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Biochemistry

CDP/*cut* is the DNA-binding subunit of histone gene transcription factor HiNF-D: A mechanism for gene regulation at the G_1/S phase cell cycle transition point independent of transcription factor E2F

(proliferation/gene expression/cyclin-dependent kinase/tumor suppressor)

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ABSTRACT **Transcription of the genes for the human histone proteins H4, H3, H2A, H2B, and H1 is activated at the G_1/S phase transition of the cell cycle. We have previously shown that the promoter complex HiNF-D, which interacts with cell cycle control elements in multiple histone genes, contains the key cell cycle factors cyclin A, CDC2, and a retinoblastoma (pRB) protein-related protein. However, an intrinsic DNA-binding subunit for HiNF-D was not identified. Many genes that are up-regulated at the G_1/S phase boundary are controlled by E2F, a transcription factor that associates with cyclin-, cyclin-dependent kinase-, and pRB-related proteins. Using gel-shift immunoassays, DNase I protection, and oligonucleotide competition analyses, we show that the homeodomain protein CDP/*cut*, not E2F, is the DNA-binding subunit of the HiNF-D complex. The HiNF-D (CDP/*cut*) complex with the H4 promoter is immunoreactive with antibodies against CDP/*cut* and pRB but not p107, whereas the CDP/*cut* complex with a nonhistone promoter (gp91-phox) reacts only with CDP and p107 antibodies. Thus, CDP/*cut* complexes at different gene promoters can associate with distinct pRB-related proteins. Transient coexpression assays show that CDP/*cut* modulates H4 promoter activity via the HiNF-D-binding site. Hence, DNA replication-dependent histone H4 genes are regulated by an E2F-independent mechanism involving a complex of CDP/*cut* with cyclin A/CDC2/RB-related proteins.**

Cell proliferation is initiated by a sequential series of growth factor-dependent events that activate cyclin-dependent kinases (CDKs), which mediate the onset of the cell cycle and progression into S phase (1, 2). There are two functional components to the G_1/S phase transition point during the cell cycle. First, initiation of DNA replication necessitates adjustments in the activities of enzymes involved in nucleotide metabolism and DNA synthesis. Second, progression into early S phase requires induction of histone gene expression, because *de novo* synthesis of histone nucleosomal proteins is essential for the ordered packaging of newly replicated DNA into chromatin (3).

Many genes that are functionally linked to cell cycle progression appear to be regulated by the E2F class of transcription factors, including genes encoding enzymes and regulatory factors involved in DNA synthesis (e.g., refs. 4–13). E2F factors are heterodimers composed of different pairs of E2F/DP proteins that are capable of forming higher order complexes with multiple cell cycle regulators including retinoblastoma protein (pRB)-related proteins (pRB/p105, pRB-2/p130, and p107), CDKs, and cyclins A and E (14–17). Variation in the composition of E2F containing multiprotein complexes may be functionally relevant for the timing and extent to which cell cycle controlled genes are activated or repressed.

Expression of the genes for the histone proteins H4, H3, H2A, H2B, and H1 is coordinately controlled at several gene regulatory levels, and transcription is up-regulated during the G_1/S phase transition of the cell cycle (18, 19). The cell cycle control element of the histone H4 gene, Site II, is a multipartite protein/DNA interaction site that binds IRF2/HiNF-M, HiNF-D, and H4TF2/HiNF-P (20–24). IRF2/HiNF-M represents a key activator of H4 gene transcription (24). Based on identification by gel-shift immunoassays, HiNF-D complexes with histone H4, H3, and H1 genes contain critical cell cycle mediators including cyclin A, CDC2, and RB-related proteins (25). Several other transcription factors, including OCT1/OTF1, (26, 27), H1TF2/HiNF-B CCAAT box-binding protein (28, 29), and SP1/HiNF-C (30), have been shown to activate distinct vertebrate histone gene classes. Although two histone H2A genes recently have been shown to be regulated by E2F (31, 32), the majority of histone promoters lack typical E2F consensus elements,

Identification of the DNA-binding subunit of the HiNF-D complex is essential for understanding the postulated role of this factor in cell cycle regulation of histone gene transcription. HiNF-D interacts with human histone H4, H3, and H1 gene promoters and recognizes complicated arrangements of consensus motifs (33, 34). These motifs are similar to sequences recognized by the cut-homology repeats of the homeodomain protein CDP/*cut* (35–37). CDP/*cut* was initially identified as a putative "CCAAT displacement protein" binding to CCAAT motifs in the sea urchin histone H2B gene (38). Human CDP/*cut* was purified and cloned (39), and shown to be homologous to the *Drosophila cut*/homeodomain protein, which is involved in cell fate determination (40). CDP/*cut* can function as a repressor for a broad spectrum of genes, including the sea urchin sperm histone H2B gene in nontesticular tissues and the human myeloid cytochrome heavy chain gene *gp91-phox* in cells where the gene is not expressed (38, 41, 42). There are similarities in the chromatographic properties, electrophoretic mobility, and biological regulation of HiNF-D and CDP/*cut* (33, 39). Furthermore, using several *Xenopus* histone gene promoters, El-Hodiri and Perry (43) have characterized gel-shift complexes that appear to be related to the *Xenopus* homolog of CDP/*cut*, and we noted that these complexes resemble HiNF-D. In this study, we show that CDP/*cut* is the DNA-binding subunit of the HiNF-D complex, and that CDP/

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Abbreviations: CDK, cyclin-dependent kinase; RB, retinoblastoma, pRB, protein RB; GST, glutathione *S*-transferase; CAT, chloramphenicol acetyltransferase.

cut is capable of modulating histone H4 gene transcription. Our data show that CDP forms complexes with different RB proteins. Thus, the targeting of pRB and other key cell cycle mediators, including cyclins and CDKs to cell cycle controlled histone genes occurs by an E2F-independent mechanism.

MATERIALS AND METHODS

Gel-Shift Assays. Gel-shift assays for detection of HiNF-D with nuclear extracts from HeLa S3 cells were performed using the same conditions as described previously (22). HiNF-D binding was monitored with DNA probes spanning the Site II element of the H4 gene (nt −97/−38), or the analogous regions of the H3 (nt −200/−20) and H1 (nt −213/−78) genes (34). Standard binding reactions (20 μl vol) for HiNF-D were incubated at room temperature and contained 10 fmol (0.4 ng) DNA probe, a mixture of nonspecific competitor DNAs [2 μg poly(dG·dC) DNA and 0.2 μg poly(dI·dC) DNA], and 3 μg HeLa nuclear protein (22). Binding of CDP/*cut* to the *gp91-phox* gene was analyzed under the same conditions using the cloned oligonucleotide FP (spanning nt −136 to −76 of the gp91-phox promoter) (39, 41). Binding reactions for E2F were carried out with an E2F consensus oligonucleotide containing the wild-type (5′-TTTCGCGCCCT) or mutated (5′-TTTCGatcCCT) E2F core motif (Santa Cruz Biotechnology) and salmon sperm double-stranded DNA (2 μg) as nonspecific competitor. E2F-1 and DP-1 proteins were synthesized by coupled *in vitro* transcription/translation using reticulocyte lysates programmed with pGEX vectors encoding E2F-1 and DP-1 (44). Competition assays were performed with oligonucleotides spanning the wild-type (TM-3, nt −93/−53) or mutant (NH-6) binding site of HiNF-D in the H4 gene (NH-6 contains several point-mutations that specifically abolish HiNF-D binding) (22), as well as with oligonucleotide FP spanning the CDP/*cut* binding site in the gp91-phox gene (39, 41). Competition assays were performed by mixing unlabeled oligonucleotides (1 pmol; i.e., 100-fold molar excess) with probe before addition of protein. Gel-shift immunoassays were performed by preincubating antibodies with nuclear protein on ice for 15 min before the addition of probe DNA. In some assays, antibodies were added for 15 min after formation of protein/DNA complexes to promote formation of "super-shift" complexes. All binding reactions were subject to electrophoresis in 4% (80:1) polyacrylamide gels using 0.5× TBE buffer (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) as described previously (22, 45).

DNase I Footprinting Analysis. Purification of glutathione *S*-transferase (GST) protein fused to the C-terminal 100-kDa portion of human CDP/*cut*, GST/CDP(CR2-Cterm) (42), was achieved by isopropyl β-D-thiogalactoside-induced expression in bacteria and isolation of the fusion protein via glutathione beads. DNase I protection assays were carried out with the GST/CDP(CR2-Cterm) fusion protein using standard procedures as described previously (33, 45). Binding reactions (50 μl) contained ≈1 pmol of protein (100 to 200 ng), 10 fmol of H4 probe (labeled on either the sense or anti-sense strand), 500 ng poly(dG·dC) DNA, and 5 μg bovine serum albumin.

Immunological Reagents. Guinea pig antiserum against CDP/*cut* was directed against the purified full-length protein isolated from human HeLa cells (39). Several mouse monoclonal antibodies directed against pRB (hybridoma supernatants XZ55, XZ133, XZ104, and C36) and cyclin A (hybridoma supernatant C160) were used in our assays (46, 47). Preimmune and nonimmune antisera, as well as negative hybridoma supernatants, were used as controls. Additional antibodies directed against E2F-1, pRB/p105, p107, and pRBR2/p130 were obtained commercially (Santa Cruz Biotechnology).

Transient Coexpression Assays. Transfection assays were carried out as described (45) in COS cells using histone H4 promoter/chloramphenicol acetyltransferase (CAT) fusion constructs that contain either a wild-type or mutant HiNF-D binding site (23, 24). Cells were cotransfected with pMT2-ATG (42), which expresses full-length CDP/*cut* under control of the cytomegalovirus promoter. Luciferase activity expressed from a cotransfected simian virus 40/luciferase reporter gene construct was used to normalize for transfection efficiency.

RESULTS

The H4-Site II Cell Cycle Regulatory Sequences of the Histone H4 Gene Do Not Interact with E2F. We investigated whether HiNF-D contains an E2F-related binding activity. E2F is a critical DNA-binding activity for many G_1/S phase related genes, and can bind its cognate sites in higher order complexes containing cyclins, CDKs, and RB-related proteins. Similarly, the HiNF-D complex contains cyclin A, CDC2, and a pRB-related protein. Although most histone genes do not contain typical E2F consensus sites, H4-Site II contains several motifs with very weak similarity to E2F (data not shown). Direct examination of protein/DNA interactions reveals that E2F/DP heterodimers do not bind to H4-Site II (Fig. 1*A*). Furthermore, while E2F does not bind H4-Site II, the E2F element does not compete for the HiNF-D/H4-Site II interaction, and E2F or DP antibodies are not immunoreactive with HiNF-D (see Fig. 3*A* and data not shown). As expected, E2F/DP heterodimers bind very strongly to a probe spanning a typical E2F consensus sequence, which provides a positive control for the functional integrity of E2F/DP binding activity in our preparations (Fig. 1*A*). Thus, these data suggest that HiNF-D binding activity is not related to E2F.

Sequence-Specific Interactions of CDP/*cut* with the HiNF-D Domain of the H4-Site II Cell Cycle Element. Similarities between HiNF-D and CDP/*cut* prompted us to determine experimentally whether these factors are related. To establish first that CDP/*cut* recognizes H4-Site II sequences, as well as analogous elements in the H3 and H1 genes, we performed gel-shift competition analyses (Fig. 1*B*) with bacterially expressed and purified GST/CDP(CR2-Cterm) fusion protein (42), which encompasses two cut-homology repeats and the homeodomain of CDP/*cut*. The results (Fig. 1*B*) demonstrate that the GST/CDP(CR2-Cterm) fusion protein is capable of binding to the H4, H3, and H1 promoters, which have previously been shown to bind HiNF-D (33, 34). Sequence specificity of these CDP/*cut* interactions with all three promoters is evidenced by competition with the unlabeled wild-type H4-Site II oligonucleotide. Furthermore, an oligonucleotide containing mutations that abolish HiNF-D binding (22) does not compete for GST/CDP(CR2-Cterm) binding to the H4, H3, and H1 genes (Fig. 1*B*), indicating strong similarities in the DNA binding specificities of CDP/*cut* and HiNF-D.

To delineate the CDP/*cut* binding site in H4-Site II relative to the HiNF-D binding domain, we performed DNase I footprinting analysis (Fig. 2). We observe that binding of GST/CDP(CR2-Cterm) results in strong DNase I protection of nt −85 to −60 and weaker protection of nt −59 to −49 on the sense strand. Similarly, strong DNase I protection of nt −91 to −60 is observed on the anti-sense strand with additional weaker protection of nt −59 to −53. The CDP/*cut* footprint spans nucleotides critical for HiNF-D binding (22, 33), and the boundaries of the footprints are comparable to the HiNF-D footprint established previously on the anti-sense strand (47). These data further support the concept that CDP and HiNF-D are related DNA binding proteins.

CDP/*cut* Is the DNA-Binding Subunit of the HiNF-D/H4-Site II Complex and Related Complexes in the Histone H3 and H1 Genes. To assess directly whether CDP/*cut* is a component of the HiNF-D complex, we performed gel-shift immunoassays using a polyclonal antibody directed against the full-length CDP/*cut* protein from HeLa cells (39). The HiNF-D/Site II

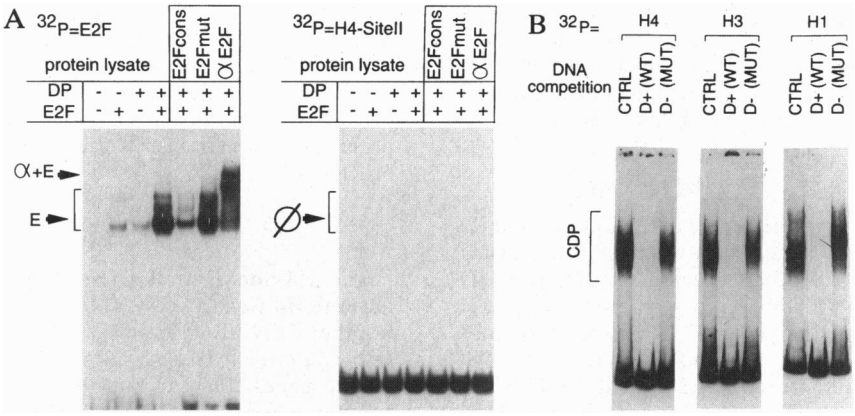

FIG. 1. CDP/*cut*, but not E2F, interacts with H4-Site II. (*A*) Gel-shift immunoassays were performed with reticulocyte lysates that were programmed with vectors encoding E2F-1 or DP-1 (+), or with vector alone (−). Lysates (2.5 μl per protein) were added to the binding reactions containing the E2F (*Left*) or H4-Site II (*Right*) probe. Competition analysis of the E2F/DP complex (*Left*, bracketed area labeled with E) was carried out with wild-type and mutant E2F binding sites (indicated above the lanes), and the E2F complex is immunoreactive with an E2F antibody (α-E2F) that mediates a supershift (*Left*, bracketed area labeled with α + E). The H4 probe does not mediate E2F/DP complexes (*Right*, ∅). (*B*) Gel-shift assays with purified GST/CDP(CR2-Cterm) fusion protein show a CDP-specific complex (indicated with bracket) with probes spanning the H4, H3, or H1 promoters (as indicated above each panel). Competition analysis reveals that these CDP/histone gene complexes compete with oligonucleotides spanning the wild-type [D + (WT); TM-3], but not mutant [D − (MUT); NH-6] HiNF-D binding sites in the H4 promoter.

complex is immunoreactive with the CDP/*cut* antibody, but not with preimmune or nonimmune serum (Fig. 3 *A* and *B*). For comparison, we used a nonhistone probe spanning a previously established high-affinity CDP/*cut* binding site that spans the duplicated CCAAT box region of the *gp91-phox* gene (39, 41). The gp91-phox oligonucleotide competes very effectively for HiNF-D binding to the H4-Site II probe (Fig. 3*C*). In the reciprocal competition experiment, H4-Site II does not compete effectively for the CDP/gp91-phox complex at 100-fold molar excess; this difference in competition efficiency suggests that CDP binds much more strongly to the *gp91-phox* gene than the H4 gene. Similar to the results obtained for the H4-Site II interaction, the HiNF-D-related protein/DNA complexes with the histone H3 and H1 promoters are also immunoreactive with the CDP antibody (Fig. 3*D*). Taken together, these results establish that CDP is the intrinsic DNA-binding subunit of the HiNF-D complexes interacting with the H4, H3, and H1 histone genes.

CDP/*cut* Represses Histone H4 Promoter Activity when Overexpressed. To address functional involvement of CDP/*cut* in the regulation of histone gene transcription, we performed transient coexpression experiments with a construct encoding full-length CDP/*cut* protein and histone H4 promoter/CAT reporter gene in COS cells (Fig. 4). The results show that expression of CDP/*cut* driven by the strong cytomegalovirus promoter reduces wild-type H4 promoter activity by approximately 3-fold. However, when the HiNF-D (CDP/*cut*) binding site is mutated, CDP/*cut* overexpression does not result in reduction of H4 promoter activity. Similar results were obtained in cotransfection experiments using ROS 17/2.8 osteosarcoma cells (data not shown). Thus, CDP/*cut* is capable of repressing H4 promoter activity through the HiNF-D (CDP/*cut*) binding site in H4-Site II.

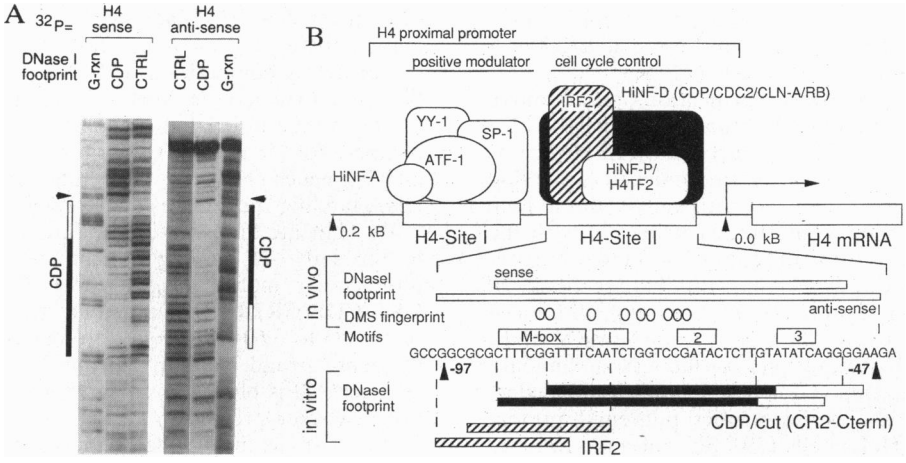

FIG. 2. DNase I footprinting analysis CDP/*cut* binding to H4-Site II. (*A*) Binding reactions were set up with the GST/CDP(CR2-Cterm) fusion protein using probes spanning the sense and anti-sense sequences of H4-Site II (as indicated above the lanes). Each set of lanes shows the location of G residues as determined by chemical sequencing (G-rxn), and DNase I footprinting reactions in the presence (CDP) or absence (CTRL) of protein. For each strand, the CDP/H4-Site II interaction displays a pattern of strong (solid thin box) and weak (open thin box) DNase I protection, as well as two sites of increased DNase I reactivity (arrowheads). (*B*) Summary of DNase I footprinting analysis. The footprints of GST/CDP(CR2-Cterm) on both strands are indicated below the sequence using the same symbols as in *A*. The footprint of IRF2/HiNF-M was previously established (33) (hatched bar below the sequence). The genomic DNase I footprint (open bars) and DMS fingerprint of H4-Site II (open ovals) determined *in vivo* (20) are indicated above the sequence. The M-box motif mediating cell cycle control (23) (open box), as well as several motifs similar to recognition elements for CDP/*cut* (35–37) (open boxes with numbers) are also indicated above the sequence. The top portion of the diagram depicts the regulatory organization of the histone H4 gene promoter (19).

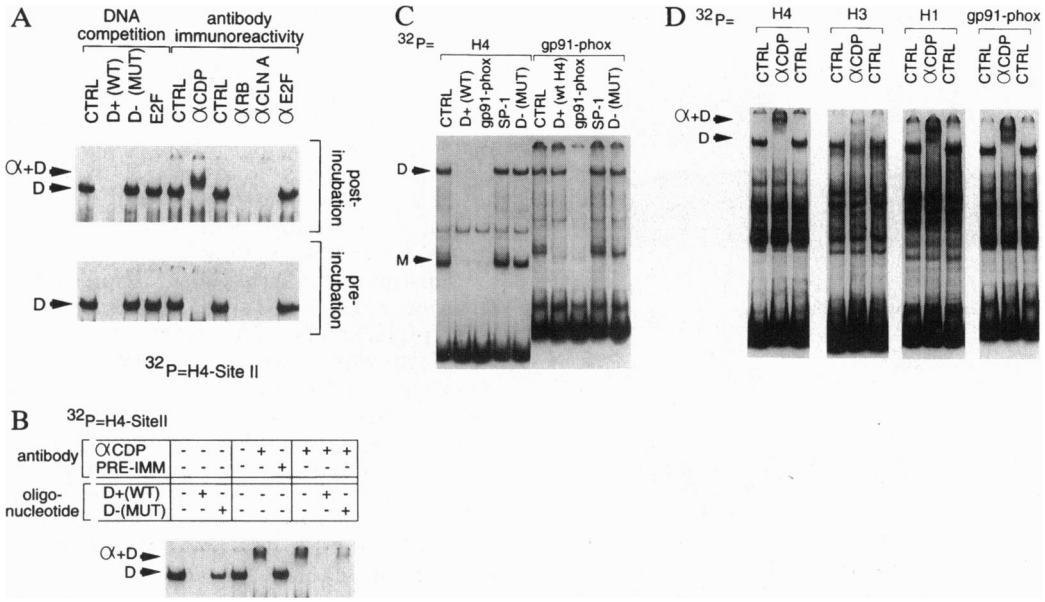

FIG. 3. CDP/*cut* is the DNA-binding subunit of the HiNF-D complex. (*A*) Gel-shift assays with the H4-Site II probe and HeLa nuclear extract (3 μg). Competition analyses (left four lanes) were performed with 100-fold molar excess of the oligonucleotides indicated described in Fig. 1 {i.e., no specific competitor (CTRL), the wild-type [D + (WT)], or mutant [D − (MUT)] HiNF-D binding sites, or the E2F binding site (E2F)}. Immunoreactivity of HiNF-D (arrowhead and D) with the CDP antiserum (α-CDP), but not with a nonimmune antiserum (1 μl each), is shown in lanes 5 and 6. Incubation of CDP antibody after formation of HiNF-D/H4-Site II complexes (postincubation) results in a supershift (α + D) (*Upper*), and preincubation of proteins with CDP antibody results in inhibition of the complex (*Lower*). For comparison and consistent with our previous report (25), immunoreactivity of HiNF-D with pRB (αRB, XZ104) and cyclin A (αCLN A, C160) antibodies is reflected by inhibition of HiNF-D binding to H4-Site II, irrespective of the order of antibody-addition. No immunoreactivity is observed with E2F-1 antibody (αE2F). (*B*) Binding reactions were complemented with CDP antiserum or preimmune serum (+). Identity of the HiNF-D supershift complex (left three lanes) was confirmed by competition analysis with oligonucleotides (described in Fig. 1) as indicated above the lanes. (*C*) Cross-competition analysis of the HiNF-D/H4 and CDP/gp91-phox complexes was carried out using wild-type and mutant HiNF-D oligonucleotides (see Fig. 1), the duplicated CCAAT box region of the gp91-phox gene ("FP" oligonucleotide) (39, 41), and the SP-1 consensus site (negative control). Two sets of binding reactions were performed with either the H4-Site II (*Left*, lanes 1–5) or the gp91-phox probe (*Right*, lanes 6–10). The H4-Site II probe detects both HiNF-D and IRF2/HiNF-M (indicated by arrowheads). The HiNF-D/H4-Site II complex comigrates with the CDP/gp91-phox complex. (*D*) Gel-shift immunoassays of HiNF-D complexes with the histone H4, H3, and H1 genes and the CDP complex with the *gp91-phox* gene (probes indicated above the lanes). The left and right control lane in each set case represent, respectively, binding reactions without CDP antiserum or preimmune serum. The HiNF-D related complexes (D) and corresponding supershift complexes (α + D) for each probe are indicated by arrowheads.

CDP/*cut* Forms Promoter-Selective Higher Order Complexes with Distinct RB-Related Proteins. To address whether CDP complexes at different promoters interact with different RB-related proteins, we used a panel of monoclonal antibodies against pRB/p105 that recognize specific epitopes dispersed throughout the pRB protein to monitor immunoreactivity of the HiNF-D/H4-Site II and CDP/gp91-phox complexes. Our data show that strong inhibition of binding ("block-shifts") is observed for the HiNF-D/H4-Site II complex with four different pRB antibodies (i.e., hybridoma supernatants XZ55, XZ104, XZ133, and C36) (Fig. 5 *A–C*). The recognition of the HiNF-D/H4-Site II complex by four different pRB specific antibodies clearly suggests that pRB or that a highly related pRB variant is a component of HiNF-D. However, the same antibody preparations have no effect on the CDP/gp91-phox complex. Therefore, we also examined the immunoreactivity of the HiNF-D (CDP/*cut*) complexes with antibodies to other members of the pRB family. We directly compared the HiNF-D/H4-Site II complex with the CDP/gp91-phox complex by using polyclonal antibodies against the C-terminal peptides of p107 and pRB-2/p130. In contrast to the HiNF-D complex, the gp91-phox gene complex is supershifted by p107, but not by pRB or pRB-2/p130 antibodies. We conclude that the endogenous CDP/*cut* complexes in HeLa nuclear extracts that interact with the H4 and gp91-phox gene promoters contain two different RB-related proteins (pRB/p105 and p107, respectively).

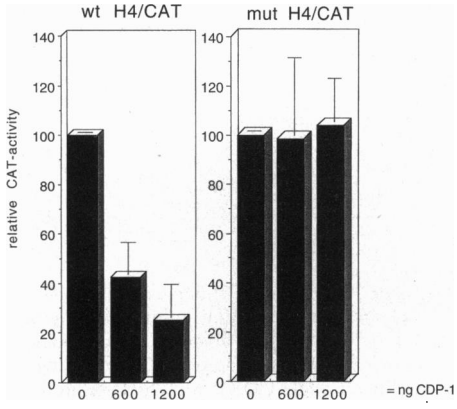

FIG. 4. Transient coexpression analysis in COS cells was performed with a wild-type human H4 promoter/CAT reporter gene construct (wtH4/CAT) or a construct with a mutation in the HiNF-D binding site (mutH4/CAT) that were each cotransfected with different amounts of CDP expression plasmid (horizontal axis; indicated in ng per 8×10^4 cells). CAT activity for each construct was normalized relative to luciferase activity (vertical axis). Statistical calculations using ANOVA indicate that the repression of H4 promoter activity is significant ($P < 0.01$).

DISCUSSION

Histone H4 Gene Regulation Occurs via an E2F Independent Mechanism. E2F is an important transcription factor for many G_1/S phase-related genes. Recently, E2F has been

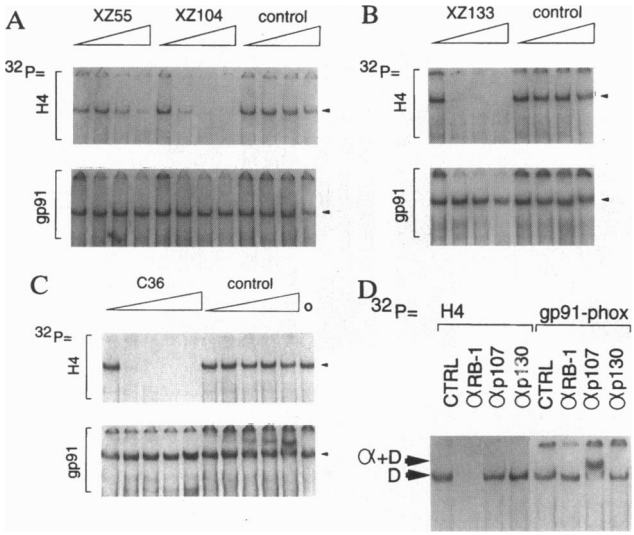

FIG. 5. CDP complexes with the histone H4 and gp91-phox gene promoters represent distinct pRB- and p107-related complexes. (*A–D*) Gel-shift immunoassays were performed by preincubating HeLa nuclear protein before addition of either the H4-Site II probe (*Upper*) or the gp91-phox probe (*Lower*) (the arrowheads point at the CDP related complexes for each probe). (*A*) Titration-curves with three different antibodies [0, 0.5, 1, and 2 μl, respectively, of anti-pRB hybridoma supernatants XZ55 and XZ104 (46), indicated by the sloped triangle]. The control curve was performed with a negative hybridoma supernatant. (*B*) Same as in *A* using hybridoma supernatant XZ133 (46). (*C*) Same as in *A*, using 0, 0.5, 1, 2, and 4 μl, respectively, of the C36 antibody and a p107-specific antibody (serving as a negative control for immunoreactivity of the HiNF-D/H4-Site II complex). The faint band observed above the CDP/gp91-phox complex (not indicated) represents a weak p107 supershift (see *D*). Lane 0 represents a binding reaction in the absence of antibody. (*D*) Gel-shift immunoassay using the H4-Site II (*Left*, lanes 1–4) and gp91-phox (*Right*, lanes 5–8) probes with a panel of antibodies directed against pRB (C36), p107, or p130 (as indicated above the lanes, 4 μl in each case). Immunoreactions were allowed to proceed for 4 hr on ice.

shown to regulate the H2A.X gene, which encodes a partially cell cycle-dependent minor variant H2A protein (31). The regulatory mechanisms controlling this gene differ from those regulating the major classes of DNA replication-dependent histone genes (3, 18, 19). E2F has also been implicated in regulation of the DNA replication-dependent H2A.1 histone gene, which also contains consensus binding sites for other transcription factors involved in cell cycle control, including OCT1/OTF-1 (26, 27) and H1TF2/HiNF-B CCAAT box-binding proteins (28, 29). Hence, the H2A.1 gene may be regulated both by E2F and by non-E2F mechanisms. Our results clearly show that E2F does not bind to the H4-Site II cell cycle sequences. Also, the promoters of most DNA replication-dependent human H3, H2B, H2A, and H1 histone genes do not contain typical E2F motifs. Therefore, we conclude that cell cycle control of most DNA replication-dependent histone genes occurs at least in part via an E2F-independent mechanism.

Control of Histone H4 Gene Transcription by IRF2 and HiNF-D (CDP/*cut*). Cell cycle control of histone H4 genes is mediated by a composite regulatory element (M-box) in the distal part of H4-Site II (23), which is required for binding of IRF2/HiNF-M, H4TF2/HiNF-P, and HiNF-D (CDP/*cut*) (22). Mutation of the IRF2 binding site in the H4 gene reduces transcription in HeLa cells, but not in nullizygous IRF2$^{-/-}$ cells, which lack IRF2 (24). In contrast, mutation of the HiNF-D-binding site reduces H4 promoter activity in nullizygous IRF2$^{-/-}$ cells, but not in HeLa cells (ref. 24; unpublished data). Thus, IRF2 and HiNF-D (CDP/*cut*) have properties of trans-activators, and the functions of these factors may be in part redundant. Furthermore, whereas overexpression of IRF2 in COS cells enhances H4 gene transcription (24), we show in this study that overexpression of CDP/*cut* represses H4 promoter activity via the HiNF-D (CDP/*cut*) site. We propose that HiNF-D (CDP/*cut*) may be bifunctional, and that HiNF-D may repress or activate H4 transcription depending on the availability of associated proteins. Taken together, our findings indicate that both HiNF-D (CDP/*cut*) and IRF2 control H4 gene transcription during the cell cycle.

Coordinate Control of Multiple DNA Replication-Dependent Histone Genes. The promoters of vertebrate histone H4, H3, H2B, H2A, and H1 genes display considerable complexity with respect to the distribution and organization of cis-acting elements recognized by a multiplicity of factors (18–34). Which factor, if any, is capable of transducing a coordinating signal to synchronize histone gene transcription rates as cells progress toward S phase in preparation for replication of chromatin? As discussed in detail (22, 34), HiNF-D (CDP/*cut*) recognizes distinct motifs in promoter domains of the H4, H3, and H1 promoters that have been shown to mediate cell cycle control. Furthermore, HiNF-D/histone gene interactions appear to be proliferation-specific and regulated with respect to S phase (e.g., refs. 48–51). Gel-shift immunoanalyses suggest that HiNF-D (CDP/*cut*) forms a complex with key cell cycle mediators, including cyclin A, CDC2, and pRB (25). Therefore, as proposed previously (22, 34), HiNF-D may be involved in coordinate control of histone genes during the cell cycle. In support of this concept and our earlier studies on HiNF-D (CDP/*cut*) binding to multiple histone gene classes (22, 34), El-Hodiri and Perry (43) have recently presented data suggesting that CDP/*cut* interacts with the five histone gene subclasses in *Xenopus*.

CDP/*cut* Interacts with Distinct RB-Related Proteins. CDP/*cut* is a ubiquitous factor that can repress the cell cycle-dependent human H4 gene and a broad spectrum of differentiation-specific genes, including the phagocyte-specific human *gp91-phox* gene (42). Similar to E2F (44), we postulate that activator or repressor activity of CDP/*cut* may depend on associated subunits, and perhaps the promoter organization of the cognate genes. During cell growth stimulation of normal diploid FDC-P1 hematopoietic progenitor cells, the HiNF-D (CDP/*cut*)/H4-Site II complex is up-regulated in conjunction with CDC2, CDK2, cyclin A, pRB, and p107 protein levels (ref.

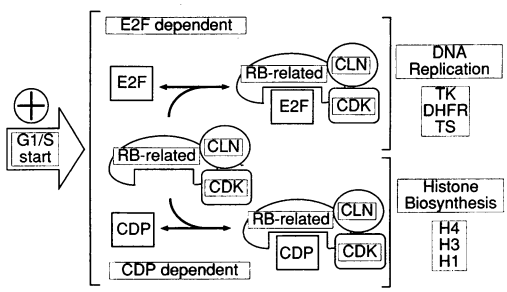

FIG. 6. Postulated E2F- and CDP-dependent mechanisms for pRB function at the G_1/S phase transition point following growth stimulation (indicated by large open arrows). The model depicts two different regulatory routes (indicated by black arrows) by which a multitude of cyclins (CLN), CDKs, and pRB-related higher order complexes may be recruited to the promoters of cell cycle-controlled genes. This recruitment of pRB-related cell cycle mediators by E2F or CDP at defined stages of the cell cycle may activate or repress gene transcription. There are several gene-encoding proteins involved in DNA synthesis or nucleotide metabolism, including thymidine kinase (TK), dihydrofolate reductase (DHFR), thymidylate synthase (TS), ribonucleotide reductase, DNA polymerase α, cdc2, cyclin A, proliferating cell nuclear antigen, c-myc, and B-myb. These genes operate at least in part via an E2F-dependent mechanism, whereas the majority of histone genes may be controlled by a CDP-dependent mechanism.

51; unpublished data). This up-regulation occurs during activation of DNA synthesis and histone gene expression, and parallels the increase in cyclin/CDK/RB-related multiprotein E2F complexes (51). We find that CDP/*cut* forms distinct complexes with different promoters; at the histone H4 promoter, CDP/*cut* associates with pRB, whereas at the gp91-phox promoter CDP/*cut* binds together with p107. The possibility arises that a multitude of variant CDP complexes may provide an E2F-independent mechanism (Fig. 6) for the cell cycle regulatory functions of RB proteins (17). This use of E2F and non-E2F mechanisms by genes subject to cell cycle control at the G_1/S phase boundary is consistent with differences, and functional redundancy, in the temporal regulation of genes encoding proteins required for DNA synthesis and the genes for histone nucleosomal proteins.

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