Chromatin-dependent E1A Activity Modulates NF-κB RelA-mediated Repression of Glucocorticoid Receptor-dependent Transcription*

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The role of chromatin-dependent regulatory mechanisms in the repression of glucocorticoid-dependent transcription from the murine mammary tumor virus (MMTV) promoter by p65 and E1A was investigated by using chromatin and transiently transfected reporters. The p65 RelA subunit of NF-κB represses MMTV expression on either transient or integrated reporters. In contrast, the viral oncoprotein E1A represses a transient but not an integrated MMTV. E1A repression is attenuated by chromatin, suggesting that E1A manipulates chromatin appropriately to inhibit the GR. Coexpression of p65 and E1A additively represses the transient MMTV but restores the transcriptional activation of the chromatin MMTV in response to glucocorticoids. This indicates that E1A has a dominant chromatin-dependent activity that attenuates repression by p65, E1A, p65, and GR, and binds the MMTV promoter, and chromatin remodeling enhances binding on both repressed and activated promoters. In addition, p65 requires Brg for repression of the integrated MMTV. This suggests that neither p65 repression nor E1A attenuation of repression results from an inhibition of remodeling that prevents transcription factor binding. Furthermore, p300/CBP is also required for both repression and attenuation by p65 and E1A. E1A and p65 mutants that do not bind p300/CBP are inactive, indicative of a requirement for p300/CBP-dependent complex formation for both repression and attenuation with chromatin. These data suggest that both the p65-dependent repression and the E1A-mediated attenuation of repression require the Brg1-dependent chromatin remodeling function and p300/CBP-dependent complex formation at a promoter assembled within chromatin.

Repression of transcription has been shown to involve the regulation of chromatin and a requirement for chromatin-modifying genes in different functional capacities (1). The chromatin structure of heterochromatin is a long term, non-gene-specific, silencing mechanism. The establishment of the heterochromatin structure is a process that requires sequential interaction of different chromatin-modifying proteins, and the heterochromatic structure blocks normal chromatin remodeling as the mechanism of action (2, 3). More simply, repression may be achieved by disrupting the function of a particular cofactor required for activation. The target cofactor may be required for chromatin regulation or for non-chromatin-related activation functions (4). Inactivation of a chromatin remodeling cofactor can repress without involving other regulatory factors as found for the Brg1 mutants (5). Alternatively, direct repressors targeted to specific genes may require active chromatin remodeling function and specific modification of histones or DNA. For example, the retinoblastoma protein requires a functional BRG1 and recruits HDACs, histone methylase, HP1, and Dmnt1 for repression (6–11). The way these cofactors interact to establish a repressed gene in euchromatin is poorly understood. Most euchromatic genes are transiently responsive to both protein activators and repressors, and a repressive chromatin structure analogous to heterochromatin has not been shown for transient repressors.

The effect of chromatin organization on transcriptional activation has been extensively studied for the MMTV promoter (12). The MMTV promoter is both functionally and structurally different when it is stably integrated compared with transiently transfected DNA, and the difference has been linked with specific chromatin structure and regulatory mechanisms. The MMTV promoter is organized as a phased array of nucleosomes when stably introduced into cells (13, 14). In contrast, transiently transfected MMTV reporters, although associated with histones, do not exhibit a normal nucleosome structure (15–17). Specific transcription factors (NF1) bind less efficiently to a stable chromatin template, in the absence of hormone, compared with a transient template and exhibit a glucocorticoid-dependent increase in binding to the chromatin template that is not apparent on the transient template (18). Following hormone treatment, the integrated MMTV promoter is modified such that sites previously resistant to DNase I and restriction digestion become hypersensitive (15, 19). BRG1 is required for remodeling and activation, and competition for BRG1 can regulate expression (5, 20–22).

The role of chromatin structure on repression of the MMTV has not been well studied. Several protein transcription factors repress glucocorticoid-dependent transcription of MMTV, including the p65 (RelA) subunit of NF-κB, adenovirus E1A, p53, Stat5, and AP1 (23–28). All of these factors activate transcription from other genes in the same cells, so repression is a gene-specific function. The specific mechanism of repression has not been identified for any of these repressors, but targeting of cofactors with chromatin modifying activities has been reported. Repression of the MMTV by p65 involves a coopera-

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1 The abbreviations used are: HDAC, histone deacetylase; MMTV, murine mammary tumor virus; CBP, cAMP-response element-binding protein-binding protein; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; GR, glucocorticoid receptor; GRE, glucocorticoid receptor element; WT, wild type; CMV, cytomegalovirus.
E1A Attenuates p65 Repression on Chromatin

The adenovirus type 5 E1A gene products are multifunctional regulators of cell growth and gene expression (34). There are two major E1A proteins, 13S and 12S, that respectively activate or repress transcription of specific genes. E1A does not encode a specific DNA binding domain but regulates cell growth and transcription via interaction with critical cellular proteins, including coactivators, corepressors, cell cycle control, and chromatin regulatory genes. E1A binds to both major categories of chromatin regulatory proteins, including histone-modifying and chromatin-remodeling proteins. E1A binds p300/CBP and may disrupt p300/CBP activation function and histone acetylation or block p300 association with other histone acetyltransferases such as pCAF (34–36). E1A may also regulate histone deacetylation function via CtBP binding (37–39). E1A expression inhibits growth in yeast, and E1A interacts with yeast homologs of human chromatin regulators. E1A targets the yGCN5p component of the SAGA complex, and expression of pCAF can overcome the growth arrest (40). E1A binds the yeast SWI/SNF protein SNF12 and inhibits SWI/SNF-dependent gene activation (41). Similarly, E1A targets human Swi/Snf proteins including p400 and TRRAP to mediate growth (42).

E1A can regulate the MMTV promoter in various ways. In mouse cells, activation of the MMTV promoter by the E1A 13S isoform occurs on both stable and transient templates, but repression by the 12S isoform is template-dependent (25). E1A can modify the effects of other transcription factors such as the human immunodeficiency virus, type 1, and the Vpr protein on the MMTV promoter (43). Similarly, p65-mediated effects on MMTV and endogenous promoters can be modified by E1A (44, 45).

This investigation explored how protein repressors use chromatin organization to influence repression. We evaluated chromatin remodeling during repression and whether chromatin regulatory cofactors are required for or lost in repression by p65 and E1A. We show that repression by p65 and E1A is mediated by chromatin context as is the attenuation of p65 repressor activity by E1A. Repression by p65 on chromatin requires a functional BRG1 and chromatin remodeling. E1A and p65 repression and chromatin-dependent attenuation of p65 repression requires a p300/CBP-dependent complex formation. Neither p65 repression nor E1A attenuation of repression results in the disruption of chromatin remodeling or loss of transcription factor binding at the promoter. This suggests that p65 repression and E1A regulation of repression depend on chromatin regulatory functions in common with activation and repression within the context of an actively remodeled promoter.

EXPERIMENTAL PROCEDURES

Cells, Cell Culture, and Treatment—UL3 and SW13 cells were maintained in Dulbecco’s modified Eagle’s medium (H21, Invitrogen), 10% fetal bovine serum, penicillin, and streptomycin at 37 °C and 5% CO2 (46). UL3 cells are derived from the human osteosarcoma cell line U2OS by the stable addition of a rat GR expression vector and a full-length MMTV promoter regulating a luciferase reporter (46, 47). SW13 cells were cotransfected with an MMTV-luciferase reporter and a neomycin-resistant cassette using Lipofectamine Plus (Invitrogen) according to the manufacturer’s protocol. Stable clones were picked and tested for stable MMTV integration by transient transfection assay using GR and Brg1 expression plasmids.

Transfection—Cells were transiently transfected using Lipofectamine Plus reagent (Invitrogen) with an efficiency of >80% as determined by β-galactosidase staining of cells transfected with 1 μg of pSPort reporter (Clontech) (48). Transient transfections routinely used a total of 1 μg of expression plasmids in 1 × 10⁶ UL3 cells or 4 μg of plasmid in SW13-MMTV cells. Expression plasmids used herein include the following: plhCAT (325 bp of proximal MMTV promoter driving a chloramphenicol acetyltransferase (CAT) reporter) (S. Nordeen, University of Colorado Medical Center, Denver); a CMV control vector expressing no protein or β-galactosidase; CMV expression vectors containing human p65 RelA and IκBα (L. McKay, NIEHS, National Institutes of Health) (29, 44); the p65 S276A expression construct (San Xia Lu, National Institutes of Health) (49); a human Brg1 and a rat GR (21). The full-length E1A (WT) was expressed from the E1A promoter (G. Preston, University of North Carolina, Chapel Hill) (50). The E1A 12S and RG2 are cDNAs for the specific isoforms (E. Moran, Temple University, Philadelphia).

In Vivo Restriction Enzyme Hypersensitivity Analysis—The sensitivity of promoters to restriction enzymes was analyzed following transfection using a standard calcium phosphate procedure with 20 μg of promoter-reporter DNA (p65, E1A, or p65/E1A) cotransfected with dexamethasone for 1 h. Nuclei were isolated and digested with SstI (100 units/ml) for 15 min at 30 °C. DNA was purified and digested to completion with HaeIII. For assaying hypersensitivity on a transient template, plhCAT plasmid was transfected with the expression vectors and left treated or untreated with dexamethasone for 1 h. Nuclei were isolated and subjected to limited digestion with SstI (100 units/ml). DNA was purified and then digested to completion with HaeIII.

The two templates were analyzed by reiterated primer extension with oligonucleotide primer (5′-TCT GGA AAG TGA AGG ATA AGT GAC GA-3′: base pairs +60 to +84), and products were separated on 8% polyacrylamide denaturing gel (18).

Chromatin Immunoprecipitation (ChIP) Assay—UL3 cells were transfected with p65 or E1A and plhCAT plasmid for ChIP of the p65 or E1A promoters or plhCAT plasmid in the presence of p65 or E1A and phhCAT plasmid for ChIP of the MMTV promoter. E1A or p65 were transfected alone for ChIP of the endogenous promoters. For native immunoprecipitation (see Fig. 7A), chromatin was prepared from cross-linked samples using substantially less starting material. For immunoprecipitation, 50-fold (for native) or 10-fold (for cross-linked) dilutions of cross-linked nuclear extracts were transfected into human 293T cells. ChIP assays from the integrated MMTV promoter. Chromatin immunoprecipitation assays were performed using a ChIP kit (Upstate Biotechnology, Inc., Lake Placid, NY). Briefly, cells were untreated or treated for 1 h with dexamethasone (10−7 M) and cross-linked with 1% formaldehyde for 30 min. Complexes were immunoprecipitated from p65-expressing cells with 10 μg of antibodies to p65, GR, or rabbit IgG for the nonspecific (NS) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). E1A-expressing cells were immunoprecipitated with E1A (Upstate Biotechnology, Inc.), GR and IgG were used as positive and negative controls. Complexes were collected using protein A-agarose/DNA beads and washed five times using low salt, high salt, LiCl, and TE buffers. Samples were divided in half for further analysis of coprecipitation with specific or MMTV promoter. After reverse cross-linking for 4 h, the genomic DNA was purified from eluted complexes with a QIAquick PCR purification kit (Qiagen, Valencia, CA). MMTV DNA was detected by PCR amplification using primers flanking the GRE (sense 5′-TTAGTTAAGTTTTTTGTTACAAACT-3′ and antisense 5′-TCTGGAAAAGTGAAGTATTACGACG-3′) and including ~300 bp of the proximal promoter. An antisense primer specific to the plhCAT plasmid (5′-TATCTCGTTCCTTCTAGTTCCGCTCCGAAAT3′) was used to detect the transient template. Primers for the GAPDH promoter (sense 5′-AAAAACGCCCAGGGAGAATTGAG-3′ and antisense 5′-CTAGCCCTC- CCGGTGGTTCTCT-3′) as the nonlinked control and the 3′-luciferase gene (sense 5′-CCCTGTTGTTTTGTTGAGC-3′ and antisense 5′-CTA- CATTGGGACTCTCCGCG-3′) as the linked control were used to verify site-specific binding. Total PCR products were quantified from ethidium bromide-stained 1.5% agarose gels using Alpha Innotech AlphaImager software. Samples were separately quantified from radiolabeled products separated on 8% polyacrylamide gels using PhosphorImager software (Amersham Biosciences). No qualitative difference was observed between the two techniques. Error bars shown on Fig. 4 represent quantitation from separate ChIP experiments.

Dimerization Assay—A dimerization assay was performed by alternative protocols using non-cross-linked or cross-linked proteins. No qualitative differences were observed between the two techniques in detection of protein association; however, protein binding was detected from cross-linked samples using substantially less starting material. For native immunoprecipitation (see Fig. 7A), UL3 cells were transfected with p65, E1A, or both and treated for 1 h with dexamethasone. Cells were washed in cold PBS and lysed in “native” buffer containing...
250 mM NaCl, 5 mM Hepes, pH 7.0, 0.1% Nonidet P-40, and protease inhibitor (Sigma). Whole cell lysates were preincubated with protein G-agarose beads. Lysates were incubated overnight at 4 °C with 10 μg of the indicated antibody. Antibody complexes were collected by incubation with protein G-agarose beads followed by four washes with native buffer. For cross-linked immunoprecipitation (see Figs. 3 and 7B), cells were treated as described for the ChIP assay. Following immunoprecipitation and washing, the protein samples were resuspended in Tris-EDTA buffer. Both native and cross-linked samples were boiled in SDS loading buffer, separated by 8 or 10% SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Invitrogen). Proteins were visualized using p65, E1A, or GR antibodies (Santa Cruz Biotechnology) with horseradish peroxidase-conjugated anti-IgG secondary antibody and chemiluminescence reagent (PerkinElmer Life Sciences).

RESULTS

E1A and p65 Repressor Activity Is Influenced by Chromatin Context—The transcriptional repressors p65 and E1A can inhibit GR-dependent transcription (23–25, 34). To explore the underlying mechanisms and to evaluate the potential role of endogenous chromatin structure in this repression, we have compared p65 and E1A on the MMTV promoter when integrated within chromatin to the promoter introduced into the cells by transient transfection. Previous experiments established that the integrated MMTV promoter exhibits an organized chromatin structure of phased nucleosomes that limit the binding of transcription factors and prevent digestion by restriction enzymes in the absence of hormone-induced chromatin remodeling (51). In contrast, the transiently transfected MMTV reporter exhibits an ill defined structure, binds transcription factors in the absence of hormone, and does not exhibit remodeling influenced hypersensitivity to restriction digestion (46).

We observed that both p65 and E1A effectively repress transient MMTV-CAT expression following treatment with the synthetic glucocorticoid dexamethasone (Fig. 1A). CAT activity in p65-expressing cells is repressed 70% in comparison to control cells transfected with an empty CMV expression vector. Similarly, CAT activity in E1A-expressing cells is repressed 85%. Basal transcription from the transient MMTV is 11% of the hormone-induced levels and both p65 and E1A-expression reduce basal transcription by 50%. In contrast to the results with the transient promoter, p65 but not E1A repressed transcription of the integrated/chromatin MMTV promoter (Fig. 1B). In this case the integrated promoter was repressed by p65 to a degree similar to the transient promoter (65 versus 70%). However, not only did the E1A fail to repress the chromatin promoter, it exhibited an increase in luciferase expression compared with controls.
The repression of the transient MMTV by both p65 and E1A suggests that repression does not require normal chromatin as a component in the mechanism of repression. However, the attenuation of E1A repression on the integrated MMTV suggests chromatin may restrict the function of a subgroup of repressor proteins and/or that E1A, in contrast to p65, is unable to effectively remodel/modify the chromatin template to repress transcription.

**E1A Regulates p65 Repression on the Chromatin MMTV Promoter**—E1A can bind directly to p65 (52). Additionally, p65 and E1A both bind to p300/CBP, although p65 binds via the p300 CH1 domain, and E1A binds to the CH3 domain (53-55). Thus E1A and p65 potentially influence the activity of one another and do so in some systems (52). The effect of coexpression of E1A and p65 on the activity of the MMTV promoter was tested in UL3 cells simultaneously transfected with p65, E1A, and phhCAT. Coexpression of p65 and E1A had an additive effect on repression of the transient promoter (Fig. 1A). Coexpression repressed transcription by 90%, 25% more than p65 alone and 5% more than E1A alone.

On the integrated MMTV, coexpression of E1A with p65 attenuates p65 repression on the chromatin template (Fig. 1B). Coexpression of E1A with p65 in equal amounts (1:1) reduced repression from 65 to 42%. E1A has a chromatin-dependent activity that attenuates the p65 repression. Therefore, E1A is active on a chromatin promoter, but within a chromatin context it has an alternative function from that found on a transient promoter.

The intermediate level of repression could be because of either a p65-E1A complex that has partial repression activity, or it could be attributed to competition for binding between complexes that contain only p65 and repress with complexes that contain E1A with or without p65 and are fully active. By increasing the amount of E1A relative to the amount of p65, the proportion of complexes that contain p65 can be saturated with E1A. Repression by p65 can be completely attenuated by increasing the amount of E1A (Fig. 1C). Thus, E1A chromatin-dependent activity is dominant in a p65-E1A complex in a chromatin context. The attenuation of p65 repression by E1A indicates p65 repression is dependent on a chromatin-modifying function that E1A can specifically disrupt.

IκB interacts directly with p65 and regulates the availability of p65. IκB alone has no effect on transient MMTV expression but completely attenuates p65 repression when coexpressed with p65 (Fig. 1A). The coexpression of IκB with p65 also attenuates repression on the integrated promoter (Fig. 1B). IκB attenuates p65 repression, but unlike E1A the activity is independent of the chromatin context of the MMTV promoter.

**GR, E1A, and p65 Associate in Vivo**—The preceding experiments suggest that E1A and/or p65 and GR may form complexes that influence transcription in different ways. A complex may activate, repress, or have partial activity depending on the components or the relative dominance of a component within the complex. Additionally, the activity of one component may require the activity of another. In this case neither p65 nor E1A have DNA binding domains to response elements in the MMTV promoter. Therefore, targeting of these factors to MMTV must occur by association with other regulatory factors, presumably the GR. Coimmunoprecipitation was used to examine the state of protein-protein interactions independent of the promoter chromatin context.

Consistent with the previous experiments, we observed that in cells transfected with p65, the GR was found associated with p65 (Fig. 2A). Similarly, the GR is also associated with E1A in cells transfected with E1A (Fig. 2B). Finally, p65 can be detected associated with E1A and GR in cells transfected with both E1A and p65 (Fig. 2C). Likewise, E1A is associated with GR in cells transfected with both p65 and E1A (Fig. 2D). The formation of complexes is not hormone-dependent (Fig. 2, C and D). Complexes that include p65 and GR, E1A and GR, or p65, E1A, and GR may all associate with the MMTV via GR binding to the hormone-response element but do not require hormone for complex formation.

**Effect of p65 and E1A on Hormone-dependent Chromatin Remodeling**—We evaluated the impact of p65 and E1A on promoter chromatin structure as revealed by chromatin remodeling and transcription factor binding assays. Transcription factor binding to the MMTV promoter and chromatin remodeling were evaluated by a restriction hypersensitivity assay to determine whether chromatin remodeling was occurring, and by the ChIP assay to determine when transcription factors bind to the MMTV promoter and whether hormone-dependent remodeling promotes binding of these repressors to the promoter.

Enhanced restriction enzyme digestion of integrated promoters is one method to detect chromatin remodeling. Hormone-dependent activation of MMTV alters chromatin by exposing restriction sites that were protected before hormone treatment, whereas a transient template is equally sensitive to digestion independent of hormone. On the transient promoter similar amounts of digestion were observed with and without hormone in CMV, p65, and E1A-expressing cells (Fig. 3B). Neither p65 nor E1A attenuated the constitutive hypersensitivity known to occur on the transient MMTV promoter.
control in the absence of hormone. Addition of hormone resulted in increased binding of p65 and GR to the MMTV. The mean fold increase in binding of p65 and GR to the integrated MMTV is shown for three or more measurements. Relative binding was normalized to nonspecific controls and compared with the unlinked GAPDH promoter or the linked luciferase gene in the same samples. The relative increase in hormone-dependent binding by p65 or GR on the chromatin MMTV was similar. Thus, p65 binds the repressed MMTV promoter, and binding is enhanced by hormone-dependent chromatin remodeling on the chromatin promoter.

On the transient MMTV promoter, E1A binds at levels higher than the nonspecific control and independent of hormone (Fig. 4B). The transient promoter is both associated with E1A and repressed by E1A as found for p65. E1A fails to repress the integrated MMTV promoter, and this failure would be readily explained if it failed to bind the chromatin promoter. However, on the integrated promoter E1A shows a hormone-dependent increase in binding, and the mean increase in E1A is similar to the mean increase in GR binding. Because the integrated promoter is not repressed by E1A, binding the promoter is not sufficient for E1A repression. Chromatin remodeling shown by hormone-dependent binding of the GR is consistent with the normal hormone-dependent activation of the integrated MMTV. The equivalent increase in E1A and GR binding suggests E1A is not excluded from the chromatin promoter but is a component of a transcriptionally active complex.

The binding of p65 and E1A in the cotransfected cells is similar to binding in cells with only a single repressor (Fig. 4C). Both p65 and E1A bind to the transient promoter independent of hormone. E1A, p65, and GR all show a hormone-dependent increase in binding to the integrated promoter. Thus, neither repressor is excluded from the chromatin promoter, and chromatin remodeling is normal. The function of E1A on chromatin that blocks expression of repression is effective on the promoter-bound p65, indicating that promoter binding alone is insufficient for repression by p65.

**Brg1 Is Required for p65 Repression of the Integrated MMTV Promoter**—The observation that p65 was able to inhibit GR transcriptional activation within the context of a remodelled promoter represents a unique separation of the GR-dependent chromatin remodeling and the activation function of the GR (56). Given that the GR remodels chromatin via its interaction with the BRG1 complex, we were curious as to the role of this remodeling complex in the p65 repression of GR activity (21). For these experiments we made use of the SW13 cell line, derived from small-cell carcinoma of the adrenal cortex, which lacks expression for both BRG1 and the alternative ATPase BRM. Specifically, the dependence of p65 repression on BRG1 activity was tested in SW13-MMTV cells that have an integrated/chromatin MMTV-luciferase reporter (5).

No repression of the MMTV promoter by p65 was found in cells that lack BRG1. Transcription was comparable in control and p65-expressing cells both before and following hormone treatment (Fig. 5). Addition of BRG1 increased transcription in control cells, but hormone-dependent transcription was repressed in p65-expressing cells. Coexpression of BRG1 and p65 repressed transcription by 90% compared with BRG1 controls. BRG1 is required for repression by p65, and p65 represses both MMTV basal and hormone-induced transcription consistent with p65 repression in UL3 cells.

Chromatin can exert a globally repressive effect on gene expression by blocking access of transcription factors to the promoter (57). Consistent with this, SW13-MMTV cells that do not express BRG1 are defective in remodeling and clearly repress GR-dependent transcription compared with BRG1-expressing
cells (Fig. 5). However, cells that express both p65 and BRG1 repress transcription more effectively than cells with no BRG1. This suggests that p65 repression is distinct from repression attributable to inactive chromatin remodeling. Indeed, the lack of BRG1 attenuated both activation and repression of the integrated MMTV suggesting the BRG1 function is required for both activation and repression of GR-dependent transcription by promoting the binding of transcription complexes.

The Role of p300/CBP Binding in Complex Formation—We examined the requirement for p300/CBP in repression by p65 and E1A and in the chromatin-dependent E1A attenuation of p65 repression by using p300/CBP-binding mutants of p65 and E1A expressed in UL3 cells. The p65 mutant (S276A) binds less efficiently to p300/CBP because it fails to expose the appropriate binding surface (49). We used two E1A variants. The 12S isoform lacks sequences required to activate transcription but binds p300/CBP and represses transcription of several genes including mouse MMTV (25). The 12S RG2 variant is a point mutant of the 12S-expressing construct that is defective for p300 binding and repression (58).

Disruption of p300 binding attenuates repression by both p65 and E1A (Fig. 6). The p65 S276A mutant represses the transient promoter less efficiently than wild type and is similarly less repressive of the chromatin promoter (Fig. 6A). Thus, p300/CBP interaction is required for p65 repression independent of chromatin structure. Both wild type E1A and the 12S isoform repress the transient MMTV promoter. E1A-dependent repression is attenuated for cells expressing the RG2 variant by demonstrating a requirement for p300 binding in E1A repression of the transient MMTV (Fig. 6B). No E1A variant

FIG. 4. Hormone enhances E1A and p65 binding on the integrated promoter. A, ChIP of p65 binding to the MMTV promoter. UL3 cells were cotransfected with p65 with or without phhCAT and untreated (−) or treated (+) with hormone (dexamethasone, Dex). MMTV sequences immunoprecipitated by p65, GR (positive binding control), or a rabbit IgG (nonspecific (NS)) (negative binding control) were detected using primers specific to the MMTV-CAT (transient MMTV) or the MMTV-luciferase (integrated MMTV) promoter. The hormone-dependent increase in binding of p65 and GR to the integrated/chromatin MMTV was calculated by (Dex+/NS+)/Dex−/NS−) from three or more determinations. Binding to GAPDH or the luciferase gene was used to control for specificity of hormone-dependent binding on the MMTV promoter. B, ChIP of E1A and GR binding to the MMTV promoter in cells transfected with E1A and under conditions used for p65. C, ChIP of p65, E1A, and GR binding to the MMTV promoter in coexpressing cells. Treatment conditions and calculation for the increase in binding follow p65 (A).
represses the integrated MMTV promoter. However, the wild type and 12S isoform attenuate p65-dependent repression but the RG2 variant does not, thus indicating a requirement for p300 interactions in E1A-dependent attenuation of p65 repression (Fig. 6C). Both E1A repression and attenuation require p300/CBP function.

The ability of E1A WT, 12S, and RG2 to bind p65, GR, and p300 was examined by coimmunoprecipitation to determine how these variants might affect assembly of repressor complexes (Fig. 7). Immunoprecipitates of GR, p65, and p300 all show binding to E1A, but the RG2 variant shows reduced binding to these cofactors (Fig. 7A). This suggests that p300/CBP may be critical for the formation of a complex containing all four cofactors, and failure to bind p300/CBP also causes disruption of E1A binding to GR and p65. Because failure to bind p300/CBP disrupts association of the repressor with GR, this would also lead to a loss of GRE targeting for both the transient and stable promoter consistent with the loss of both chromatin-dependent and -independent functions.

The wild type E1A expresses different isoforms, and differences in the immunoprecipitation could be attributed to higher amounts of total isoforms or alternative isoform-specific binding. In reciprocal coimmunoprecipitation assays, the binding of p65 and GR to the 12S isoform was tested by comparing the relative binding of GR and p65 to cells transfected with different amounts (1–4 μg) of the 12S or RG2 (Fig. 7B). Cells transfected with the 12S isoform efficiently bind both the GR and p65. Cells expressing the RG2 variant bind the GR much less efficiently than the 12S variant. The 12S isoform also binds to p65 more efficiently than the RG2 variant, although RG2 retains some p65 binding. However, E1A can bind p65 directly or indirectly via p300/CBP, and direct binding may be unaffected in the RG2 mutant (Fig. 7B).

**DISCUSSION**

We have evaluated the chromatin regulatory mechanisms that participate in p65 and E1A repression, and E1A-dependent attenuation of p65 repression of GR-dependent activation of the MMTV promoter in vivo. We observed that repression occurred without disruption of chromatin regulatory functions required for activation but that the normal chromatin structure of an integrated MMTV promoter permitted repression by...
p65 but not E1A. In contrast, we found that repression by both p65 and E1A occurs readily on a transient MMTV promoter that does not have a normal chromatin structure. This suggests that active chromatin regulation is required for repression as well as for activation on a chromatin promoter.

In order to determine what chromatin regulatory functions are required for repression on chromatin, we measured functions normally required for activation, i.e., chromatin remodeling, transcription complex assembly, and transcription factor binding. We observed that all three of these functions are fully active on the repressed promoter, and disruption of these functions abrogates repression. An additional function required only for repression was implicated in the E1A-dependent attenuation of repression.

Many proteins repress when bound to specific promoters. Promoter binding could regulate the activity of repressor complexes, and chromatin remodeling could be required to enhance binding. In yeast, mutation of Swi2 increased the expression of many genes suggesting repression is regulated by chromatin remodeling (59). Swi/Snf proteins have been shown to be components of repressor complexes and required for repressor function (6, 60, 61). We observed that both E1A and p65 exhibit binding to the promoter in order to repress transcription. Repression by p65 is accompanied by active chromatin remodeling measured by restriction hypersensitivity, and p65 repression is Brg1-dependent. Brg1 serves as a coactivator for hormone-induced transcription specifically requiring the ATPase and chromatin remodeling activity for expression on the chromatin MMTV (5). Brg1 serves as a corepressor with p65 on the integrated MMTV, suggesting chromatin remodeling is both a component of and required for repression on the chromatin promoter.

The p300/CBP protein is a central component of many tran-
scription complexes and has functions required for activation of chromatin templates (62, 63). p300/CBP functions as a coactivator for GR-dependent MMTV activation, and it has also been shown to act as a corepressor with p65 on the MMTV promoter (24). We found that mutants with diminished p300/CBP binding activity have reduced ability to repress the MMTV. The loss of repression is not confined to the chromatin promoter but equally affects the transient promoter. Assembly of the repressor with other transcription factors, notably GR, is abrogated by the loss of p300/CBP binding. Neither p65 nor E1A have direct DNA-binding sites in the MMTV promoter and are bound to the GRE region of the promoter, presumably via their interactions with the GR. Loss of GR binding to the repressor would result in the loss of a mechanism for targeting the repressor to the MMTV consistent with the loss of repression on both transient and chromatin promoters.

E1A is known to interact with p65, IxB, components of the Swi/Snf complex, and various histone acetylases and deacetylases (34, 41, 52). Wild type E1A has an activating 12S and a repressive 12S variant. Chromatin structure potentially could affect which variant is expressed. However we found that attenuation of repression was an activity of the 12S “repressive” isoform and does not result from E1A activation specific to chromatin. E1A can affect the p65-IxB interaction thus enhancing p65 activation (52). However, direct regulation of either p65 or IxB is inconsistent with the activity of E1A on the MMTV promoter. IxB regulation of p65 on MMTV is template-independent, but E1A has different activities depending on chromatin context. Additionally, p65 and E1A repress additively on a transient template, suggesting E1A does not block p65 repression only p65 repression on chromatin. We also found that the E1A RG2 mutant can bind p65 in the absence of p300/CBP binding with no effect on p65 repression.

E1A can bind the Smn12 protein and block the activation of yeast genes that are dependent on Swi/Snf activity (41). E1A also binds to the Swi/Snf-like p400 complex via the interacting TRRAP protein (42). A general inactivation of the human Swi/Snf is not consistent with E1A activity on MMTV because the lack of Brg1 attenuated MMTV activation as well as attenuating p65 repression. MMTV chromatin remodeling is fully active when MMTV is repressed by p65. Also E1A does not block activation of the chromatin promoter, and activation requires remodeling. Repression by p65 and E1A attenuation of p65 repression both clearly require p300/CBP binding and may require a chromatin-specific p300/CBP activity that E1A disrupts. E1A binds the p300 C/H3 domain that overlaps with the p300 histone acetylation domain, and binding can modify the histone acetyltransferase (HAT) function of p300 or p/CAF (64–66). In contrast, p65 binds p300 at the C/H1 domain. If E1A binding modifies the specific histone acetyltransferase activity in a p65-containing complex, then repression that is dependent on acetylation would be blocked in the complex. CBP/p300 acetylation of E1A can affect the binding of the corepressor CtBP and associated HDACs to E1A, and this may also affect repression (67). Glucocorticoid-dependent repression of p65 promoters has been reported to be dependent on both p300 histone acetylation and recruitment of histone deacetylases (29, 33). p300-dependent acetylation of specific transcription factor sites in NF-xB p50 and p65 are also important in regulation (68, 69). The specific acetylation of chromatin or transcription factors in the repression complex may contribute independently or cooperatively to either remodeling or repression.

Although we have identified two important proteins and three functions in this process, other chromatin-modifying and non-chromatin proteins and activities may be involved. In particular, recruitment of specific HDACs, histone, or DNA methyltransferases may be involved. However, the function of these proteins may differ depending on the specific promoter. For example, Sin3 forms a complex with HDACs, NeoR/SMRT, and nuclear hormone receptors to bind and repress specific promoters (70). Sin3, RB (retinoblastoma), and CtBP recruit HDACs, which are proposed to repress by altering chromatin structure such that transcription factors cannot bind (37, 71). However, histone acetylation declines on the MMTV promoter during activation treatment with the HDAC inhibitor trichostatin A is inhibitory rather than stimulatory, suggesting mechanisms distinct from those attributed to HDAC activity in other systems (72–74).

Most interestingly, within chromatin, GR-dependent MMTV promoter activity is elevated in cells expressing E1A compared with the CMV control. E1A appears to preferentially block repression but not activation. If transcriptional activity results from the net effect of activation and repression complexes acting independently on the promoter, then preferential disruption of repression could result in enhanced activation. The E1A attenuation of repression by endogenous repressors could account for the enhanced activation. In this context the E1A block of repression may represent a mechanism for enhancing activation by attenuating the activity of endogenous repressors. A previous study (25) found that E1A activated the transient and integrated MMTV in mouse cells, an activation attributed to the activation function of the 13S isoform. In human UL3 cells, we observed no activation by E1A, suggesting the human cells lack elements critical to activation. However, template-dependent repression by the 12S isoform in mouse is similar to E1A activity in human cells. Thus E1A exhibits two alternative mechanisms to regulate the MMTV, direct activation and attenuation of repression, and both promote activation.

In this investigation we have shown that chromatin-dependent mechanisms affect both the ability of p65 to repress GR-dependent activation of transcription and the subsequent attenuation of p65 repression by E1A. p65 repression takes place in the context of an actively remodeled promoter and is dependent on BRG1 and CBP/p300. E1A uses a CBP/p300-dependent activity on chromatin to disrupt p65 repression, again within the context of an actively remodeled promoter. Neither repression nor chromatin-specific loss of repression is attributable to a repressive chromatin structure that affects transcription factor binding or remodeling. Targeted disruption of p65-dependent repression represents a novel mechanism by which E1A can regulate gene expression. These studies expand our view of the mechanisms by which chromatin structure may act to regulate gene repression from one of a simple barrier to a dynamic process involving active remodeling and modification of chromatin.

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