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Cytosine methylation transforms an E2F site in the retinoblastoma gene promoter into a binding site for the general repressor methylcytosine-binding protein 2 (MeCP2)

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ABSTRACT

The CpG-rich promoter of the retinoblastoma tumor suppressor gene (*Rb-1*) is normally unmethylated. However, aberrant methylation of CpG dinucleotides within the *Rb-1* promoter has been depicted in certain tumors, which determines transcriptional inactivity of the gene and absence of the pRb retinoblastoma protein. Here we have concentrated on an E2F-binding site in the *Rb-1* promoter. We show that the E2F site is required for cell-cycle regulated *Rb-1* transcription in non-transformed cells. The function of the E2F site is associated with its ability to interact with several activating factors of the E2F family. In contrast, *in vitro* methylation of two tandemly arranged CpGs in the E2F recognition site prevents binding by E2F factors, and determines instead the recruitment of the general repressor methylcytosine-binding protein 2 (MeCP2). These results suggest that the interaction of MeCP2 with the methylated version of the E2F site may represent a step towards *Rb-1* promoter inactivity in tumor cells.

INTRODUCTION

E2F factors regulate transcription of many cell cycle genes and exert their activity by binding promoter elements harboring TTTSSCGC or related sequences. The E2F DNA-binding activity is shared by heterodimeric complexes, composed of one protein synthesized by one of five related E2F-encoding genes (a sixth member of recent identification is regarded as atypical, because it carries a DNA-binding, yet no transactivation, domain), and one of three related dimerization partners, DP (1–3). Promoters harboring E2F-binding sites can be subjected to positive or negative control of transcription depending on the type of recruited transcriptional complex. E2F/DP heterodimers mostly act as activators; however, their transactivation ability is neutralized by the interaction with members of the pocket protein family, including the pRb product of the *Rb-1* retinoblastoma gene and the related p130 and p107 proteins. Specific interactions are established between pocket proteins and E2F/DP complexes. Cell cycle gene transcription is controlled by the molecular balance between E2F/DP activators and repressors of the pocket family (reviewed in 4).

With the remarkable exception of Sp1, most mammalian transcription factors are sensitive to CpG methylation in their recognition sequence. Target elements of E2F factors (most characterized sequences are listed in 3,5) contain one, and in certain genes two, CpG dinucleotides, which can act as substrates of methyltransferases and become methylated in mammalian cells. The promoters of many tumor suppressor genes lie within CpG-rich DNA sequences that are normally unmethylated. However, aberrant CpG methylation has been reported to occur within the promoters of several genes encoding tumor suppressor proteins (reviewed in 6,7), including pRb, the p16 and p15 inhibitors of cyclin-dependent kinases, the Von Hippel–Landau protein and E-cadherin, in transformed cells and tumors. The *Rb-1* gene is a major tumor suppressor gene, whose disruption is associated with tumor cell growth (8). Functional pRb is essential for negative control of proliferation; conversely, cell cycle progression requires pRb inactivation by phosphorylation by cyclin-dependent kinases (reviewed in 9,10). Several tumors are characterized by loss-of-function mutations at both alleles of the *Rb-1* gene: in most cases, a germline mutation of one allele is followed by a somatic mutation affecting the homologous allele (11). In certain tumors, one *Rb-1* allele carries an aberrantly methylated promoter (12), while the homologous one carries a 'conventional' structural mutation. In those cases, aberrant methylation of the *Rb-1* promoter acts as an epigenetic mutation that can be functionally equated to mutational inactivation of the *Rb-1* gene (7).

The *Rb-1* promoter harbors a potential site for E2F factors in the region of transcription initiation. The functional role of that site in *Rb-1* transcription has been the object of conflicting reports (13–16) and remains as yet uncertain. The site contains two tandemly arranged CpG dinucleotides. Previous experiments

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showed that a methylated E2 oligonucleotide was unable to compete for factor(s) binding to the wild-type Adenovirus *E2* promoter element, implying that cytosine methylation inhibits DNA binding by E2F factors (17). However, the binding features of the methylated E2F site have not been addressed. Since the *Rb-1* promoter, including the E2F site, can be methylated in tumors, it was of interest to examine the interaction of factors with the methylated version of the E2F site.

We show here that the E2F site contributes to up-regulate the *Rb-1* promoter at the G1/S transition. The E2F element is bound by E2F factors *in vitro*. In contrast, a methylated version of the site is not only refractory to binding by E2F family members, but specifically assembles nucleoprotein complexes with distinct factors. Model promoters carrying methylated CpGs undergo repression mediated by methylcytosine-binding protein 2 (MeCP2) (18). However, the binding of MeCP2 from cell extracts to genomic regulatory sequences has not been demonstrated as yet. We report here that MeCP2 complexes interact with the methylated version of the *Rb-1*-derived E2F site. Thus, CpG methylation transforms the E2F site into a binding site for MeCP2. These results suggest that inactivity of the methylated *Rb-1* promoter, such as seen in tumors, does not simply reflect the loss of interaction with activating factors, but may involve novel interaction(s) with MeCP2.

MATERIALS AND METHODS

Cell cultures and FACS analysis

Murine NIH 3T3 embryo fibroblasts (ATCC CRL 1658) were cultured in Dulbecco's modified Eagle medium. To induce cell cycle synchronization, cells were brought to quiescence in medium containing 0.5% fetal calf serum (FCS) for at least 48 h. The serum concentration was then raised to 15%, and cells were collected at regular intervals after restimulation. To monitor S phase, cells were incubated with bromo-deoxyuridine (BrdU) for 30 min before harvesting. Cell samples were subjected to either FACS analysis of the DNA content only, or biparametric analysis of the DNA content and of BrdU incorporation as described (19).

Plasmid construction

The pSV0t2CAT vector was obtained by inserting the polylinker from pCAT-basic (Promega) into the pSV0tCAT vector (20); for a detailed description, see the web site: http://mercury.itbm.rm.cnr.it . The human *Rb-1* promoter-CAT reporter construct, pRb-CAT(–227 to –175) was obtained by PCR amplification of human *Rb-1* promoter sequences comprised between –227 and –175 relative to the start of translation. A *Hin*dIII-containing tail was fused to the 5' end of the upstream primer (5'-taag**aagctt**CGGGAGCCTCGCGGACGTG, corresponding to the sequence spanning from –227 to –209); a *Xba*I-containing tail was fused to the 5' end of the downstream primer (5'-gata**tctagA**ACCGCGGGAAAACGTCAC corresponding to the sequence spanning from –175 to –193). Sequences in capital letters are from the *Rb-1* promoter, sequences in boldface indicate the *Hin*dIII and *Xba*I sites. The PCR amplification template was the HRP-CAT (–509 to –85) construct (described in 21). The PCR product was digested with *Hin*dIII and *Xba*I and cloned into pSV0t2CAT. The E2F-mutated *Rb-1* promoter-CAT reporter construct, pRb-CAT(–227 to –175)xE2F, was constructed as described for pRb-CAT(–227 to –175); to generate the mutant E2F site, the following downstream primer was used: 5'-gata**tctagA**ACCGTAGGAAAACGTCAC, in which nucleotides G –180 and C –181 were changed to T and A, respectively. The upstream primer was the same used to obtain the wild-type –227 to –175 sequence. The pTS-A clone, carrying the cell cycle-dependent *RanBP1* promoter upstream of the CAT gene, was described previously (22).

Transfection experiments

Cells were transfected as described in (23) using 4 μg of CAT reporter construct and 1 μg of CMV-*lacZ* control plasmid. The medium was replaced 6 h after transfection with 0.5% FCS-containing medium to induce growth arrest. To induce synchronous cell cycle progression, cells were serum-starved for 48 h, restimulated to cycle by adding 15% FCS, and collected at regular intervals thereafter. Synthesized CAT enzyme was measured by CAT enzyme-linked immunosorbent assay (Boehringer Mannheim) and normalized relative to the levels of β-galactosidase from the cotransfected construct, measured by β-galactosidase enzyme-linked immunosorbent assay (Boehringer Mannheim).

Gel shift mobility assays

Protein extracts were prepared from NIH 3T3 cells as described in (23). E2F-1-enriched extracts were prepared 48 h after transfection of the pCMV-E2F1 and pCMV-DP1 expression constructs as previously described (23). The following oligonucleotides, and their reverse complementary strand, were used: wild-type *Rb-1* E2F site, 5'-AGTGACGTTTTCCCGCGGTTGGA-3'; methylated *Rb-1* E2F site, 5'-AGTGA^{m}CGTTTTCC^{m}CG^{m}CGGTTGGA-3', where ^{m}C indicates 5-methylcytosine; E2F sites from the *RanBP1* promoter: b-E2F, 5'-CATCGCCGCGGGCGTTTTGGCGGGAAGCGC-3', and c-E2F, 5'-AATTCGCGTTTCCCGCCGCTG-3'; E2F site from the Adenovirus 5 *E2* promoter, 5'-TAGTTTTCGCGCTTAAATTT-3'. Mutagenized versions of the *RanBP1*-derived E2F sites include the DW oligonucleotide, harboring a mutated E2F-b site (underlined) and clustered CpGs on either side: 5'-GGCCGGCATCGCCGCGGGCGTTTTACTCAGAAGCGCGGGGGCG-3', and the EA fragment, harboring a mutated E2F-c site (underlined), with sparse CpGs throughout the fragment: 5'-CTACACTGGTTTTGAATCACTGCGCGTTTACTCACGCTGGGGTCAGGGGTCGGGTTCGGGTGGGGGGGCGGAGG-3'. Both fragments were methylated *in vitro* using the *Sss*I methylase and S-adenosyl-L-methionine; one-tenth of the reaction was incubated with radioactive S-adenosyl-L-[methyl-^{3}H]methionine and the incorporation of [^{3}H]-methyl groups was measured as described (24). Poly(dI-dC), poly(dA-dT) (both from Sigma) and poly[d(5-methyl)C-dG] (Amersham Pharmacia Biotech) were used as non-specific competitors. Gel-shift reactions were set up as described in (23,25). For supershift experiments, the following antibodies (0.1–0.2 μg/μl of reaction) were added for 3 h on ice: pRb (C-15); p107 (SD-9); p130 (C-20); E2F-1 (KH95); E2F-3 (C-18); E2F-4 (C-20 sc866); E2F-5 (C-20) (Santa Cruz Biotechnology). Anti-MeCP2 antibodies [674 and 670 clones, see (26) for details] were a gift from A. Bird and were used in a 1:10 dilution.

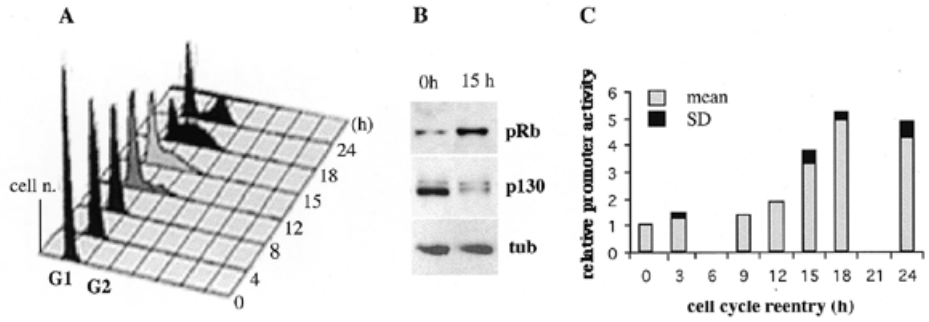

Figure 1. Cell cycle expression of the *Rb-1* promoter. (**A**) FACS analysis of NIH 3T3 cultures brought to growth arrest by serum starvation and restimulated to cycle. Cell numbers are plotted on the *y* axis, the DNA content is plotted on the *x* axis, cell cycle progression (in hours from the time of restimulation) is indicated on the *z* axis. (**B**) Western blot experiments with protein extract (40 µg/lane) from cells harvested after 0 (growth-arrested) and 15 h (G1/S transition) of cell cycle re-entry. Filters were incubated with antibodies against pRb, p130 and tubulin as a loading control. (**C**) Activity of the wild-type *Rb-1* promoter (pRb-CAT construct) during cell cycle progression. Promoter activity was calculated by measuring synthesized CAT enzyme at each time point, and normalizing relative to the amount of β-galactosidase from a cotransfected CMV-dependent plasmid. Values are expressed relative to the basal level recorded in growth-arrested (0 h), which was taken as 1; mean and SD values were calculated from four independent experiments.

Western immunoblotting

Extracts were prepared from serum starved and restimulated NIH 3T3 cells, resolved through SDS–PAGE and electroblotted as described in (25). After blocking in 5% low-fat milk, membranes were incubated for 2 h with the following antibodies: anti-tubulin (Amersham Pharmacia Biotech), 0.05 µg/ml; anti-E2F-1, anti-E2F-4, anti-pRb, anti-p130 (Santa Cruz Biotechnology, see above), 1 µg/ml each. Bands were detected using horseradish–peroxidase conjugated secondary antibodies and revealed using the enhanced chemioluminescence system (ECL-plus, Amersham Pharmacia Biotech).

RESULTS

An E2F promoter element contributes to S-phase up-regulation of *Rb-1* gene transcription

Several studies have examined the retinoblastoma protein during the cell cycle and have depicted increased levels in S phase (reviewed in 4,9,10), reflecting an increased abundance of *Rb-1* mRNA (16). However, no parallel time-course analysis of the *Rb-1* promoter has been carried out. In this study we have used murine NIH 3T3 cells, whose effective response to serum starvation/restimulation methods (19) enabled us to address that question. As shown in Figure 1A, cells collected prior to serum stimulation were arrested in the G0/G1 state and, after serum refeeding, progressed synchronously through the cell cycle. We controlled that pRb protein levels were up-regulated in S phase cells by western blotting assays (Fig. 1B); S phase up-regulation was specific, as shown by comparison with the p130 retinoblastoma-related protein encoded by the *Rb-2* gene, which was instead abundant in G0 cells and decreased during S phase, as expected (4). We next examined promoter activity during cell cycle progression. The pRb-CAT construct contains the minimal *Rb-1* promoter, carrying all elements required for transcription, upstream of the CAT gene. This construct was transfected in NIH 3T3 cell cultures which were induced to synchronously progress through the cell cycle. Results in Figure 1C show that the *Rb-1* promoter was up-regulated after 15 h of cell cycle re-entry, corresponding to the G1/S boundary in our experiments (Fig. 1A). From 15 to 18 h of restimulation, i.e. when cells were traversing S phase, the *Rb-1* promoter underwent a 5-fold induction; for comparison the *E1A* cell cycle-independent promoter was induced by 1.3-fold [data not shown; also see (22)]. Thus, the S phase-dependent increase in pRb levels in Figure 1B truly reflects transcriptional up-regulation of the *Rb-1* gene.

The *Rb-1* promoter harbors several transcription factor-binding sites. Elements recognized by E4TF1 and CREB/ATF factors were previously found to be essential for transcription (13,14,27). A consensus E2F site is located in the region of transcription initiation in the human (14) and murine (13) genes. Primer extension experiments with the pRb-CAT construct (data not shown) confirmed that mRNA transcription in the plasmid vector is also initiated at two sites flanking the E2F sequence (summarized in Fig. 2A). Previous studies of the E2F site in the *Rb-1* promoter indicated either a negative (15) or, on the contrary, a positive (16) role in cells of various origin. Most available evidence derives from transformed cell lines. We were interested to assess whether the E2F site contributed to *Rb-1* promoter up-regulation during a normal cell cycle, in which the endogenous pRb undergoes S phase-dependent up-regulation. To that aim, the *Rb-1* promoter construct was subjected to site-directed mutagenesis to inactivate the E2F site. The wild-type (pRb-CAT) and E2F-mutagenized (pRb-CATxE2F) constructs (Fig. 2A) were transfected in NIH 3T3 cultures. For comparison, we also analyzed the cell cycle-dependent pTS-A construct, carrying the *RanBP1* gene promoter (22,23). Transfected cell cultures were growth-arrested by serum starvation and subsequently restimulated to cycle; a biparametric FACS analysis of the DNA content and of BrdU incorporation showed that 15 h after serum restimulation cells reached the G1/S transition (19). At that time, up-regulation of transcription was comparable for both the *Rb-1* and the pTS-A promoters, yielding a 3-fold increase (Fig. 2B). In contrast, the E2F-defective *Rb-1* promoter failed to undergo G1/S-dependent activation, and transcriptional activity did not significantly differ from the basal level measured during growth arrest.

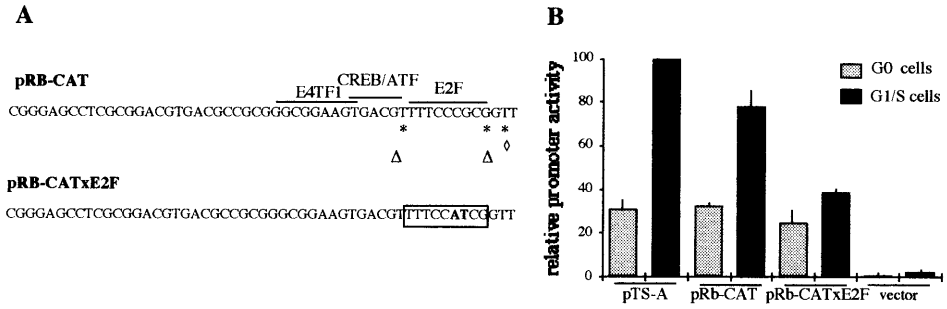

Figure 2. Activity of the wild-type and mutated *Rb-1* promoter constructs in growth-arrested and G1/S cells. (**A**) Sequence of the human *Rb-1* promoter in the pRb-CAT construct; relevant factor-binding sites are indicated; symbols are as follows: asterisks, murine transcription starts (13); lozenge, transcription starts in the endogenous human gene (14); triangles, transcription starts in the transfected pRb-CAT plasmid (our unpublished results). Below, the E2F mutation in the pRB-CATxE2F construct is boxed; mutagenized bases are in boldface. (**B**) Transient expression assays of the wild-type (pRb-CAT) and E2F-mutated (pRb-CATxE2F) constructs in growth-arrested and in G1/S cells. Parallel cultures were transfected with the cell cycle-dependent *RanBP1* promoter (pTS-A construct) and with vector alone. CAT activities were measured and normalized relative to β-galactosidase from a cotransfected plasmid as decribed for Figure 1. Histograms represent the activity of each construct relative to that of pTS-A in S-phase cells, which was taken as 100%; mean and SD values (bars) were calculated from three independent experiments.

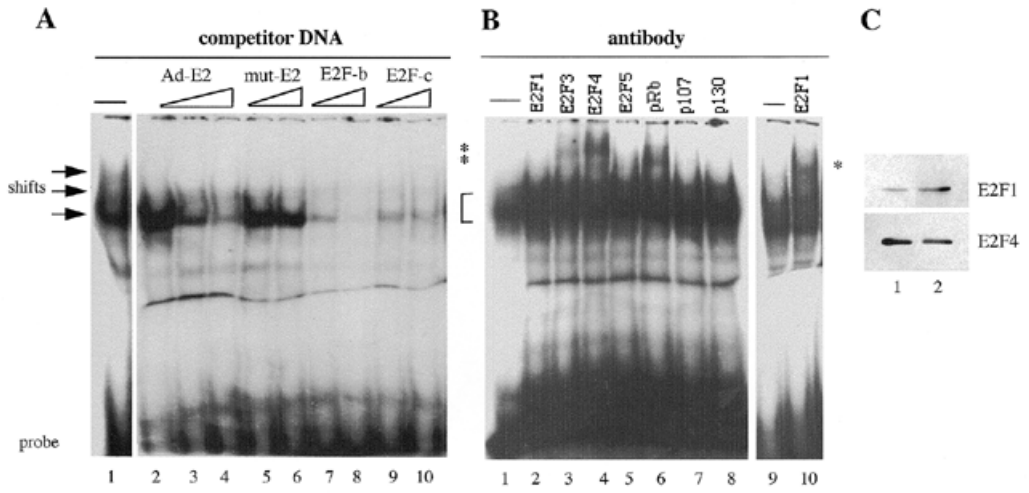

Figure 3. Gel-shift assay of the E2F site from the *Rb-1* promoter. (**A**) Competition assays with characterized E2F sites. Nucleoprotein complexes are arrowed; competitor E2F sites are from the Adenovirus *E2* (Ad-E2), and *RanBP1* (sites E2F-b and E2F-c), promoters; mut-E2 is a mutagenized version of site E2F-b. (**B**) Immunological characterization of protein complexes interacting with the *Rb-1*-derived E2F site; 10 μg of total protein extract from cycling cells were incubated with no antibody (lanes 1 and 9), antibodies against E2F factors except E2F-2 (lanes 2–5), and pRb-related proteins (lanes 6–8). In lanes 9 and 10, extracts were enriched in E2F-1/DP1 proteins. Retarded complexes are indicated by a bracket and supershifts by asterisks. (**C**) Western immunoblotting of the endogenous E2F-1 and E2F-4 factors in whole extracts from standard cell cultures (lane 1) and after transfection with E2F-1 and DP1 expression constructs (lane 2).

These results indicate that integrity of the E2F element is required for G1/S-dependent up-regulation of *Rb-1* transcription in NIH 3T3 cells.

The E2F site in the *Rb-1* promoter interacts with transactivating members of the E2F family

The protein-binding features of the *Rb-1*-derived E2F element were examined in gel-shift experiments using extracts from proliferating NIH 3T3 cells. Assembled nucleoprotein complexes were competitively inhibited by characterized E2F sites, but not by a mutated E2F sequence (Fig. 3A), nor by a CREB/ATF oligonucleotide (data not shown); the latter was included for control, because the E2F site in the *Rb-1* promoter is immediately flanked by a site for CREB/ATF factors, the most 3' bases of which are included in the E2F gel-shift probe. Thus, the *Rb-1* E2F element is a bona fide E2F-binding site. E2F sites with slight sequence variations act as targets for different E2F members *in vivo* (23,28,29) and *in vitro* (30). To identify components interacting with the *Rb-1*-derived E2F site, we used specific antibodies against E2F and retinoblastoma-related proteins. Nucleoprotein complexes were reactive to antibodies against E2F-3, E2F-4 and pRb, but not against the retinoblastoma-related p130 and p107 proteins (Fig. 3B). pRb preferentially interacts with, and antagonizes transactivation by, E2F-1, E2F-2 and E2F-3 members (reviewed in 1,4). In our experiments, E2F-3 effectively binds the E2F site (Fig. 3B, lane 3) and thus may bridge at least part of the pRb protein to the *Rb-1* promoter. The E2F-2 antibody gave no reaction using various sources of

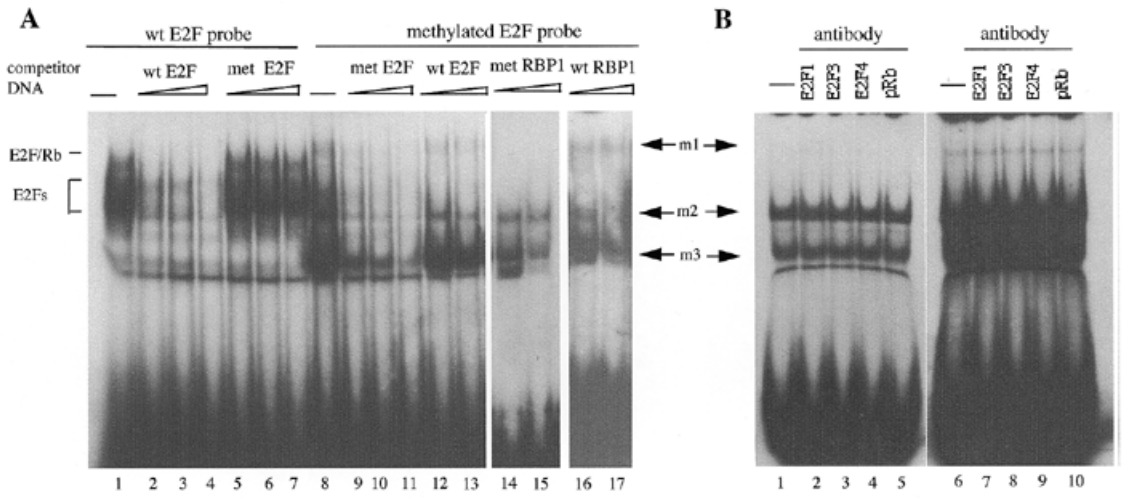

Figure 4. Specific nucleoprotein complexes interact with the methylated E2F site. (**A**) Gel shift assays using the unmethylated (wt) E2F probe with no competitor (lane 1), and with increasing amounts of homologous (lanes 2–4), or methylated (lanes 5–7) E2F site; E2F complexes and complexes containing pRb are indicated. Lane 8, gel shift assays using the methylated (met) E2F probe without competitor DNA, and with increasing amounts of methylated (lanes 9–11) or unmethylated (lanes 12 and 13) E2F site, and of methylated (lanes 14 and 15) or unmethylated (lanes 16 and 17) EA fragment from the *RanBP1* (RBP1) promoter. Nucleoprotein complexes are indicated as m1, m2 and m3. (**B**) Supershift assays of the binding reaction with methylated E2F probe using antibodies against E2F and pRb factors. Two exposures of the same experiment are shown. The short exposure shows that methylcytosine-binding complexes (lane 1) are unaffected by the antibodies (lanes 2–5); no trace of supershifted complexes is seen even after overexposure of the autoradiograph (lanes 6–10).

protein extract (data not shown). The anti-E2F-1 antibody also apparently failed to give a distinct supershift, despite the many demonstrations that E2F-1 biologically acts as the preferred pRb target (1–4). In previous experiments, we found that the absence of a discrete supershift reflects a low relative abundance of E2F-1 compared to other E2F species in NIH 3T3 cells (23). Western blot experiments in Figure 3C (lane 1) show that E2F-1 is indeed significantly less represented than E2F-4 in whole cell extracts. To establish whether E2F-1 can actually interact with the *Rb-1*-derived E2F site, we transfected cycling cells with constructs expressing E2F-1 and its DP1 dimerization partner, and repeated the experiments using protein extracts from transfected cells. Under these conditions, the relative abundance of E2F-1 and E2F-4 was comparable as revealed by western immunoblotting (Fig. 3C, lane 2), and a complex reactive to anti-E2F-1 was clearly visualized in binding assays (Fig. 3B, lane 10). That result was specific, since overexpressed E2F-1/DP1 complexes do not bind all consensus E2F sites, but show a clear preference for particular DNA sequences (23). In summary, therefore, the *Rb-1*-derived E2F site is recognized by several E2F family members, and, among pocket proteins, by pRb.

Cytosine methylation abolishes binding by E2F factors and targets unrelated DNA-binding activities to the E2F site

The *Rb-1* promoter is aberrantly methylated in tumor cells (12). DNA footprinting experiments with the Adenovirus-derived *E2* elements previously showed that a methylated site failed to compete for factors interacting with the unmethylated site (17). However, the sensitivity of E2F factors to CpG methylation was not directly assessed. To address that question, we synthesized an E2F oligonucleotide in which both tandemly repeated CpGs, characteristic of the *Rb-1* site, were methylated and examined it in gel-shift assays. The experiments in Figure 4A confirmed that the methylated oligonucleotide did not compete for factors binding to the wild-type E2F site (lanes 1–7). When the methylated oligonucleotide was used as the probe, three novel DNA-binding complex(es) were detected (Fig. 4A, lane 8): m1 and m3 indicate the complexes of lowest and highest electrophoretic mobility, respectively, while m2 migrates with an intermediate mobility. All three complexes were inhibited by homologous competitor DNA (Fig. 4A, lanes 9–11). Complexes m1 and m3 were unaffected by the addition of unmethylated E2F competitor (lanes 12 and 13), and thus represent true methylcytosine-specific complexes. In addition, both m1 and m3 were sensitive to competition by a uniformly methylated fragment from the CG-rich *RanBP1* promoter carrying a mutated E2F site (25), but not by its unmethylated counterpart (lanes 14–17); this result indicates that the presence of methylated CpGs, rather than a specific sequence, is important for assembly of both m1 and m3. The complex of intermediate mobility, m2, had the lowest DNA-binding specificity, as its assembly was perturbed by both methylated (lanes 9–11, 14 and 15) and unmethylated (lanes 12, 13, 16 and 17) DNAs. The m2 complex migrates close to, though not quite at the identical position of, E2F complexes (compare lanes 7 and 8). To unambiguously rule out the possibility that m2 reflected residual binding by E2F factors to the methylated site, the reaction was challenged with E2F-specific antibodies. As shown in Figure 4B, m2 was totally unaffected in the presence of anti-E2F or anti-pRb antibodies (lanes 1–5); overexposure of the gel-shift autoradiograph enabled us to rule out that minor amounts of complex(es) were supershifted (lanes 6–10), indicating that no residual binding of E2F factors to the methylated site took place. These results clearly indicate that cytosine methylation prevents the assembly of all E2F complexes. Finally, we examined the stability of complexes assembled with the methylated and unmethylated E2F sites by assaying

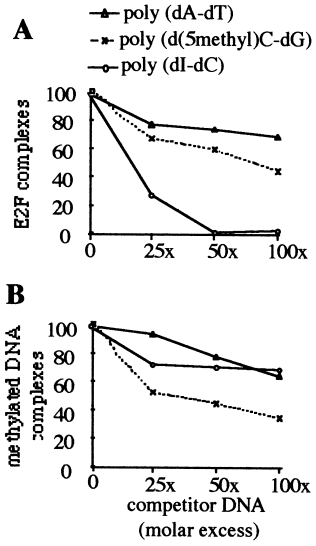

Figure 5. Stability of the complexes interacting with unmethylated (**A**) and methylated (**B**) E2F site, in the presence of synthetic competitor DNAs. Molar excesses are indicated on the *x* axis. The radioactivity associated to free and complexed probe was measured by microdensitometry. In (A) each point represents the value obtained for retarded E2F complexes/input probe (i.e. E2F complexes + free E2F probe); (B) shows the values obtained for (m1 + m3)/input probe (i.e. m1 + m2 + m3 + free probe); in both panels, the value obtained in the absence of competitor DNA was taken as 100%. Mean values from two independent experiments are shown.

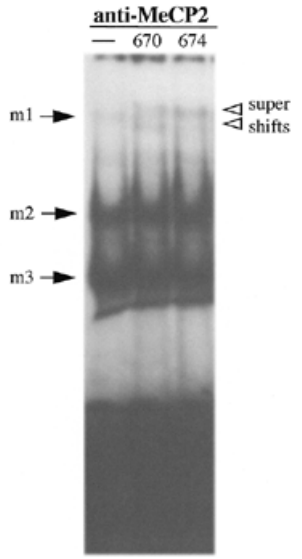

Figure 6. Immunological detection of MeCP2 in nucleoprotein complexes with methylated E2F probe. Gel-shift reactions with methylated E2F probe were set up after preincubation with either 640 or 647 anti-MeCP2 antisera. Methylated DNA-binding complexes (m1, m2 and m3) are arrowed; open triangles indicate the supershifted complexes generated by anti-MeCP2 antibody.

their sensitivity to competition by synthetic DNA polymers: factors binding to the wild-type E2F oligonucleotide were displaced in the presence of increasing amounts of poly(dI-dC), as expected of genuine E2F factors (Fig. 5A). Factors binding to the methylated E2F site were instead highly sensitive to competition by poly[d(5-methyl)C-dG], while remaining stable in the presence of either poly(dI-dC), or poly(dA-dT) (Fig. 5B). Together, these results indicate that distinct factors are recruited in stable complexes with the methylated, versus the unmethylated, E2F site.

MeCP2 binds the methylated version of the E2F site

Results thus far indicate that both m1 and m3 complexes contain methyl-cytosine binding protein(s) (Figs 4 and 5). Two such proteins are extensively characterized: MeCP1 (31) and MeCP2 (32,33). MeCP1 is a large protein requiring several methylated CpGs to bind DNA, and is therefore unlikely to bind the methylated E2F oligonucleotide. MeCP2 is a general transcriptional repressor (18) and, in the purified form, can bind synthetic DNA fragments carrying as little as one single CpG (34). To assess the presence of MeCP2 in m1/m3 complexes, we used two antibodies (described in 26), which respectively recognize residues 1–390, including the DNA-binding domain (antiserum 674), and the C-terminal region (residues 207–492), containing the transcriptional repression domain (antiserum 670), of MeCP2. Extract preincubation with either antibody yielded supershifted complexes, indicating that MeCP2 is indeed part of the complexes that bind the methylated E2F site (Fig. 6). Densitometric scanning of three independent gel-shift experiments showed that antibody addition supershifted virtually all of the m1 complex, of lower abundance, and a variable proportion of the more abundant m3 complex. Preincubation of protein extract with sodium deoxycholate, which disrupts weak protein–protein interactions, prevented the assembly of complex m1, while leaving m3 unaffected (data not shown). Together, these observations indicate that MeCP2 is indeed present both in m3 and, as a multiprotein complex, in m1.

DISCUSSION

The *Rb-1* gene encodes the pRb tumor suppressor protein, whose role in negative control of cell growth is extensively documented (4,8–10). The pRb protein acts largely, though not exclusively, as a repressor of transcription of cell cycle genes by antagonizing several activating factors (1–3), including members of the E2F family. Here we have determined the timing of activation of the *Rb-1* promoter and have mapped it to the G1/S boundary (Fig. 1). Transcriptional activation is reflected by increased levels of pRb protein in S phase cells. It is well established that cell cycle progression requires pRb inactivation by cyclin-dependent kinases, which phosphorylate pRb and neutralize its biological activity (9). Thus, the time-course analysis presented here contributes to define the cell cycle window during which the balance between *Rb-1* gene transcription and pRb inactivation is crucial for cell cycle control.

Previous studies of the *Rb-1* promoter consistently identified two essential regulatory elements, harboring sites for RBF/E4TF1 and CREB/ATF factors, respectively (13,14,27). An E2F site in the region of transcription initiation may represent a third regulatory element. Functional studies of that site yielded conflicting results. Certain *Rb-1* derivatives carrying particular E2F mutations, TTTAAACC (13) or TTTTCTT (15),

were more effectively expressed than their wild-type counterpart. These results were taken to indicate that the E2F site negatively controls transcription. However, due to the location of the E2F site in the region of transcription initiation (Fig. 2A), particular mutations may in fact favor the recruitment of initiation factors to the *Rb-1* promoter, which lacks a TATA box. Most TATA-less promoters indirectly recruit the TATA-binding protein via initiator-binding factors (reviewed in 35). By comparison with characterized initiator sequences (36), certain E2F mutagenized versions may coincidentally increase transcription initiation from the *Rb-1* promoter. In fact, an independent mutation that changed the E2F sequence to TTTACCACG, bearing no TATA or initiator resemblance, impaired *Rb-1* promoter activity and responsiveness to exogenous E2F-1 (16). Different results obtained with different cell types may also reflect the ability of the E2F site to interact with different factors in particular cellular contexts. Several *Rb-1* promoter studies were carried out in transformed cells, in which the pool of transcriptional regulators may be altered. These experiments depict mechanisms whose control is lost during tumorigenesis, yet may not necessarily identify those mechanisms which normally ensure cell cycle-dependent transcription of the *Rb-1* gene.

Here we have used NIH 3T3 cells that were synchronously progressing through the cell cycle and have addressed the role of the E2F site in S phase-dependent *Rb-1* transcription. In our study, E2F mutation to TTTCCATCG impaired up-regulation of the *Rb-1* promoter at the G1/S boundary (Fig. 2). The E2F site recruits several E2F family members *in vitro* (Fig. 3). Among those, E2F-4 is functionally regulated by compartmentalization, being nuclear in G0 cells and exiting the nucleus at the G1/S boundary (23,37,38). The binding of E2F-4 depicted here is likely to reflect the high abundance of E2F-4 in whole cell extracts (Fig. 3C), yet does not necessarily indicate a biologically relevant interaction, since E2F-4 is not present in the nucleus in the cycle phase during which *Rb-1* transcription is up-regulated *in vivo*. Among factors that bind the *Rb-1*-derived E2F site *in vitro* (Fig. 3B), both E2F-1 and E2F-3 are candidate *trans*-activators of the *Rb-1* promoter in S-phase cells *in vivo*, based on cell cycle distribution of E2F family members (4).We have also found that enforced pRb expression yields *Rb-1* promoter repression in both growth-arrested and S-phase NIH 3T3 cells (data not shown), consistent with previous indications that pRb controls an auto-regulatory loop by regulating the activity of its own promoter (14–16,39). Exogenous pRb can repress both the wild-type *Rb-1* promoter and various mutated derivatives (our unpublished data and 14,15), indicating that pRb regulates its own promoter through several elements besides the E2F site. It has recently been shown that repression of transcription by pRb is exerted, at least in part, through the interaction with histone deacetylases, which remodel chromatin in an inactive conformation (40–42). E2F factors bound to target promoter elements play a pivotal role in recruiting pRb-bound deacetylases to E2F-driven promoters (43).

In certain tumors, the *Rb-1* gene promoter undergoes aberrant methylation (12). Several studies of CpG-rich promoters suggest that inactivation of key factor-binding sites following mutation, or methylation, trigger transcriptional inactivity, which will ultimately be 'sealed' by methylation spreading throughout the surrounding CG-rich sequences. A previous study using methylases with different substrate specificities

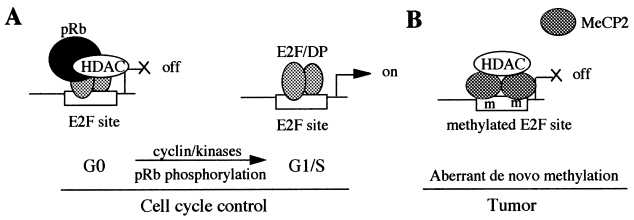

Figure 7. Hypothetical model of the interactions between proteins and the *Rb-1*-derived E2F site. In (**A**) the E2F site is occupied by E2F/DP complexes that are blocked in the interaction with pRb, and hence are transcriptionally ineffective; pRb bridges deacetylase (HDAC) molecules to the promoter which is inactive (left panel). As cells approach the G1/S transition (right panel), pRb is inactivated through phosphorylation by cyclin-dependent kinases. These mechanisms ensure cell cycle-regulated *Rb-1* promoter activity. In (**B**) methylated CpGs in the E2F site are indicated with m. If aberrant methylation takes place within the E2F site, E2F factors are excluded whereas MeCP2 is stably recruited. MeCP2 bridges deacetylase (HDAC) molecules near the transcription start and remodel the surrounding chromatin in an inactive state.

helps to understand how methylation affects *Rb-1* transcription (44). Activity of the *Rb-1* promoter was unaffected by *Hpa*II methylase, whose recognition sites fall outside genetically identified promoter elements, i.e. RBF/E4TF1, CREB/ATF and E2F. In contrast, promoter activity was abolished after methylation by the mammalian CpG methyltransferase, and was drastically reduced (24% of the wild-type level) by *Fnu*DII methylase. These results indicate that methylation of CpG dinucleotides differentially affects the *Rb-1* promoter, depending on whether it does, or does not, interfere with the interaction of transcription factors with regulatory elements. Examination of the *Rb-1* promoter sequence reveals that neither the CREB/ATF element, nor the site recognized by RBF/E4TF1—as defined in a study of RBF1/E4TF1 binding requirements (45)—contain methylatable sites by *Fnu*DII (CGCG). The E2F element contains instead one *Fnu*DII site. Thus, changes in the methylation status of the E2F site can be expected to yield significant functional consequences. We have found that not only the methylated E2F site becomes refractory to binding by E2F factors, but also acts as a binding site for MeCP2 (Fig. 6).

The binding of MeCP2 to genuine promoter elements had not been previously examined, because most binding studies with methylated DNA have made use of random oligonucleotides. Here we show for the first time that the same promoter element can act as a binding-site for E2F activators, or for MeCP2, depending on its methylation status. These alternative possibilities are schematized in Figure 7. MeCP2 binding to the *Rb-1*-derived E2F site may be stabilized due to the presence of two tandem CpG dinucleotides; such a tandem repetition does not occur in all identified E2F sites (3,5). These findings suggest that once aberrant methylation takes place within the E2F element of the *Rb-1* promoter, which was mimicked here by using a methylcytosine-substituted oligonucleotide, E2F factors are excluded, while MeCP2 is stably recruited. Thus, the present observations suggest that inactivity of the *Rb-1* gene in tumors is not only due to failure of promoter binding by transcription factors, but also to MeCP2 recruitment to the promoter.

MeCP2 interacts with methylated DNA in chromatin complexes containing the Sin3 co-repressor and histone deacetylases

(26,46). This suggests that MeCP2 acts as a bridge between methylated promoter sequences and proteins favoring nucleosome deacetylation, which inhibit transcription. Furthermore, repression of aberrantly methylated tumor suppressor genes cannot be relieved by exposing tumor cells to histone deacetylase inhibitors, suggesting that DNA methylation and nucleosome deacetylation synergize in imposing a transcriptionally inactive chromatin organization (47). In situations in which the *Rb-1* promoter becomes aberrantly methylated, MeCP2 bound to the methylated E2F element may recruit proteins that mediate histone deacetylation in the region of transcription initiation. This would remodel the nucleosomal organization in an inactive state and contribute to *Rb-1* transcriptional repression. Other sequences in the *Rb-1* promoter may also contribute to MeCP2 recruitment; however, the presence of two tandem CpG dinucleotides in the E2F site, and its proximity to the transcription starts, suggest that the E2F site may be important for integrating different regulatory signals which determine the transcriptional state of the *Rb-1* gene. Once methylation-linked repression is established at the *Rb-1* promoter, it will override the cyclic control of transcription which normally relies on cell cycle-regulated interactions between promoter elements and 'free', or pRb-bound, E2F complexes (Fig. 7). It is interesting to note that both MeCP2, interacting with methylated promoters, and pRb, physiologically regulating repression of cell cycle-regulated promoters, ultimately recruit the same class of histone deceatylases to inactivate transcription.

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