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CDP and AP-2 Mediated Repression Mechanism of the Replication-Dependent Hamster Histone H3.2 Promoter

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Abstract The replication-dependent hamster histone H3.2 promoter contains two tandem CCAAT repeats located upstream of the TATA element. It has been shown that the NF-Y/CBF complex binds to a single CCAAT motif with high affinity, whereas the CCAAT displacement protein (CDP) binds to at least two CCAAT motifs in close proximity. Here, we report that the two CCAAT motifs within the H3.2 promoter confer transcriptional repression of the promoter during the cell cycle. While we cannot detect direct association of CDP with Rb in vitro, we discover that CDP can bind AP-2, a ubiquitous factor that interacts with Rb. The interaction domains between CDP and AP-2 are mapped to the highly conserved cut repeats of CDP as well as the basic and dimerization region of AP-2. Further, in transfection assays, CDP and AP-2 act synergistically to suppress the H3.2 promoter. Together, these data support a repression mechanism mediated by CDP and AP-2 that regulates H3.2 gene expression during the mammalian cell cycle. J. Cell. Biochem. 84: 699–707, 2002.

Key words: AP-2; CCAAT displacement protein; hamster histone H3.2 promoter; gene regulation

The transcription of the replication-dependent hamster histone H3.2 promoter is activated during the G1/S transition. In contrast to many replication-dependent promoters, the H3.2 promoter does not contain E2F binding sites implicated in cell cycle control [Slansky and Farnham, 1996]. Instead, a distal *cis*-regulatory domain referred to as the H3core is required for its S-phase induction of transcription [Artishevsky et al., 1987]. The H3core domain has a bipartite structure that includes both the AP-1-like and AP-2 binding sites [Naeve et al., 1992; Wu and Lee, 1998]. AP-2, a retinoic acid-inducible and developmentally regulated transcription factor, negatively regulates transcription of the H3.2 promoter through the H3core and interacts with the retinoblastoma (Rb) tumor suppressor protein [Williams et al., 1988; Mitchell et al., 1991; Buettner et al., 1993; Schorle et al., 1996; Zhang et al., 1996; Wu and Lee, 1998, 2001]. Interestingly, the proximal region of the H3.2 promoter displays features of a TATA box and two tandem CCAAT repeats similar to elements in the human thymidine kinase (TK) promoter [Arcot et al., 1989; Kim and Lee, 1991; Arcot and Deininger, 1992]. However, the significance of the tandem CCAAT motifs and their cognate binding factors in the control of the H3.2 promoter transcription during the cell cycle has not been defined.

The promoters of many of the cell growth regulated genes have multiple CCAAT motifs. Among the various CCAAT-binding factors, NF-Y/CBF binds with high affinity to the CCAAT and related sequences. It has been documented that the mammalian CCAAT displacement protein (CDP) recognizes a duplicate CCAAT-like box [Neufeld et al., 1992; Barberis et al., 1995; Nepveu, 2001]. The mammalian CDP is a counterpart of *Drosophila* cut protein which is involved in cell fate determination [Neufeld et al., 1992; Nepveu, 2001]. The full-length CDP is a 180 kDa nuclear protein consisting of six evolutionarily conserved domains. It is characterized by a region predicted to form a coiled-coil structure toward the N-terminus, three related regions called cut repeats, a distinctive

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homeodomain, and a recently identified alanine-rich region functioning as a repression domain in the C-terminus of the CDP protein. A hallmark feature of the CDP/cut proteins is three conserved cut repeats (CR1, 2, and 3) composed of about 70 amino acids each. Each cut repeat exhibits DNA-binding properties independently of the homeodomain with broad and overlapping specificities [Andres et al., 1994; Aufiero et al., 1994; Harada et al., 1995]. Protein kinases can efficiently phosphorylate cut repeats, causing reduction of DNA binding and interfering with its transcriptional repression function. [Coqueret et al., 1996, 1998]

CDP has been implicated in the regulation of differentiation, cell growth, and development [Nepveu, 2001]. One mechanism is through its repression of transcription of target genes such as the human myelomonocyte-specific cytochrome heavy chain gene *gp91-phox* [Skalnik et al., 1991], human c-Myc [Dufort and Nepveu, 1994], and vertebrate histone genes [van Wijnen et al., 1994, 1996; el-Hodiri and Perry, 1995; Aziz et al., 1998; Last et al., 1998, 1999; van Gurp et al., 1999]. The human histone H1 and H3 promoters contain two consensus CCAAT boxes, whereas the H4 promoter contains one consensus CCAAT box and two other sites that vary from the consensus by a single base mismatch. It has been shown that HiNF-D is a CDP-containing complex, which recognizes distinct motifs in human H1, H3, and H4 promoters [van Wijnen et al., 1994, 1996]. The HiNF-D (CDP) binding activity appears to be associated with cyclin A, cdc2, and Rb [van Wijnen et al., 1994; Aziz et al., 1998; van Gurp et al., 1999]. Further, the human CDP represses H4 promoter activity via the HiNF-D-binding site in transient transfection assays [van Wijnen et al., 1996]. CDP also binds the two inverted CCAAT motifs in the human TK promoter and suppresses its transcription [Kim et al., 1997]. Here we report that CDP is a repressor of the H3.2 promoter acting through the two CCAAT motifs. CDP can directly associate with AP-2 and functionally synergizes with it in repression of the H3.2 promoter activity.

MATERIALS AND METHODS

Construction of Plasmids

The construction of the expression plasmids of the wild-type (WT) H3.2 promoter (spanning from −254 to −6) linked to a chloramphenicol acetyl transferase (CAT) or luciferase (Luc) reporter gene (pH3[−254]CAT or pH3[−254]Luc) has been described previously [Naeve et al., 1992; Wu and Lee, 2001]. The 5′ mutagenic oligonucleotide primer, 5′-ctagctcgAGCAGAACCTT*ttatta*TCAGCCAGCCGC-3′, was used to generate the mutated H3.2 promoter in the AP-1-like site within the H3core region (AP-1^m) by polymerase chain reaction (PCR) amplification. The lower case denotes polylinker sequences and the lower case in italics denotes mutated bases. The pH3[−254-$AP1^m$]CAT and pH3[−254$AP1^m$]Luc were constructed by subcloning the mutated promoter sequence into a pBLCAT3 vector (gift of Dr. D. Ann, University of Southern California, Los Angeles, CA) or into a pGL-basic Luc vector (Promega, Madison, WI), respectively. The pH3[−254-$2C^m$]CAT and pH3[−254-$AP1^m$/$2C^m$]Luc were generated by site-directed mutagenesis using the QuickChange kit (Stratagene, La Jolla, CA) and the mutagenic oligonucleotide primer 5′-CGGGTCCAAG*agtga*GGGCGAGGCGGGATGGGTGACGC CCGGG*agtga*GACCGGACAG-3′. The orientation and sequence of the WT and mutated H3.2 promoter constructs were confirmed by DNA sequencing.

Cell Culture and Transfection Assays

The K12 Chinese hamster fibroblasts were grown in DMEM with 10% bovine calf serum (Gemini, Calabasas, CA) and 1% antibiotics (including 50 μg/ml penicillin and streptomycin, 100 μg/ml neomycin). Conditions for calcium phosphate transfection of DNA into K12 cells were previously described [Resendez et al., 1985]. K12 cell synchronization, selection of stable transfectants, and CAT or Luc activity assays have been described previously [Wu and Lee, 1998, 2001]. The expression vector pCMV/mAP-2β was a gift of Dr. R. Buettner (University of Regensburg, Germany). The pCMV/CDP expression plasmid was a gift of Dr. A. Nepveu (McGill University, Quebec, Canada). The reporter gene activity was normalized to β-galactosidase activity. Each transfection experiment was repeated independently two to three times.

Glutathione *S*-Transferase (GST) Pull-Down Assays

The GST-fusion proteins used were as follows: GST, GST-AP-2A (gift of Dr. P. Kannan, University of Texas at Houston, Houston, TX);

GST-AP-2β(DBD) containing only the C-terminal DNA binding domain (DBD) of the murine AP-2β (gift of Dr. R. Buettner, University of Regensberg, Regensberg, Germany); GST-TBP and GST-Rb(379-928) (gifts of Dr. D. Dean, Washington University, St. Louis, MO); GST-Rb-A/B(379-792) and GST-Rb-C(768-928) (gifts of Dr. J.Y. Wang, University of California, San Diego, CA); GST-Rb-B/C(646-928) (gift of Dr. Y.K. Fung, University of Southern California, Los Angeles, CA); GST-c-Myc(259-439) (gift of Dr. K. Calame, Columbia University, New York, NY); GST-H-Ras (gift of Dr. D. Broek, University of Southern California, Los Angeles, CA); and GST-CR1 and GST-CR3HD (gifts of Dr. A. Nepveu, McGill University, Quebec, Canada). GST or GST-fusion proteins were induced in *Escherichia coli* with IPTG and purified by affinity chromatography. Protein yields were quantitated by Coomassie blue staining with BSA as control after SDS–PAGE. Protein–protein interactions were assayed with equal amount of in vitro translated and ^{35}S-labeled AP-2 or CDP proteins and purified GST proteins bound onto beads as described previously [Wu and Lee, 1998]. The interacting proteins were released from beads, resolved on 10% SDS–PAGE and detected by autoradiography.

RESULTS

Functional Role of Tandem CCAAT Motifs in the H3.2 Promoter

The hamster H3.2 promoter contains a TATA element, two tandem CCAAT repeats, an inverted YY1 binding site flanked by GC rich sequences, and the H3core domain (Fig. 1). The proximal promoter region of replication-dependent human TK gene also contains two tandem CCAAT motifs, the YY1 core binding site, and GC-rich sequences in between the CCAAT sequences (Fig. 1). Previously, we have shown that NF-Y/CBF binds to the proximal and distal CCAAT motifs and CDP binding is mediated through the tandem CCAAT motifs [Kim et al., 1997]. By using binding site competition and antigenic immunoreactivity in EMSAs, we observed that NF-Y/CBF binds the single CCAAT motifs in the H3.2 promoter (data not shown). Based on the similarity between the H3.2 and the human TK promoter, we predict that the two tandem CCAAT repeats of the H3.2 promoter may also be regulated by CDP.

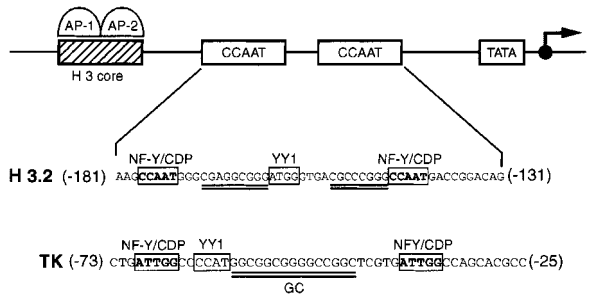

Fig. 1. Schematic drawing of the hamster histone H3.2 promoter. The upstream *cis*-regulatory elements of the hamster H3.2 promoter spanning from −254 to −6 are indicated on top. The H3core binds transcription factors AP-1 and AP-2. Transcriptional initiation site is indicated by an arrow. The proximal promoter sequence spanning from −181 to −131 containing two tandem CCAAT repeats and an inverted core YY1 binding site (ATGG) flanked by GC rich sequence is shown below. The proximal sequence of the human TK promoter spanning from −73 to −25 with features similar to the H3.2 promoter is shown. For both promoters, the CCAAT sequence binds NF-Y. Tandem CCAAT sequence motifs are putative CDP binding sites.

To determine the effect of CDP on the H3.2 promoter in vivo, the WT pH3[−254]CAT reporter plasmid was cotransfected in K12 cells with various amounts of CDP expression vector. In all transfections, the total amount of DNA was adjusted to be the same with empty vector DNA. In agreement with earlier results obtained with the human TK promoter [Kim et al., 1997], overexpression of human CDP resulted in repression of the H3.2 promoter activity in a dose-dependent manner (Fig. 2B). These results predicted that if CDP acts through the tandem CCAAT motifs, simultaneous mutation of the both CCAAT sequences should result in derepression of the H3.2 promoter activity. To test this idea, reporter constructs harboring the WT or the mutated H3.2 promoters were used to direct the CAT gene expression. The pH3[−254-2C^{m}]CAT is identical to the WT promoter construct except that the two CCAAT motifs are mutated by site-directed mutagenesis. The pH3[−254-AP1^{m}]CAT has a mutation in the AP-1-like site within the H3core sequence (Fig. 2A). The various constructs were transiently transfected into K12 cells and the promoter activity was measured in exponentially growing cells. In support of the CDP repression of the H3.2 promoter through interactions with the CCAAT sequences, mutation of the two CCAAT motifs resulted in an increase of the H3.2 promoter activity in transient transfections (Fig. 2C). In contrast, mutation of the

AP-1-like site within the H3core in pH3[−254-AP1^m]CAT caused reduction of H3.2 promoter activity (Fig. 2C).

To determine the functional role of the tandem CCAAT motifs during the cell cycle and to rule out interference of the AP1-like activating site, we created a Luc reporter construct pH3[−254-AP1^m/2C^m]Luc. This construct has eliminated both the tandem CCAAT motifs and the AP1-like site within the H3core. The constructs pH3[−254]Luc, pH3[−254-AP1^m]Luc and pH3[−254-AP1^m/2C^m]Luc were stably introduced into hamster K12 cells by cotransfection with a neomycin resistance gene. Independent pools of stable transfectants were selected and expanded. The pooled transfectants of each construct were synchronized by serum deprivation. After serum release, the promoter activities during the cell cycle were determined by Luc assays in synchronized K12 cells established from several independent pools of stable transfectants. As a control, the cell cycle profile of the synchronized K12 cells was determined by [^{3}H]thymidine incorporation across the cell cycle (data not shown). The Luc activity of the WT H3.2 promoter (pH3[−254]Luc) remained unchanged in quiescent and G1 phase cells through 8–10 h prior to the G1/S transition. There was a significant increase in its Luc activity during the G1/S-phase transition (at 10–12 h) following readdition of serum. The Luc activity of the WT H3.2 promoter increased rapidly when cells entered S-phase and reached a plateau at 20 h (Fig. 3).

Previously, we showed that mutation of an AP-2 site (pH3[−254-AP2^m]Luc) caused an increase in H3.2 promoter activity in quiescent cells and an up-regulation of its activity prior to the G1/S-phase transition [Wu and Lee, 2001]. Mutation of the AP-1-like site located within the H3core region (pH3[−254-AP1^m]Luc) reduced both the levels of Luc activity in quiescent K12 cells and its induction in S-phase cells as compared with that of the WT promoter (Fig. 3

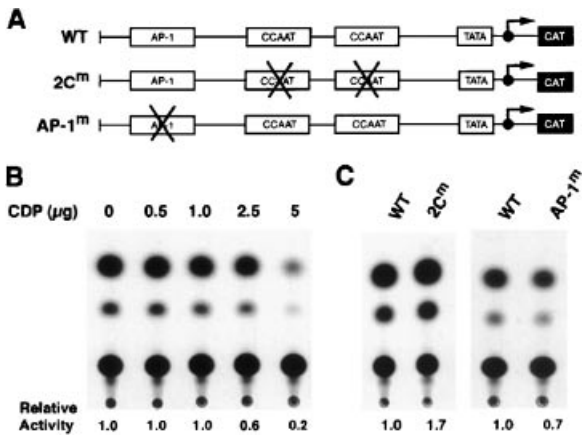

Fig. 2. Effects of CDP overexpression and CCAAT motif mutations on activity of the H3.2 promoter. (**A**) Schematic drawings of CAT reporter genes driven by the WT or mutated H3.2 promoter. WT refers to pH3[−254]CAT containing the WT H3.2 promoter (from −254 to −6) linked to CAT; 2C^m refers to pH3[−254-2C^m]CAT with both CCAAT sequence mutated; and AP1^m refers to pH3[−254-AP1^m]CAT containing a mutated AP1 sequence. (**B**) The WT pH3[−254]CAT reporter plasmid (5 μg) was transiently cotransfected in K12 cells with different amount of pCMV/CDP as indicated on top (0–5 μg). In all transfections, the total amount of DNA was adjusted to be the same with empty vector DNA. The H3.2 promoter activity in the presence of empty vector was set as one. The relative promoter activity in the presence of pCMV/CDP coexpression is indicated below the autoradiogram. (**C**) The WT pH3[−254]CAT, and mutated pH3[−254-2C^m]CAT (2C^m) or pH3[−254-AP1^m]CAT (AP1^m) reporter plasmid (5 μg of each) were transiently transfected in K12 cells. The CAT activity of the WT H3.2 promoter expression plasmid was set as one. The relative promoter activity is shown below the autoradiogram.

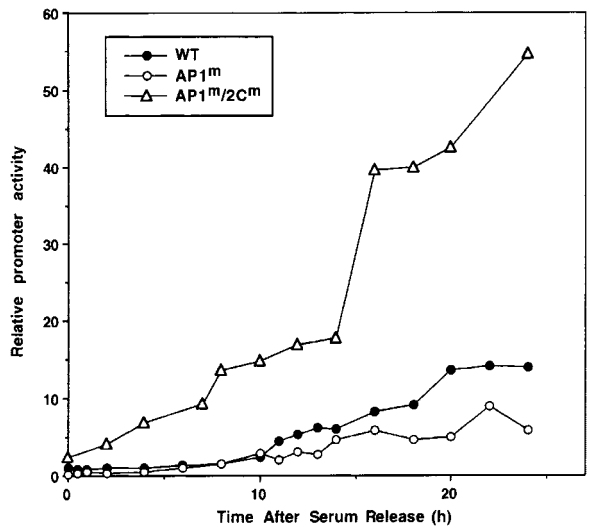

Fig. 3. Effect of CCAAT and AP-1 binding site mutations on the H3.2 promoter activity during the cell cycle. The ET H3.2 or mutant promoters (AP-1^m or AP-1^m/2C^m) linked to a Luc reporter gene were stably transfected into K12 cells. Stable transfectants of K12 cells were synchronized by serum deprivation. The Luc activities were determined from equal number of cells at each time point. The Luc activity of the WT H3.2 promoter from the quiescent cells without addition of serum (at Time 0) was set as one. The relative promoter activities were plotted as a function of time after serum release. pH3[−254]Luc (-●-) is the WT H3.2 promoter construct; the pH3[−254-AP1^m]Luc (-○-) is a construct with mutation of the AP-1-like site within the H3core sequence; and the pH3[−254-AP1^m/2C^m]Luc (-Δ-) is a construct with mutations in both the AP-1-like site and the two CCAAT motifs.

and data not shown). Mutations of the two CCAAT motifs and the AP-1-like site (pH3 [−254-AP1^{m}/2C^{m}]CAT) resulted in elevated Luc activity (about 2.5-fold) in quiescent cells. A strong derepression of the H3.2 promoter activity was observed at 6–12 h in mid-to-late G1 phase prior to the G1/S transition. The promoter activity of pH3[−254-AP1^{m}/2C^{m}]Luc was further increased to about 40-fold just as cells entered S-phase (Fig. 3). Together, these findings provide genetic evidence that the two CCAAT elements confer repression upon the H3.2 promoter, especially in the G1 phase and G1/S boundary of the cell cycle. In addition, our results suggest that activators other than that binding to the AP1-like site contribute to the S-phase induction of transcription of the H3.2 gene.

Physical Association of CDP With AP-2 In Vitro

It has been reported that a CDP-containing complex, HiNF-D, associates with Rb and the related protein p107 in EMSAs, and is involved in an E2F-independent regulation of histone gene expression [van Wijnen et al., 1996, 1997]. To address the issue of whether there is a direct physical association between CDP and Rb, GST pull-down assays were performed with GST-Rb fusion proteins and in vitro translated ^{35}S-labeled full-length human CDP. As shown in Figure 4, no direct in vitro physical interaction between CDP and GST-Rb (379–928) was detected under these assay conditions. Nor did CDP interact with a series of other proteins including GST, GST-TBP, GST-H-Ras, and GST-c-Myc (259–439) (Fig. 4A). Further, none of the GST-Rb domains tested showed any specific interaction with radiolabeled CDP (Fig. 4B), whereas this same set of Rb domains interacted with both AP-2A and AP-2β as previously reported (Fig. 4C,D). Unexpectedly, a physical interaction was detected between the radiolabeled full-length CDP and GST-AP-2A or GST-AP-2β(DBD) (Fig. 4A).

Further experiments to map their interaction domains were performed with various radiolabeled and C-terminal truncated CDP fragments, which were generated by restriction enzyme digestions of pCMV/CDP template DNAs prior to the in vitro transcription and translation. These CDP subfragments have either one or two cut repeats toward the N-terminus without the C-terminal homeodomain

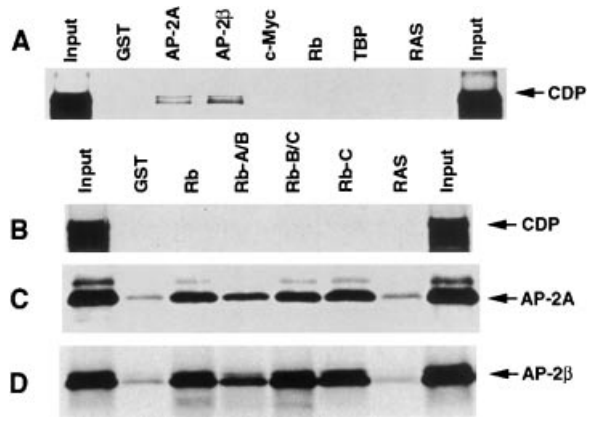

Fig. 4. Physical interaction of CDP with AP-2 but not with Rb in vitro. GST pull-down assays were performed by using the purified GST or GST-fusion proteins and by mixing with in vitro translated and ^{35}S-labeled full-length CDP. The proteins bound onto the GST-beads were eluted, applied to a 10% SDS–PAGE, and detected by autoradiography; (**A**) Interactions of ^{35}S-labeled CDP with AP-2A and AP-2β; (**B**) negative interaction of CDP with various Rb domains; (**C**) interaction of AP-2A; and (**D**) AP-2β with various Rb domains. A 10% of total radiolabeled proteins was shown in each of the input lanes. GST-fusion proteins used are indicated on top, that include GST-AP-2A, GST-AP-2β(DBD), GST-c-Myc(259-439), GST-TBP, GST-H-Ras, GST-Rb(379-928), GST-Rb-A/B(379-792), GST-Rb-B/C(646-928), GST-Rb-C(768-928).

and the alanine-rich repression domain. Preliminary data showed that GST-AP-2A interacted with the C-terminal partially deleted CDP fragments (data not shown). These observations suggest that the cut-repeat region of CDP is involved in its association with AP-2. To test this idea, GST pull-down assays were performed by using various ^{35}S-labeled AP-2A subfragments and GST-fused cut repeats GST-CR1 or GST-CR3HD which include the C-terminal homeodomain [Harada et al., 1994, 1995]. Both GST-CR1 and GST-CR3HD, but not GST or GST-H-Ras interacted specifically with the full-length AP-2A (Fig. 5 and data not shown). However, an N-terminal subfragment of AP-2A(N262) containing part of the C-terminal basic region showed a reduced interaction with GST-CR1 or GST-CR3HD. This interaction was intact when an intact basic region and the dimerization domain in the C-terminus of AP-2A were present (ΔN165 and full-length AP-2A). For ΔN227, which has partially lost its basic region, its ability to bind CR1 was much reduced and binding to CR3HD was minimal. For ΔC390, containing a deletion in the dimerization domain, its binding to CR3HD was reduced and binding to CR1 was minimal (Fig. 5B,C).

Together, these data demonstrated that CDP can physically interact with AP-2A in vitro and the interaction occurs through the cut repeats of CDP and the basic region and dimerization domain of AP-2 (Fig. 5D).

Cooperative Effect of CDP and AP-2 on H3.2 Promoter

To analyze the collaborative effect of CDP and AP-2 in vivo, expression vectors for CDP (2.5 μg) and AP-2β (0.25 μg) alone or in combination were cotransfected in K12 cells with the WT pH3[−254]CAT reporter construct. Under these conditions, AP-2β exerted about 60% reduction of H3.2 promoter activity, while CDP had marginal effect. When both CDP and AP-2β were coexpressed, significant synergistic effect was observed in WT H3.2 promoter activity that resulted in about seven-fold reduction of its transcription (Fig. 6A). In addition, no obvious synergism between CDP and AP-2 was observed on the control promoters pCMV-CAT (Fig. 6B) and pSV2-CAT (Fig. 6C). Thus, the cooperative effect of repression mediated by CDP and AP-2 is relatively specific for the H3.2 promoter.

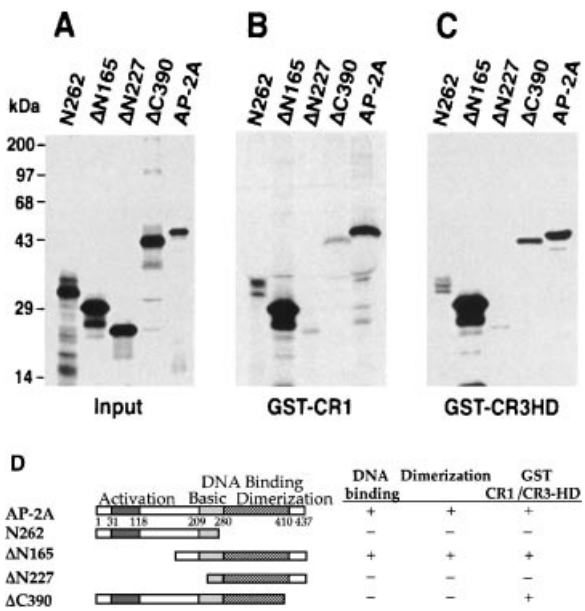

Fig. 5. Mapping the interaction domains of CDP and AP-2. The AP-2A subfragments (N262, ΔN165, ΔN227, or ΔC390) and the full-length AP-2A were in vitro translated and ^{35}S-labeled and a 10% of total input proteins is shown (**A**). GST pull-down assays were carried out by using the various radiolabeled AP-2A proteins and the GST-CR1 (**B**) or GST-CR3HD (**C**). The proteins bound onto the GST-beads were eluted, applied to a 10% SDS–PAGE and detected by autoradiography. The positions of protein size markers (in kDa) run in parallel are indicated in the left. The schematic drawing of AP-2A structural domains and their interactions with CDP cut repeats are summarized (**D**).

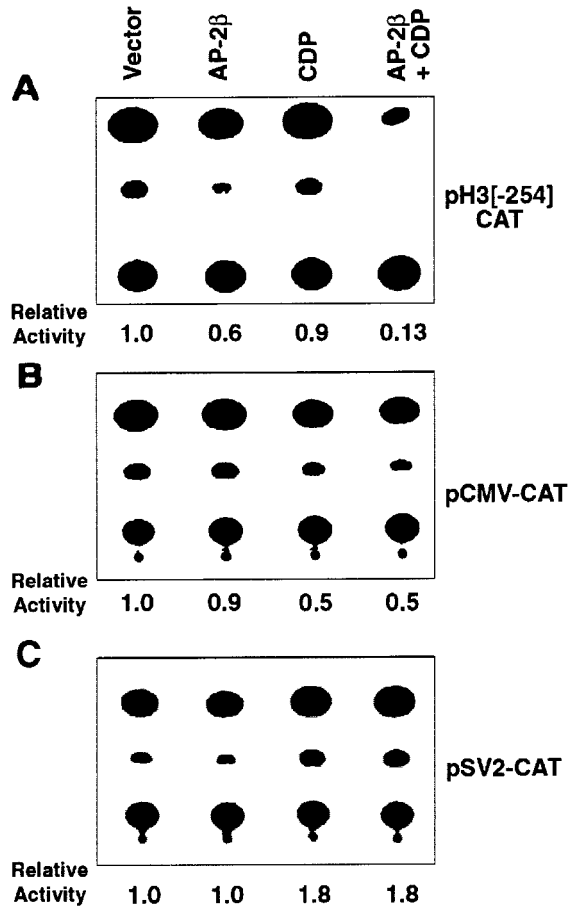

Fig. 6. Synergism between CDP and AP-2 in repression of the H3.2 promoter. K12 cells were transiently transfected with the reporter constructs (**A**) pH3[−254]CAT, (**B**) pCMV-CAT or (**C**) pSV2-CAT (5 μg of each). Expression plasmid pCMV/CDP (2.5 μg) or pCMV/mAP-2β (0.25 μg) alone or in combination was included in the cotransfections as indicated on top. In all transfections, the total amount of DNA was adjusted to be the same with empty vector DNA. The autoradiograms of the CAT assays are shown. The relative CAT activity is shown below the autoradiogram.

DISCUSSION

The CCAAT motif is commonly found in the promoters of many mammalian genes. It has been shown that a large number of CCAAT motifs bind the abundant and ubiquitously expressed NF-Y/CBF factor, which contributes to the strength of most, if not all, CCAAT-containing promoters [Maity and de Crombrugghe, 1998]. For the human TK gene, this is primarily demonstrated by downregulation of the TK promoter activity by mutation of single

CCAAT motifs or by a dominant negative mutant of NF-Y in transient transfections [Arcot et al., 1989; Arcot and Deininger, 1992; Pang and Chen, 1993; el-Hodiri and Perry, 1995; Kim et al., 1997]. However, further analysis of the TK promoter reveals that the CCAAT elements are present in tandem and CDP can act as a repressor mediated by the tandem CCAAT motifs [Kim et al., 1997]. Our results show that the tandem CCAAT motifs also serve as a binding site for a potent repressor of the H3.2 promoter and that binding of NF-Y to the single CCAAT sites may not be obligatory for the S-phase induction of the H3.2 promoter. The results from the double mutation of AP-1 and the tandem CCAAT motifs further implies that there are additional activating sites responsible for the stimulation of H3.2 promoter during the cell cycle. Previously, we have established that an inverted YY1 binding site located between the two tandem CCAAT repeats is required for the S-phase induction of H3.2 gene transcription [Wu and Lee, 2001]. The importance of the GC-rich Sp1 site in replication-dependent gene activation of transcription has been shown in some E2F-dependent promoters as well [Fridovich-Keil et al., 1993].

It has been established that CDP binding requires two CCAAT motifs in close proximity. CDP has been shown in EMSAs to form a HiNF-D complex with Rb and related p107 protein, suggesting that a CDP-dependent, but E2F-independent mechanism may be involved in histone gene regulation [van Wijnen et al., 1996, 1997]. While we cannot detect the direct association of CDP with Rb in GST pull-down assays, we discovered that CDP interacted with AP-2A, a protein that has been reported to associate with Rb, especially with the critical growth suppression region of Rb-C domain [Wu and Lee, 1998]. Further, the interaction domains of CDP and AP-2 are mapped to the highly conserved cut repeats of CDP, and the basic region and dimerization domain of AP-2A. Currently, it is unknown whether the HiNF-D/CDP complex on human H4 promoter contains an AP-2 protein, which may serve as a bridge protein for the association of CDP with Rb [van Wijnen et al., 1994, 1996]. Our discoveries of AP-2/Rb and AP-2/CDP interactions present the complexity of the intricate network for controlling the mammalian G1/S transition (Fig. 7). It is well established that the histone deacetylase (HDAC) containing complexes such as Mad/Max-mSin3A-HDAC, Rb-Rbp48-HDAC or HDAC-Rb-hSwi/SNF, and MeCP2-mSin3A-HDAC are implicated in transcriptional repression of many cell-cycle control genes [Nan et al., 1998; Ayer, 1999; Dang, 1999; Harbour and Dean, 2000]. This repression mechanism may apply to promoters containing E2F binding sites mediated by E2F/DP1 and Rb interactions (Fig. 7A). Our work on the TK and H3.2 promoters supports a regulatory mechanism independent of E2F for their repression in quiescent cells and activation at the onset of S-phase. Here we propose that CDP/AP-2-Rb-HDAC-mSin3A complex may be involved in transcriptional repression of the hamster H3.2 and human TK gene expression (Fig. 7B). This repression mechanism could be used to prevent misfiring of the histone promoter during the cell cycle and for turning off the promoter at the end of the S-phase. The physical interaction between CDP and AP-2 supports their cooperation in suppression of H3.2 promoter activity. When the AP-2 site within the H3core region was mutated, the promoter activity was elevated in late G1 phase prior to the G1/S transition in stable transfectants [Wu and Lee, 2001]. The mutation of the tandem CCAAT motifs displays a similar pattern of deregulation of H3.2 promoter activity. We further show that CDP and AP-2 synergistically repress the H3.2 promoter in vivo. Some of the CDP mutant phenotypes were observed in the nervous system and the Malpighian tubules in *Drosophila*, and in equivalent parts of AP-2 null mice [Schorle et al., 1996; Zhang et al., 1996]. Interestingly, loss of heterozygosity and reduced expression of the CDP gene is

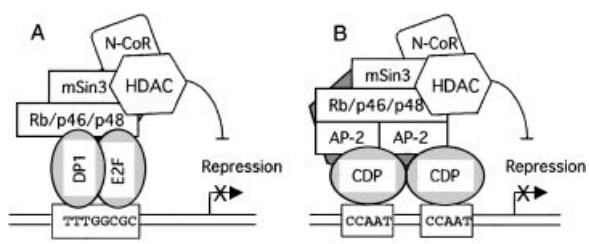

Fig. 7. Model of chromatin-remodeling complexes in E2F-dependent and E2F-independent transcriptional repression. The diagrams depict the association of (**A**) the E2F/DP heterodimer with the E2F binding site (5′-TTTGGCGC-3′), and (**B**) the proposed CDP complex with the two CCAAT repeats, with mSin3, N-CoR, and HDAC. The model predicts that the association of E2F/Rb and CDP/AP-2/Rb complex mediates E2F-dependent (A) and E2F-independent (B) repression mechanisms in transcription.

observed in human uterine leiomyomas and breast cancer, providing the first evidence that CDP/cut could act as potential tumor suppressors [Zeng et al., 1997; Neville et al., 2001]. Future studies on the precise mechanism for CDP suppression may have important implications in cell and cancer biology.

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REFERENCES

Andres V, Chiara MD, Mahdavi V. 1994. A new bipartite DNA-binding domain: Cooperative interaction between the cut repeat and homeo domain of the cut homeo proteins. Genes Dev 8:245–257.

Arcot SS, Deininger PL. 1992. Protein binding sites within the human thymidine kinase promoter. Gene 111:249–254.

Arcot SS, Flemington EK, Deininger PL. 1989. The human thymidine kinase gene promoter. Deletion analysis and specific protein binding. J Biol Chem 264:2343–2349.

Artishevsky A, Wooden S, Sharma A, Resendez E Jr., Lee AS. 1987. Cell-cycle regulatory sequences in a hamster histone promoter and their interactions with cellular factors. Nature 328:823–827.

Aufiero B, Neufeld EJ, Orkin SH. 1994. Sequence-specific DNA binding of individual cut repeats of the human CCAAT displacement/cut homeodomain protein. Proc Natl Acad Sci U S A 91:7757–7761.

Ayer DE. 1999. Histone deacetylases: Transcriptional repression with SINers and NuRDs. Trends Cell Biol 9: 193–198.

Aziz F, van Wijnen AJ, Stein JL, Stein GS. 1998. HiNF-D (CDP-cut/CDC2/cyclin A/pRB-complex) influences the timing of IRF-2-dependent cell cycle activation of human histone H4 gene transcription at the G1/S phase transition. J Cell Physiol 177:453–464.

Barberis A, Pearlberg J, Simkovich N, Farrell S, Reinagel P, Bamdad C, Sigal G, Ptashne M. 1995. Contact with a component of the polymerase II holoenzyme suffices for gene activation. Cell 81:359–368.

Buettner R, Kannan P, Imhof A, Bauer R, Yim SO, Glockshuber R, Van Dyke MW, Tainsky MA. 1993. An alternatively spliced mRNA from the AP-2 gene encodes a negative regulator of transcriptional activation by AP-2. Mol Cell Biol 13:4174–4185.

Coqueret O, Berube G, Nepveu A. 1996. DNA binding by cut homeodomain proteins is down-modulated by protein kinase C. J Biol Chem 271:24862–24868.

Coqueret O, Martin N, Berube G, Rabbat M, Litchfield DW, Nepveu A. 1998. DNA binding by cut homeodomain proteins is down-modulated by casein kinase II. J Biol Chem 273:2561–2566.

Dang CV. 1999. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol 19:1–11.

Dufort D, Nepveu A. 1994. The human cut homeodomain protein represses transcription from the *c-myc* promoter. Mol Cell Biol 14:4251–4257.

el-Hodiri HM, Perry M. 1995. Interaction of the CCAAT displacement protein with shared regulatory elements required for transcription of paired histone genes. Mol Cell Biol 15:3587–3596.

Fridovich-Keil JL, Markell PJ, Gudas JM, Pardee AB. 1993. DNA sequences required for serum-responsive regulation of expression from the mouse thymidine kinase promoter. Cell Growth Differ 4:679–687.

Harada R, Dufort D, Denis-Larose C, Nepveu A. 1994. Conserved cut repeats in the human cut homeodomain protein function as DNA binding domains. J Biol Chem 269:2062–2067.

Harada R, Berube G, Tamplin OJ, Denis-Larose C, Nepveu A. 1995. DNA-binding specificity of the cut repeats from the human cut-like protein. Mol Cell Biol 15:129–140.

Harbour JW, Dean DC. 2000. The Rb/E2F pathway: Expanding roles and emerging paradigms. Genes Dev 14:2393–2409.

Kim YK, Lee AS. 1991. Identification of a 70-base-pair cell cycle regulatory unit within the promoter of the human thymidine kinase gene and its interaction with cellular factors. Mol Cell Biol 11:2296–2302.

Kim EC, Lau JS, Rawlings S, Lee AS. 1997. Positive and negative regulation of the human thymidine kinase promoter mediated by CCAAT binding transcription factors NF-Y/CBF, dbpA, and CDP/cut. Cell Growth Differ 8:1329–1338.

Last TJ, Birnbaum M, van Wijnen AJ, Stein GS, Stein JL. 1998. Repressor elements in the coding region of the human histone H4 gene interact with the transcription factor CDP/cut. Gene 221:267–277.

Last TJ, van Wijnen AJ, de Ridder MC, Stein GS, Stein JL. 1999. The homeodomain transcription factor CDP/cut interacts with the cell cycle regulatory element of histone H4 genes packaged into nucleosomes. Mol Biol Rep 26:185–194.

Maity SN, de Crombrugghe B. 1998. Role of the CCAAT-binding protein CBF/NF-Y in transcription. Trends Biochem Sci 23:174–178.

Mitchell PJ, Timmons PM, Hebert JM, Rigby PW, Tjian R. 1991. Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. Gene Dev 5:105–119.

Naeve GS, Sharma A, Lee AS. 1992. Identification of a 10-base pair protein binding site in the promoter of the hamster H3.2 gene required for the S phase dependent increase in transcription and its interaction with a Jun-like nuclear factor. Cell Growth Differ 3:919–928.

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A. 1998. Tanscriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393:386–389.

Nepveu A. 2001. Role of the multifunctional CDP/Cut/Cux homeodomain transcription factor in regulating differentiation, cell growth and development. Gene 270:1–15.

Neufeld EJ, Skalnik DG, Lievens PM, Orkin SH. 1992. Human CCAAT displacement protein is homologous to the Drosophila homeoprotein, *cut*. Nat Genet 1:50–55.

Neville PJ, Thomas N, Campbell IG. 2001. Loss of heterozygosity at 7q22 and mutation analysis of the CDP gene in human epithelial ovarian tumors. Int J Cancer 91:345–349.

Pang JH, Chen KY. 1993. A specific CCAAT-binding protein, CBP/tk may be involved in the regulation of thymidine kinase gene expression in human IMR-90 diploid fibroblasts during senescence. J Biol Chem 268:2909–2916.

Resendez E, Jr., Attenello JW, Grafsky A, Chang CS, Lee AS. 1985. Calcium ionophore A23187 induces expression of glucose-regulated genes and their heterologous fusion genes. Mol Cell Biol 5:1212–1219.

Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ. 1996. Transcription factor AP-2 essential for cranial closure and craniofacial development. Nature 381:235–238.

Skalnik DG, Strauss EC, Orkin SH. 1991. CCAAT displacement protein as a repressor of the myelomonocytic-specific gp91-phox gene promoter. J Biol Chem 266:16736–16744.

Slansky JE, Farnham PJ. 1996. Introduction to the E2F family: Protein structure and gene regulation. Curr Topics Microbiol Immunol 208:1–30.

van Gurp MF, Pratap J, Luong M, Javed A, Hoffmann H, Giordano A, Stein JL, Neufeld EJ, Lian JB, Stein GS, van Wijnen AJ. 1999. The CCAAT displacement protein/cut homeodomain protein osteocalcin gene transcription and forms complexes with the retinoblastoma protein-related protein p107 and cyclin A. Cancer Res 59:5980–5988.

van Wijnen AJ, Aziz F, Grana X, De Luca A, Desai RK, Jaarsveld K, Last TJ, Soprano K, Giordano A, Lian JB, Stein JL, Stein GS. 1994. Transcription of histone H4, H3, and H1 cell cycle genes: Promoter factor HiNF-D contains CDC2, cyclin A, and an RB-related protein. Proc Natl Acad Sci U S A 91:12882–12886.

van Wijnen AJ, van Gurp MF, de Ridder MC, Tufarelli C, Last TJ, Birnbaum M, Vaughan PS, Giordano A, Krek W, Neufeld EJ, Stein JL, Stein GS. 1996. CDP/*cut* is the DNA-binding subunit of histone gene transcription factor HiNF-D: A mechanism for gene regulation at the G1/S phase cell cycle transition point independent of transcription factor E2F. Proc Natl Acad Sci U S A 93:11516–11521.

van Wijnen AJ, Cooper C, Odgren P, Aziz F, De Luca A, Shakoori PA, Giordano A, Quesenberry PJ, Lian JB, Stein GS, Stein JL. 1997. Cell cycle-dependent modifications in activities of pRb-related tumor suppressors and proliferation-specific CDP/cut homeodomain factors in murine hematopoietic progenitor cells. J Cell Biochem 66:512–523.

Williams T, Admon A, Luscher B, Tjian R. 1988. Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. Genes Dev 2:1557–1569.

Wu F, Lee AS. 1998. Identification of AP-2 as an interactive target of Rb and a regulator of the G1/S control element of the hamster histone H3.2 promoter. Nucleic Acids Res 26:4837–4845.

Wu F, Lee AS. 2001. YY1 as a regulator of replication-dependent hamster histone H3.2 promoter and an interactive partner with AP-2. J Biol Chem 276:28–34.

Zeng WR, Scherer SW, Koutsilieris M, Huizenga JJ, Filteau F, Tsui LC, Nepveu A. 1997. Loss of heterozygosity and reduced expression of the CUTL1 gene in uterine leiomyomas. Oncogene 14:2355–2365.

Zhang J, Hagopian-Donaldson S, Serbedzija G, Elsemore J, Plehn-Dujowich D, McMahon AP, Flavell RA, Williams T. 1996. Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. Nature 381:238–241.