y values are shown in box plots. The maximum cycle number difference between methylated and nonmethylated DNA, based on the digestion and real-time PCR experiments of panels F–I, ranged from 21 to 26 with the exception of the “no-transfection” control in panel I, which averaged about 33 cycles. This in turn indicates (i) that little or no demethylation at this site occurs during the period of DNA transfection and RFX expression; (ii) that about half of the DNA is demethylated at the Hgal site in the absence of RFX; and (iii) that about half of the DNA is demethylated at the luciferase AvaI site in the presence or absence of RFX. In summary, these data indicate that RFX expression does not facilitate pDRA demethylation.
cleotide immediately adjacent to the X1 element in the HLA-DRA pro-
moter (Fig. 4E), or with AvaI. Neither HgaI nor AvaI cleave DNA when
the C at the cleavage site is methylated. The digested DNA was then
assayed by real-time PCR using pDRA-specific primers on either side of
the pDRA promoter HgaI site or luciferase coding sequence primers on
either side of an AvaI site located in the luciferase coding region (Fig.
4D). The methylation sensitive PCR results indicated that nonmethyl-
ated DNA was significantly more sensitive than the methylated DNA to
both of the restriction enzymes. Furthermore, the results indicated that
RFX expression did not lead to an increase in the restriction enzyme
sensitivity of the methylated DNA (i.e. did not lead to an increase in
DNA demethylation) compared with the empty vector (Fig. 4, F–I).
Thus, the activation of the methylated pDRA by RFX (Figs. 2 and 3)
cannot be explained by RFX-mediated DNA demethylation.

Interestingly, the results of the methylation sensitive PCR also indi-
cated that RFX expression stabilized the methylation of the C in the
HgaI-CpG dinucleotide that is adjacent to the RFX binding site on non-
methylated DNA (Fig. 4, D and E). RFX expression had no apparent
effect on the durability of the methylation of the C in the AvaI site distal
to the RFX binding site (Fig. 4, F–I). These results, coupled with the
knowledge that RFX5 is a member of a methyl-DNA binding family of
proteins, suggest that the HgaI site C, adjacent to but not included in the
RFX contact points in nonmethylated DNA (37), contacts RFX in meth-
ylated DNA.

**RFX-defective B-cells Have a Reduced Capacity to Activate Methyl-
ated pDRA**—To determine whether the absence of endogenous RFX
would limit the activation of methylated pDRA, we transfected methyl-
lated and mock methylated pDRA into T5-1 cells, expressing wild-type
RFX, and into a T5-1-derived, chemically mutated subclone, 6.1.6, which
lacks the RFXAP subunit of the RFX trimer (38) and consequently has no RFX DNA binding activity. 6.1.6 does not express the
MHC class II genes, including the *HLA-DRA* gene. This experiment

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**FIGURE 5.** Transfection of Me-pDRA into RFX-positive and -negative B cells and MS-PCR and bisulfite sequencing of the HLA-DRA promoter in RFX-positive and RFX-
defective B-cells. **Panel A**, transfection of methylated and mock methylated pDRA into RFX-positive T5-1 cells. Luciferase activity is set at unity for the mock methylated pDRA as indicated on the y axis. **Panel B**, transfection of methylated and mock methylated pDRA into RFX-negative 6.1.6 cells. **Panel C**, real-time PCR analysis of DNA prepared from T5-1 and 6.1.6 as indicated. Real-time PCR was performed on Hgal- (Fig. 4E) digested and mock digested DNA. Results from mock digested DNA were subtracted from the results obtained for Hgal-digested DNA. Data are thus presented as increasing number of cycles to detect the Hgal-digested DNA. (See also Fig. 4, F–I, and the associated legend.) **Panel D**, results of the bisulfite sequencing analyses for T5-1, 6.1.6, and SJO as indicated. RFX-pos refers to RFX-positive and RFX-neg refers to RFX-negative. Arrow indicates T5-1 C residue conversion to a T residue.
revealed that methylation of pDRA caused an approximate 50% reduction in pDRA activity in T5-1 (Fig. 5A) but an 80% reduction in activity in 6.1.6 (Fig. 5B), consistent with a role for endogenous RFX in activating the methylated HLA-DRA promoter.

**RFX-defective B-cells Have a Methylated CpG Immediately Adjacent to the Canonical RFX-binding Site**—To determine whether the cytosine in the CpG dinucleotide immediately 3′ of the canonical RFX binding site (Fig. 4E) is methylated in the endogenous promoter, in cells lacking RFX, we employed MS-PCR and bisulfite sequencing analysis. For both approaches, endogenous DNA was prepared from T5-1 B-cells, 6.1.6 B-cells, and SJO B-cells, which lack RFX5 (39) and thus have no RFX DNA binding activity. SJO cells were derived from a bare lymphocyte syndrome patient, and SJO cells do not express any of the MHC class II genes. MS-PCR was performed with T5-1 and 6.1.6 DNA (Fig. 5C). In this analysis, the T5-1 DNA was more sensitive to Hgal digestion than was the DNA from 6.1.6 (Fig. 5C) or the DNA from SJO cells (data not shown).

To verify and extend the MS-PCR analysis, we conducted a bisulfite sequencing analysis of the T5-1, 6.1.6, and SJO DNA. In this analysis, unmethylated C residues are converted to uracil as a result of the bisulfite treatment. (The uracil is converted to thymidine during the subsequent PCR step.) Whereas the C residue immediately 3′ of the canonical RFX binding site was converted to a T residue in T5-1, the C residues in 6.1.6 and SJO, respectively, were not converted to T residues (Fig. 5D).

**RFX Binds to Methylated pDRA**—To examine whether RFX can bind to methylated DNA in vivo, we transfected 5637 cells with the His-tagged RFX expression vectors along with the methylated or unmethylated pDRA luciferase construct (Fig. 4D). Extracts were prepared and exogenous RFX was immunoprecipitated with an anti-His tag antibody and its association with the HLA-DRA promoter assayed by PCR with proximal primers (primers a and b in Fig. 4D). The promoter fragment could not be detected in cells transfected with the RFX or pDRA alone (Fig. 6). Similarly, there was no fragment in extracts representing RFX co-transfected with the unmethylated pDRA promoter. A band corresponding to the promoter was detected in cells transfected with RFX and methylated pDRA (Fig. 6). There was a comparable amount of the promoter in the input lanes, showing that the transfection efficiency was similar in all the samples. Taken together, these results indicate that RFX can indeed bind preferentially to the methylated HLA-DRA promoter sequences.

Because RFX does not facilitate the CIITA activation of nonmethylated DNA or an increase in pDRA demethylation (Fig. 4), the RFX activation of methylated pDRA must be because of RFX binding to methylated pDRA and to the RFX-mediated recruitment of CIITA to the methylated pDRA (Figs. 6 and 8, A and B). These results represent the first description of a molecular mechanism indicating how a specific, methylated promoter can transition from a repressed to an activated state. Most importantly, the process of RFX-mediated enhancerosome formation on the methylated HLA-DRA promoter indicates that the transition from methylated, repressed DNA to methylated, activated DNA does not require a nonspecific transition or demethylation over long regions of heterochromatin.

**The Oct-1/YY1 Repression Mechanism Does Not Function to Repress Either Inactive or Transcriptionally Competent Methylated pDRA**—We have previously reported that both Oct-1 and YY1 repress the IFN-γ activation of pDRA (14, 40, 41). To determine whether this mechanism(s) functions to repress methylated pDRA, we transfected methylated pDRA or the previously described (14, 40, 41), methylated pDRAOct or methylated pDRAΔYY1 into IFN-γ-treated 5637 cells. The latter two pDRA luciferase constructs lack the Oct-1 and YY1 binding sites, respectively. We also transfected nonmethylated pDRA or pDRAOct or pDRAΔYY1 into IFN-γ-treated 5637 cells, a transfection series that serves as a positive control for the detection of Oct-1- or YY1-mediated repression. Results indicate that the nonmethylated DNA is repressed by Oct-1 and YY1, exactly as previously and extensively reported (Fig. 7A). The repression is revealed by an increase in luciferase activity when comparing the activity of either pDRAOct or pDRAΔYY1 to pDRA. However, the lack of these sites does not lead to increased activity when pDRA is methylated, with or without exogenous RFX expression (Fig. 7B).
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DISCUSSION

The data above indicate for the first time that a sequence-specific DNA-binding protein can facilitate enhanceosome formation on, and transcriptional activation of methylated promoter DNA; that a highly localized, sequence-specific transition from repressed methylated DNA to transcriptionally active DNA is possible; that RFX facilitates activation of a methylated HLA-DRA promoter; and that function of the Oct-1 and YY1 repression mechanisms requires demethylated DNA.

The RFX transactivator may lead to methylated DNA promoter activation by either displacing or preventing the binding of MBD proteins that form a repressosome complex (Fig. 8, A and B). A direct role for DNA methylation in the function of RFX may be possible because (i) the RFX5 subunit of RFX belongs to a family of methyl-DNA-binding proteins; (ii) C residue methylation is known not to inhibit the binding of RFX5 to a collagen gene promoter (28); and (iii) RFX protects the methylation of the C residue immediately adjacent to its previously described HLA-DRA promoter binding site (Fig. 4, E–G). The contact points of RFX on methylated DNA are unknown, however, RFX does not contact the C residue of the HLA-DRA X2 element when the promoter is not methylated (37).

Alternatively, RFX may activate a methylated promoter because the methylated promoter is in nucleosomal form. RFX has a higher affinity for nucleosomes compared with naked DNA (42), and as noted in the Introduction, DNA methylation leads to the formation of deacetylated nucleosomes.

Either RFX-mediated displacement of repressive MBD proteins (Fig. 8, A and B), or RFX affinity for nucleosomes could be consistent with the well established cooperativity of the HLA-DRA promoter-binding proteins. For example, the displacement of an MBD repressive complex, or binding to nucleosomes may require the cooperative assembly of the RFX-CREB-NF-Y enhanceosome (17–23). However, the cooperativity of the canonical enhanceosome has only been demonstrated on non-methylated DNA. Thus, it is also possible that RFX functions without the previously described enhanceosome to mediate promoter activation on methylated DNA. In short, it is now of considerable interest to determine the structure of the enhanceosome that forms on methylated HLA-DRA promoter sequences.

The above described mechanism of transitioning from a repressed methylated promoter to an activated promoter raises the question of whether demethylation must occur prior to transcription? Whereas our data indicate that RFX does not facilitate demethylation, there has not been a determination of whether demethylation occurs following the addition of CIITA, which in the above described system regulates the initiation of transcription. CIITA recruitment to the enhanceosome is concomitant with histone acetyltransferase activity in the enhanceosome (32, 33). The histone acetyltransferase activity could be sufficient for the nucleosome remodeling that is necessary for pre-initiation complex formation and transcription of methylated DNA, consistent with other systems where methylation per se does not interfere with transcription (9–11). The fact that Oct-1 and YY1 appear to be required for a full activation of methylated DNA by RFX (Fig. 7B) suggest that it is the methylated DNA that is being transcribed. If there were an essentially immediate transition to demethylated DNA with CIITA addition, lack of Oct-1 or YY1 should lead to a de-repression, as in Fig. 7A.

Presumably at some point, there is a transition from methylated DNA to demethylated DNA, raising the question of whether this transition is instigated by one or multiple rounds of mRNA transcription? If transcription is required for demethylation in the above described RFX-HLA-DRA system, the role of transcription in facilitating DNA demethylation would be reminiscent of other covalent DNA modifications that are stimulated by transcription, such as immunoglobulin class switching and DNA repair (43–45).

Our conclusions also raise the question, which proteins or class of proteins might mediate the DNA sequence specificity for other methylated promoters? The HLA-DRA promoter binding specificity for the RFX interaction with condensed chromatin and methylated DNA requires all three subunits (Fig. 3) (46). Multiple RFX5 family members (RFX1–5) have well studied and related “RFX5-type” DNA binding domains (39) and related DNA binding sites. In summary, it is not likely that significant promoter specificity resides exclusively in the RFX5-type DNA binding domain common to the RFX5 family members. The other two RFX subunits, RFXAP (38) and RFXB/RFXANK/Tvl-1 (46–48), the functions of which are not well understood, are required for HLA-DRA promoter specificity of the RFX trimer. It seems likely that other, currently unknown proteins substitute for RFXAP and RFXB in establishing sequence specificity for other methylated promoters. However, at this point, it is formally possible that all other promoters that undergo a transition from repression by methylation to activation interact with the same RFX trimer that mediates the activation of the methylated HLA-DRA promoter.

Recently, RFXAP has been shown to interact with the chromatin remodeling factor BRG-1 (49), very likely able to facilitate the decondensation of chromatin that would be expected to occur as part of a transition from methylated DNA to transcriptionally active DNA.
Our data and conclusions have implications for transitions between different repressosomes, and in particular indicate that demethylation of DNA is likely to represent a mechanism of transitioning between repressosomes. For example, demethylation of the HLA-DRA promoter represents a transition from the MBD protein-related repressosome that regulates methylated HLA-DRA DNA (Fig. 1) to the DRAN (Oct-1 related) (14, 40, 41) or YY1-HDAC repressosomes (14) that regulate demethylated HLA-DRA DNA (Figs. 7 and 9).

Acknowledgments—We thank members of the Eichler and Solomonson laboratories (University of South Florida) for real-time PCR advice, Larry Solomonson and Barbara Smith (Boston University) for helpful discussions, and the Moffitt Cancer Center Molecular Biology core facility for help with the bisulfite sequencing.

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