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Cell Cycle-Dependent Modifications in Activities of pRb-Related Tumor Suppressors and Proliferation-Specific CDP/*cut* Homeodomain Factors in Murine Hematopoietic Progenitor Cells

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Abstract The histone H4 gene promoter provides a paradigm for defining transcriptional control operative at the G_1/S phase transition point in the cell cycle. Transcription of the cell cycle-dependent histone H4 gene is upregulated at the onset of S phase, and the cell cycle control element that mediates this activation has been functionally mapped to a proximal promoter domain designated Site II. Activity of Site II is regulated by an E2F-independent mechanism involving binding of the oncoprotein IRF2 and the multisubunit protein HiNF-D, which contains the homeodomain CDP/*cut,* CDC2, cyclin A, and the tumor suppressor pRb. To address mechanisms that define interactions of Site II regulatory factors with this cell cycle control element, we have investigated these determinants of transcriptional regulation at the G_1/S phase transition in FDC-P1 hematopoietic progenitor cells. The representation and activities of histone gene regulatory factors were examined as a function of FDC-P1 growth stimulation. We find striking differences in expression of the pRb-related growth regulatory proteins (pRb/p105, pRb2/p130, and p107) following the onset of proliferation. pRb2/p130 is present at elevated levels in quiescent cells and declines following growth stimulation. By contrast, pRb and p107 are minimally represented in quiescent FDC-P1 cells but are upregulated at the G_1/S phase transition point. We also observe a dramatic upregulation of the cellular levels of pRb2/p130-associated protein kinase activity when S phase is initiated. Selective interactions of pRb and p107 with CDP/*cut* are observed during the FDC-P1 cell cycle and suggest functional linkage to competency for DNA binding and/or transcriptional activity. These results are particularly significant in the context of hematopoietic differentiation where stringent control of the cell cycle program is requisite for expanding the stem cell population during development and tissue renewal. J. Cell. Biochem. 66:512–523, 1997. © 1997 Wiley-Liss, Inc.

Key words: cell cycle control; H4 gene promoter; G_1/S phase transition point; CDP/*cut*; interferon regulatory factor 2

A pool of primitive hematopoietic stem cells (PHSCs) resides in the adult bone marrow as a reservoir of uncommitted and undifferentiated blood cell precursors. In order to maintain an appropriately balanced system, these cells must respond to a complex network of signals from the extracellular environment by proliferation and subsequent differentiation into mature cell types. Regulation of hematopoietic cell differentiation is strongly affected by cell cycle position, with cells being most receptive to differentiation signals during G_1 [Hui et al., 1993; Caroll et al., 1995]. Cytokines that act to arrest cells in G_1 or to prolong this phase generally promote differentiation [Hestdal et al., 1993; Larden et al., 1994; Johnson et al., 1993; Caroll et al.,

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1995], while conditions that shorten G_1 lower the probability that differentiation will occur [Kato et al., 1993; Caroll et al., 1995].

Competency for proliferation and threshold requirements for cell cycle progression are mediated by growth regulatory factors, which include cyclins, cyclin-dependent kinases, cyclin inhibitors, tumor suppressors, and E2F/DP-related factors. These modulators of growth control function in part by activating and repressing transcription of specific genes at defined cell cycle stages [reviewed in Sherr, 1994; Morgan, 1995; Weinberg, 1995; MacLachlan et al., 1995, Lam and La Thangue, 1994]. A complex and interdependent series of cellular signaling pathways impinge on promoter elements of genes that are selectively expressed during the cell cycle, contributing to transcriptional control by influencing options for occupancy by a broad spectrum of both positive and negative regulatory factors. Equally important, occupancy by specific growth regulatory factors supports the integration of activities via protein–DNA and protein–protein interactions within as well as between promoter domains.

The histone gene promoter provides a paradigm for defining transcriptional control operative at the G_1/S phase transition point in the cell cycle [Stein et al., 1994]. The regulatory element of cell cycle-dependent H4 histone genes that controls transcriptional upregulation at the onset of S phase has been functionally mapped to a promoter domain designated Site II [Ramsey-Ewing et al., 1994]. Site II represents a genomic in vivo protein–DNA interaction domain [Koreger et al., 1995; Pauli et al., 1987] that contains motifs that interact with several histone gene transcription factors (HiNF's) [van Wijnen et al., 1992]. Activity of Site II is responsive to an E2F-independent cell cycle regulatory mechanism that involves binding of IRF2 to the distal HiNF-M motif [Vaughan et al., 1995; van Wijnen et al., 1991] and binding of CDP/*cut* to the proximal HiNF-D motif [van Wijnen et al., 1996]. The phosphorylation-dependent protein–DNA and protein–protein interactions at the Site II cell cycle regulatory element include CDC2, cyclin A, and an RB-related protein, as components of the HiNF-D complex [van Wijnen et al., 1994]. A mechanism for coordinate transcriptional control of cell cycle-dependent H4, H3, and H1 histone genes is supported by similar Site II promoter regulatory sequences and cognate transcription factors [van den Ent et al., 1994].

While a series of promoter elements and factors contribute to control of histone gene transcription have been defined, the rate-limiting regulatory steps in transcriptional control in cells of different lineages and with distinct phenotypic properties remain to be established. This is particularly significant in hematopoietic progenitor cells, where regulation of cell cycle position is a key factor in the ability of cells to respond to differentiation factors. Because most differentiated blood cells are short-lived and must be continuously replenished over the life of the organism, stringent control of the cell cycle program is critical for maintaining adequate levels of mature, functional cells in all lineages [Quesenberry et al., 1994]. This requires sequential activation of PHSCs in G_0, expansion of the progenitor pool and an appropriate response to cues that direct cells in G_1 transit to exit the cell cycle and undergo differentiation. Competency for differentiation along the hematopoietic lineages cannot be compromised.

This report experimentally addresses parameters of cell cycle control in hematopoietic progenitor cells that are determinants of transcriptional regulation at the G_1/S phase transition point. Using FDC-P1 murine hematopoietic progenitor cells, we examined the representation and activities of two classes of regulatory factors (pRb-related proteins and CDP/*cut*) that influence the histone gene cell cycle regulatory element at the initiation of S phase, as well as assessed phosphorylation-mediated mechanisms involving pRb-related proteins during the cell cycle in FDC-P1 cells.

MATERIALS AND METHODS

Cell Synchronization

FDC-P1 cells [Dexter et al., 1980] were maintained in RPMI 1640 with 25% WEHI-3 conditioned medium (WEHIcm) and 10% fetal calf serum (FCS). FDC-P1 cells were synchronized by isoleucine deprivation, which arrests cells in early G_1 phase (Reddy et al., 1992). Actively growing cells (in early to mid log-phase) were pre-cultured for 36 h in isoleucine-deficient medium (i.e., isoleucine-deficient DMEM supplemented with 10% dialyzed WEHIcm and 10% FCS). At time zero, cells were transferred to complete growth medium as described above. Samples were withdrawn over the next 48 h at

selected time points for analysis of ^{3}H-thymidine incorporation [Reddy et al., 1992]. Additionally, cell growth and viability were monitored by cell counts and trypan blue exclusion. Cell culture samples for protein analysis were taken at 2- or 4-h intervals following release, and the FDC-P1 cells were harvested by centrifugation. The resulting cell pellets were rapidly frozen in liquid nitrogen, and stored at −70°C.

Nuclear Protein Preparations

Nuclear protein preparations were obtained by lysing frozen pellets of FDC-P1 cells (1×10^8 cells or approximately 200–300 µl wet packed cell volume) in 4 ml ice-cold buffer R (10 mM KCl, 10 mM Hepes/NaOH, pH 7.5, 0.5% Triton, 300 mM sucrose, 3 mM $MgCl_2$), complemented with a broad spectrum of protease inhibitors (Boehringer Mannheim, Indiana, IN) and additives (0.2 mM PMSF, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 10 µg/ml trypsin inhibitor, 2 µg/ml TPCK, 40 µg/ml bestatin, 17 µg/ml calpain inhibitor, 1 µg/ml E64, 1.0 mM EGTA, 0.2 mM EDTA, 1 mM DTT, 0.75 mM spermidine, 0.15 mM spermine). Cells were mechanically lysed on ice in a homogenizer tube using a Teflon pestle attached to an electric drill. After centrifugation of the lysates at 1,500 rpm using a low-speed swing-out centrifuge (IEC), nuclear pellets were resuspended in 1,000 µl buffer A (10 mM Hepes, pH 7.5, 10 mM KCl) plus the above additives. Samples were transferred to 1.5-ml microfuge tubes and subjected to centrifugation at 6,000 rpm for 1 min. The nuclear pellets were extracted with 400 µl buffer C (0.4 M KCl, 25 mM Hepes, pH 7.5, 25% glycerol plus protease inhibitors and additives) for 30 min on ice. Nuclear homogenates were centrifuged for 10 min at 4°C at and the supernatant (nuclear extract) was frozen in liquid nitrogen without dialysis. The concentration of nuclear proteins was determined by Coomassie Blue staining using a multiwell plate reader. Absorbance readings were obtained in duplicate for a range of volumes for each sample (protein volume titration), and the protein concentrations were calculated using bovine serum albumin (BSA) as the external protein standard.

Protein–DNA Interaction Assays

HiNF-D binding was monitored with DNA probes spanning the Site II element of the H4 gene (nt −97/−38). Standard binding reactions (20 µl vol) for HiNF-D were performed at room temperature and contain 10 fmole (=0.4 ng) DNA probe, a mixture of non-specific competitor DNAs (2 µg poly G/C DNA and 0.2 µg poly I/C DNA), and 3 µg HeLa nuclear protein. Binding reactions for AP-1 and SP-1/SP-3 probes were carried out in the presence of 2 µg poly I/C DNA as nonspecific competitor. Gel shift assays for detection of HiNF-D, SP-1/SP-3, AP-1 and CDP/*cut* were performed using the same conditions as described previously [Shakoori et al., 1994; Birnbaum et al., 1995; Skalnik et al., 1991]. Competition assays were performed with the wild-type HiNF-D binding site oligonucleotide (TM-3, spanning nt −93 to −53 of the H4 gene) or a mutant DNA fragment (NH-6; containing point-mutations that specifically abolish HiNF-D binding) [van Wijnen et al., 1992]. Binding of CDP/*cut* to a nonhistone gene was studied using a probe spanning the duplicated CCAAT-box region of the gp91-phox gene (FP oligonucleotide) [Skalnik et al., 1991]. Oligonucleotide competition assays were performed by mixing unlabeled competitor DNA oligonucleotide (1 pmole; i.e., 100-fold molar excess) with probe prior to the inclusion of protein. Electophoretic fractionation of protein–DNA complexes was performed in 4% (80 : 1) polyacrylamide gels, using $0.5 \times$ TBE as buffer [Ausubel et al., 1987].

Gel Shift Immunoassays

Immunoreactions were performed by preincubating antibodies with nuclear protein on ice for 15 min prior to the addition of probe DNA, although in some experiments antibodies were added for 15 min after the formation of protein–DNA complexes (post-incubation) to promote formation of supershift complexes. The following immunological reagents were used in our assays: mouse monoclonal antibodies (MAb) directed against pRb (hybridoma supernatant XZ104) [Hu et al., 1991] and cyclin A (hybridoma supernatant C160) [Giordano et al., 1989], a guinea pig antiserum against CDP/*cut* [Neufeld et al., 1992], and a rabbit antiserum against CDC2 (G6) [Draetta and Beach, 1989]. Nonimmune sera and negative hybridoma supernatants were used as control reagents in our assays.

Western Blot Analysis

Western blot analysis was performed as described previously [Odgren et al., 1996]. In brief, protein samples were electrophoretically frac-

tionated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels [Ausubel et al., 1989] and transferred onto Immobilon P PVDF membranes (Millipore) by semidry electroblotting with a current of 2.5 mA/cm^2. To verify complete transfer of proteins, gels were stained with Coomassie blue after transfer. Membranes were incubated in the presence of antibodies against pRb/p105 (C36) (Pharmingen, San Diego, CA), p107(C15), pRb2/p130(C20) (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were processed using the Immun-Lite system (BioRad, Hercules, CA) according to the instructions of the manufacturer. Immunoreactive proteins were visualized by chemiluminescence using either AMPPD (Bio-Rad, Hercules, CA) as substrate, followed by autoradiography.

Protein Kinase Assays

Protein kinase activity associated with RB-related complexes was determined by immunoprecipitation of pRb2/p130 and incubation of the precipitates in the presence of histone H1 (as the exogenous substrate) as described previously [Baldi et al., 1995; Krek et al., 1994].

RESULTS AND DISCUSSION

Homeodomain Protein CDP/*cut* and the Tumor Suppressors pRb and p107 Form Multiple Higher-Order Complexes in FDC-P1 Cells

The Site II cell cycle element of a histone H4 gene [Ramsey-Ewing et al., 1994] interacts with the transcriptional regulator HiNF-D. Using gel-shift immunoassays, oligonucleotide competition analysis, and DNase I footprinting with bacterially expressed CDP/*cut* fusion proteins, we have previously shown that CDP/*cut* is the DNA binding subunit of HiNF-D [van Wijnen et al., 1996]. In addition, the multisubunit HiNF-D protein contains the cell cycle regulators pRb, cyclin A and CDC2 [van Wijnen et al., 1994, 1996; Shakoori et al., 1995]. Gel-shift assays performed with HeLa S3 cervical carcinoma cells typically detect a single HiNF-D complex, which is completely immunoreactive with antibodies against pRB, CDC2, cyclin A, and CDP/*cut.* However, there appear to be two types of HiNF-D complexes in normal diploid calvarial osteoblasts [van Wijnen et al., 1994]. FDC-P1 cells are hematopoietic progenitor cells derived from long-term bone marrow culture [Dexter et al., 1980]. Results from gel-shift immunoassays (Fig. 1) suggest that at least two types of HiNF-D complexes recognize Site II in FDC-P1 hematopoietic progenitor cells. These complexes display subtle differences in electrophoretic mobility and are immunoreactive with a polyclonal antibody against CDP/*cut,* as evidenced by formation of a ternary DNA–protein/antibody complex (supershift). In addition, formation of these complexes is inhibited by a monoclonal hybridoma supernatant (C36) directed against the pocket of pRb (Fig. 1) and by a cyclin A antibody [Shakoori et al., 1995; data not shown]. By contrast, only a subset of these complexes forms a supershift in the presence of a polyclonal antibody directed against the C-terminus of CDC2 (Fig. 1A). This partial supershift is observed in the presence of excess

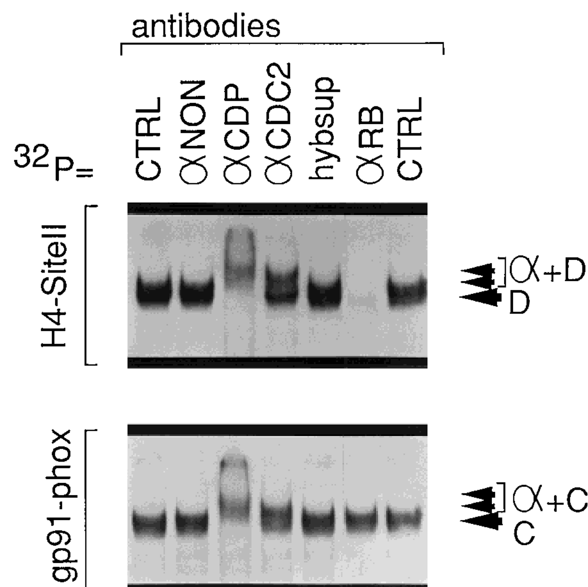

Fig. 1. Characterization of HiNF-D-related protein/DNA complexes mediated by CDP/*cut* from FDC-P1 cells with probes ($^{32}P^{=}$) spanning the histone H4 (D-complex) (*top*) and gp91-phox (C-complex) (*bottom*) genes. Gel-shift immunoassays were performed with antibodies directed against CDP/*cut* (αCDP), CDC2 (αCDC2), and pRb/p105 (αRB), as indicated above the lanes. Control reactions were performed in the absence of antibody (CTRL) or in the presence of non-immune serum (αNON) or a negative hybridoma supernatant (hybsup). Binding reactions were performed with the H4 or gp91-phox probes in the presence of FDC-P1 nuclear protein from exponentially growing cells. Incubation of complexes with the CDP or CDC2 antibodies for each probe results in supershift complexes (α + D and α + C) with different mobilities (*double arrowhead with bracket*). The CDP band in the control lanes represents at least two different types of complexes. In lane 3 (top and bottom) the entire band supershifts with αCDP, but only the upper part of this band forms a supershift with the αCDC2 antibody. Similar results have been observed with CDP complexes from osteoblasts [van Wijnen et al., 1994].

antibody. The observation that there are two types of CDP/*cut* complexes that differ in electrophoretic mobility and CDC2 immunoreactivity is consistent with observations from several other cell types, including normal diploid rat osteoblasts [van Wijnen et al., 1994], WI38 fibroblasts, and ROS 17/2.8 osteosarcoma cells (M. van Gurp, J.L. Stein, G.S. Stein, and A.J. van Wijnen, unpublished observations).

We conclude that the Site II complexes in FDC-P1 cells contain CDP/*cut* as the DNA binding protein, which directly contacts the Site II sequences. Furthermore, CDP/Site II complexes from FDC-P1 cells also contain the tumor suppressor protein pRb (Fig. 1) and cyclin A [Shakoori et al., 1995], which is a modulator of cyclin-dependent kinases. The key distinction between Site II complexes is that only one type of complex contains the cyclin-dependent kinase CDC2 as an additional regulatory subunit. The differences in the composition of the two types of HiNF-D/Site II complexes suggest that these complexes may perform distinct functions in the activation or repression of H4 gene transcription at different stages during the cell cycle. In addition, the cell type-related variations in HiNF-D complexes may reflect cell type-specific differences in cell cycle mechanisms and/or cell growth phenotypic properties (Holthuis et al., 1990).

We also analyzed complexes of CDP/*cut* with a nonhistone promoter (i.e., the gp91-phox gene is expressed only in differentiated phagocytes) [Skalnik et al., 1991]. The probe used in our assays spans the two duplicated CCAAT-boxes of the gp91-phox gene and has previously been shown to bind CDP/*cut* [Skalnik et al., 1991]. Similar to the Site II cell cycle element, this nonhistone promoter fragment mediates binding of two types of complexes in nuclear extracts from FDC-P1, which form supershifts with the CDP/*cut* antibody, and only one of these types is immunoreactive with the CDC2 antibody (Fig. 1B). Strikingly, unlike the histone Site II element, neither of these two types of gp91-phox complexes is immunoreactive with the pRb antibody (Fig. 1B). These results are consistent with the observation that the interactions of CDP/*cut* proteins (e.g., from HeLa cervical carcinoma and ROS 17/2.8 osteosarcoma cells) with differentiation-specific genes, including the gp91-phox gene and the bone-related osteocalcin gene occur in conjunction with p107, rather than pRb/p105 [van Wijnen et al., 1994, 1996; unpublished data]. Taken together, our results indicate that there are at least four distinct CDP/*cut* complexes in FDC-P1 cells that differ in the type of tumor suppressor protein (pRb versus p107) and in the presence or absence of CDC2 in each complex (Fig. 1). Interestingly, a similar diversity exists in the complexes formed by E2F, which is associated with a multiplicity of different cyclins, CDK's and pRb-related proteins [Helin and Harlow, 1993; Lam and LaThangue, 1994; Claudio et al., 1994, 1996; Paggi et al., 1996]. This similarity with higher order E2F complexes raises the possibility that CDP/*cut* may function in a manner analogous to E2F by recruiting distinct cell cycle mediators (pRb proteins, cyclins, and CDK's) to cognate promoters.

Apart from binding to the histone H4 gene promoter, the HiNF-D (CDP/*cut*) complex also interacts with other cell cycle-controlled histone gene promoters [van den Ent et al., 1994; van Wijnen et al., 1994]. In each case, we observe that these complexes are associated with pRb and that the cognate promoters are all actively transcribed in proliferating cells with maximal transcription occurring during the G_1/S phase transition. By contrast, the promoters of an unrelated differentiation-specific gene (gp91-phox), which is normally not transcribed in proliferating cells, interacts with CDP/*cut* in conjunction with the pRb related protein p107 [van Wijnen et al., 1996]. These results are consistent with a functional division in the regulatory properties of CDP/*cut* complexes containing pRb versus p107.

Cell Cycle-Dependent Modifications of CDP/*cut* Interactions With Site II in FDC-P1 Cells

To assess modifications in the formation of higher-order CDP/*cut* complexes during cell cycle progression in FDC-P1 cells, we monitored the levels by gel-shift assays with nuclear proteins from cells harvested at regular intervals following release from isoleucine deprivation. Under our conditions, DNA synthesis rates in FDC-P1 cells begin to increase dramatically at 10–12 h after release, which reflects progression through the G_1/S phase transition. DNA synthesis rates are maximal at 16–20 h (Fig. 2), which indicates that the majority of the cell population is in mid-S phase during this time interval. The gel-shift assay results (Fig. 3A) show that the total levels of the pRb-related HiNF-D (CDP/*cut*) complexes with the histone

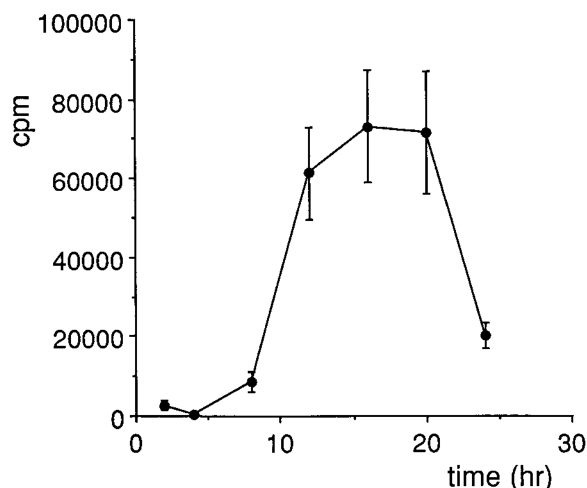

Fig. 2. DNA synthesis time course following cytokine stimulation of FDC-P1 cells after cell cycle inhibition by isoleucine deprivation. DNA synthesis rates were determined by ^{3}H-thymidine incorporation into acid-precipitable material and expressed as cpm. At 0 h, FDC-P1 cells were supplemented with amino acids in the presence of conditioned medium (WEHIcm).

H4 promoter increase dramatically when cells progress into S phase. However, FDC-P1 cells in the G_1 phase (2 h after release) show only the high-mobility complex, whereas the CDC2 containing the low-mobility complex is only present when DNA synthesis is maximal. Similarly, the p107-containing CDP/*cut* complexes with a nonhistone (gp91-phox) promoter are upregulated during S phase, and the CDC2-containing complex is detected only after the G_1/S phase transition (Fig. 3B). In contrast to the tight modulation of CDP/*cut* complexes as cells progress from G_1 to S phase, the levels of SP-1 and related proteins (e.g., SP-3) remain constitutive during cell cycle progression (Fig. 4). Also, the levels of AP-1 proteins (e.g., heterodimers formed between c-*fos,* fra-1, fra-2, c-*jun,* jun-B and jun-D) are regulated differently as compared to CDP/*cut* complexes.

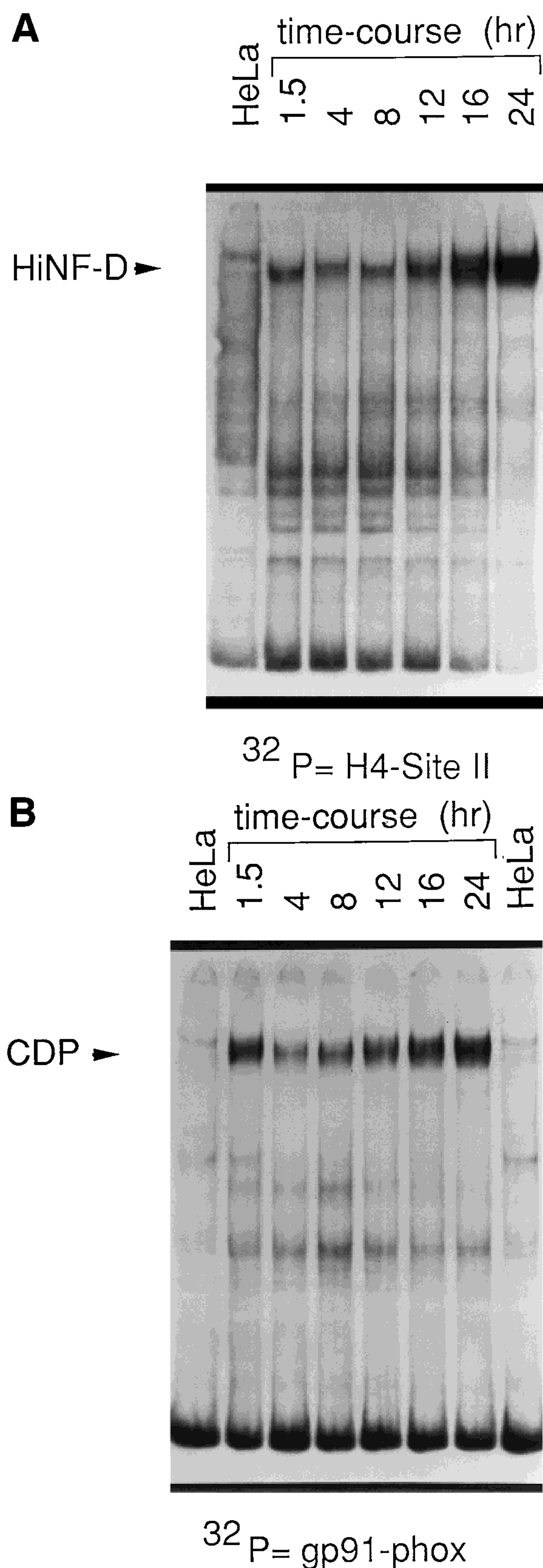

Fig. 3. Gel-shift analysis of the interactions of HiNF-D with probes spanning H4-Site II (**A**) and CDP with the gp91-phox promoter (**B**) in cytokine-stimulated FDC-P1 cells following cell growth inhibition by isoleucine deprivation. Nuclear proteins were isolated at the indicated times after refeeding growth inhibited cells in the presence of cytokine-containing conditioned medium (WEHIcm) supplemented with amino acids.

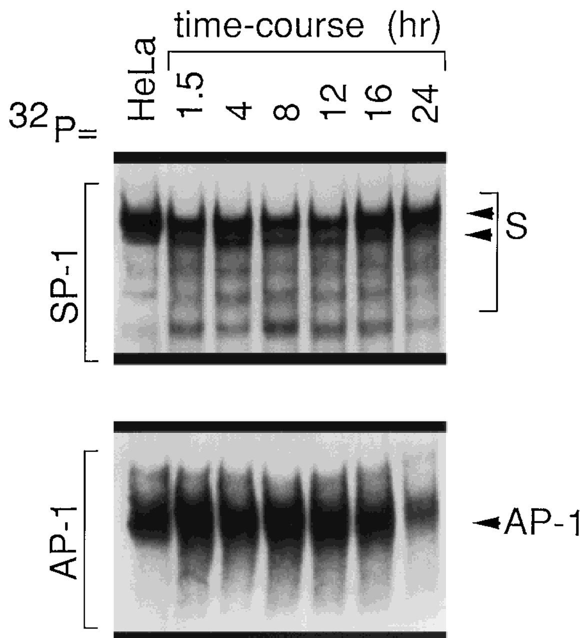

Fig. 4. Gel-shift analysis of ubiquitous SP-1, SP-3, and AP-1 binding activities following release of FDC-P1 cells from cytokine and isoleucine deprivation. Binding reactions were performed with nuclear proteins from exponentially growing HeLa cells (HeLa), or FDC-P1 cells isolated at the indicated times (hr) after cell growth stimulation (time course). (Top) The SP-1 probe (^{32}P = SP-1), mediates formation of SP-1 and SP-3 complexes (jointly designated with S and bracket on right); *double arrowhead,* complexes formed by SP-3 (*upper arrowhead*) and SP-1 (*lower arrowhead*) [Birnbaum et al., 1995]. (*Bottom*) Results obtained with the AP-1 probe; *arrowhead,* complex mediated by AP-1.

Cellular Representation of CDP/*cut* Is Constitutive During the FDC-P1 Cell Cycle

To address whether modifications in the representation of higher-order CDP/*cut* complexes are directly related to changes in the levels of the CDP/*cut* DNA binding protein during the cell cycle, we performed Western blot analysis with total cell lysates from FDC-P1 cells harvested at multiple time points after release from isoleucine deprivation (Fig. 5). We find that the levels of CDP/*cut* remain relatively constant throughout the cell cycle (Fig. 5A). Thus, changes in CDP/*cut* protein levels are not responsible for the modulations in the binding activities of the pRb and p107-related higher-order CDP/*cut* complexes during the cell cycle. It appears that the interactions of CDP/*cut* complexes with the histone gene promoter are regulated during the cell cycle by modifications in protein/protein interactions, perhaps facilitated by alterations in the phosphorylation status of CDP/*cut* or its associated subunits.

Modulations in Representation and Phosphorylation Status of Tumor Suppressors pRb/p105, pRb2/p130, and p107 During the FDC-P1 Cell Cycle

Because pRb and p107 are principal subunits of higher order CDP/*cut* complexes, we analyzed the levels of these tumor suppressors during the FDC-P1 cell cycle to assess whether the levels of pRb-related proteins are linked to modulation of CDP/*cut* binding activity. The results (Fig. 5B,C) show that the level of pRb and p107 is upregulated when FDC-P1 cells pass through the G_1/S phase transition (8–12 h after release from isoleucine block). By contrast, pRb2/p130 is present at elevated levels during G_1 and is downregulated at the G_1/S phase boundary (Fig. 5B,C). We previously showed that progression into S phase in FDC-P1 cells occurs concomitant with selective upregulation of cyclin A and CDC2, which are also key subunits of S phase-related higher-order CDP/*cut* complexes. Taken together, these data suggest that the availability of these subunits (i.e., pRb, p107, cyclin A, and CDC2) is rate-limiting for formation of higher-order CDP/*cut* complexes.

To begin to address the question whether upregulation of pRb-containing histone gene transcription factor complexes at the G_1/S phase transition is functionally coupled to modifications in the phosphorylation status of pRb, we performed western blotting analyses using electrophoretic conditions permitting resolution of the hypo- and hyperphosphorylated forms of pRb. We find that while pRb is present in G_1 phase cells at low levels, as expected the predominant pRb species is the hypophosphorylated variant (Fig. 5D). However, the elevated levels of pRb present in S phase occur primarily in the hyperphosphorylated form. Thus, modifications in pRb phosphorylation status at the G_1/S phase boundary coincide with upregulation of pRb containing CDP/*cut* transcription factor complexes. We propose that both modulation of pRb levels and phosphorylation of pRb contribute to enhanced formation of CDP/*cut* complexes with the histone H4 cell cycle domain, with consequential cell cycle stage-specific regulatory effects on histone H4 gene transcription.

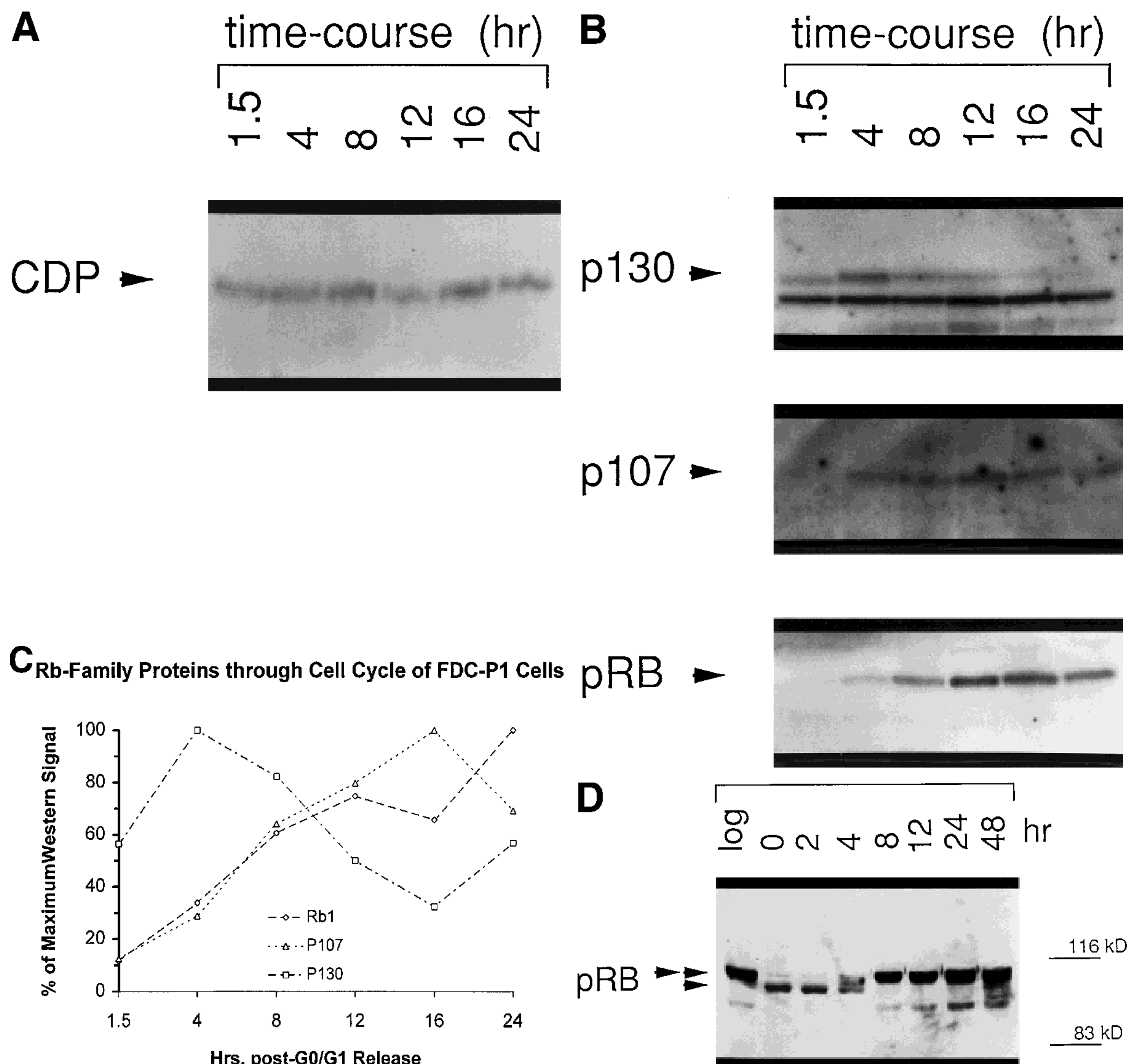

Fig. 5. Western blot analysis of CDP/*cut* (**A**) and pRb-related proteins (**B**) during the cell cycle in cytokine-stimulated FDC-P1 cells. Total cell lysates were prepared from cells isolated at the indicated times after release of the cells from isoleucine deprivation. The blots were incubated with antibodies against pRb2/p130 (*top*), p107 (*middle*) and pRb/p105 (*bottom*). **C**: Quantitation of the results presented in B. **D**: Western blot analysis of pRb/p105 during the FDC-P1 cell cycle using electrophoretic conditions permitting detection of hypophosphorylated (*single arrowhead*) and hyperphosphorylated (*double arrowhead*) pRb variants.

Modifications in p130-Associated Protein Kinase Activity at the G_1/S Phase Transition in FDC-P1 Cells

Because cell cycle-dependent modifications in the phosphorylation status of key cell cycle mediators may be important for formation of pRb-related transcription factor complexes, we initiated studies aimed at characterizing the activities of protein kinases associated with pRb related proteins. Protein kinase activity was monitored during the FDC-P1 cell cycle, using immunoprecipitations with antibodies directed against the pRb family of proteins followed by assaying protein kinase activity in the immunoprecipitate with histone H1 as the exogenous substrate. Our results indicate that pRb2/p130-associated kinase activity is upregulated during the G_1/S phase transition (Fig. 6A,B). However, this upregulation occurs while the level of pRb2/p130 is decreasing (Fig. 5B,C). This result suggests that the increase in pRb2/p130-associated protein kinase activity involves another rate-limiting component, perhaps a specific CDK inhibitor (CDI) interacting

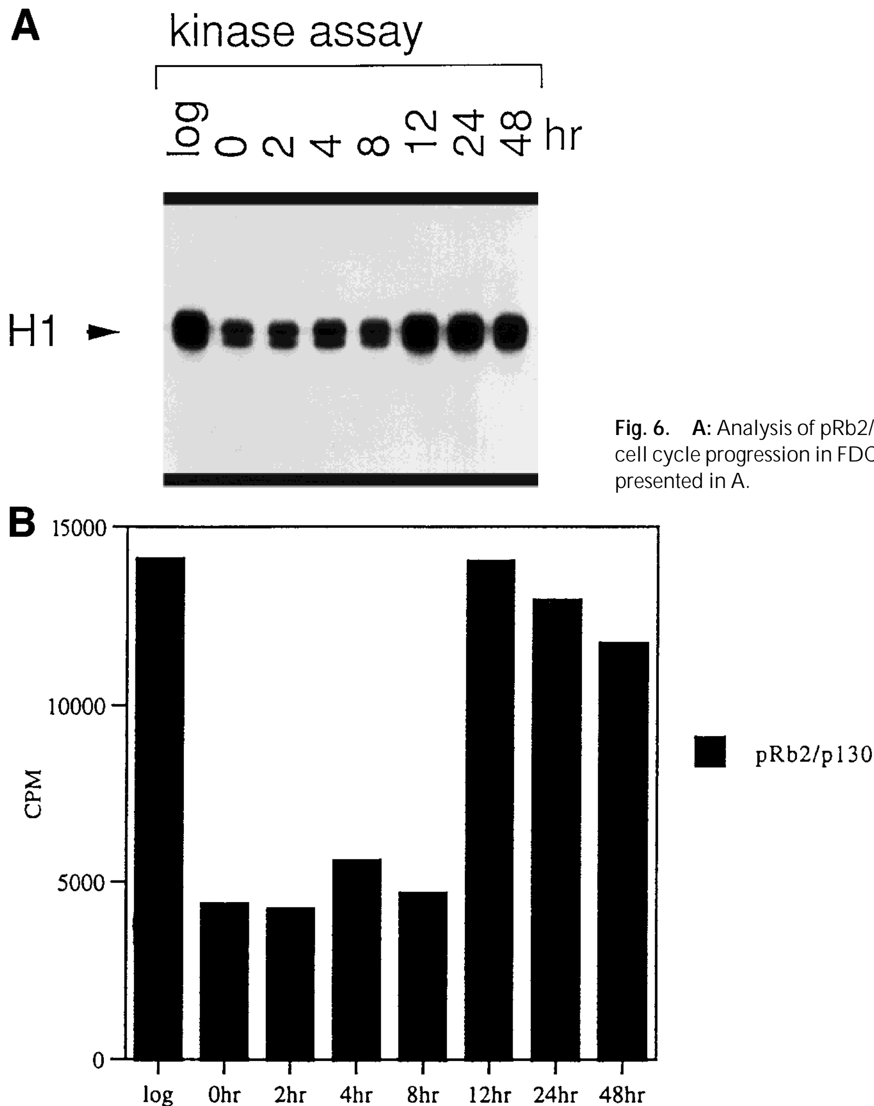

Fig. 6. **A:** Analysis of pRb2/p130-associated kinase activity during cell cycle progression in FDC-P1 cells. **B:** Quantitation of the results presented in A.

with the kinase complex. It has been shown that pRb-related proteins are associated with functional CDK/cyclin kinase complexes, and activity of these complexes is controlled by a multiplicity of CDK inhibitors (CDI's), which can physically interact with these kinase complexes [Sherr et al., 1994; Morgan et al., 1995]. We postulate that as the levels of pRb2/p130 decline modestly, the inhibitory function of the putative associated CDI is completely inactivated, resulting in a net increase in protein kinase activity at the G_1/S phase boundary. The possibility arises that the observed activation of protein kinases associated with pRb-related proteins may be functionally involved in modulating the activities of transcription factors at the G_1/S phase transition. Furthermore, these cell cycle-regulated kinase activities may be tightly controlled by the presence of CDI's in FDC-P1 cells.

CONCLUSIONS

Multiple distinct regulatory processes occur during cytokine-dependent stimulation of cell proliferation in hematopoietic progenitor cells [Carroll and May, 1994; Gold et al., 1994; Mayo et al., 1994; McCubrey et al., 1994; Quesenberry et al., 1996; Reddy et al., 1992; Redner et al., 1992]. In this and previous studies [Shakoori et al., 1995; Vaughan et al., 1995], we have monitored the regulatory mechanisms controlling histone gene transcription factors and associated regulatory proteins that mediate cell cycle progression in FDC-P1 cells following cytokine stimulation. Our results indicate that an intricate gene-regulatory program governs cell cycle

entry and subsequent traverse toward the G_1/S phase transition in FDC-P1 cells. The immediate early response following cytokine stimulation involves downregulation of the tumor suppressor protein pRb2/p130. This event may mark changes in the DNA binding activity and composition of E2F complexes observed during cell cycle entry observed in other cell types [Helin and Harlow, 1993; Lam and LaThangue, 1994; Dou et al., 1994; Li et al., 1993]. During late G_1, the activities of ATF proteins and the oncoprotein encoded transcription factors IRF2 and AP-1 are upregulated [Vaughan et al., 1995; Shakoori et al., 1995]. We postulate that these and other transcription factors which are maximal in late G_1 may drive the expression of genes required for the preparative events preceding the G_1/S phase transition.

Events critical for commitment to S phase entry in FDC-P1 cells occur at the G_1/S phase transition. We have shown that transition of the G_1/S phase boundary occurs in conjunction with upregulation of multiple cell cycle regulators, including pRb/p105, p107, cyclin A, CDC2, and CDK2, as well as recruitment of these factors to histone gene promoters in multisubunit complexes that contain the CDP/*cut* homeodomain protein as the DNA binding subunit. In addition, higher-order complexes of E2F and cyclin/CDK/pRb-related proteins are also upregulated at the G_1/S phase boundary in FDC-P1 cells [Shakoori et al., 1995]. Thus, the tumor suppressor protein pRb and p107 are recruited to G_1/S phase-specific gene promoters by both E2F- and CDP/*cut*-dependent mechanisms. This recruitment occurs in conjunction with upregulation of protein kinase activities associated with pRb-related proteins and with hyperphosphorylation of pRb. Thus, a staged cascade of cell cycle regulatory steps involving critical phosphorylation events culminates in the modulation of histone gene transcription and other G_1/S phase related genes and involves recrutement of pRb and p107 proteins to cell cycle-controlled promoters by two distinct molecular mechanisms.

In summary, we have shown that cell cycle control of histone gene transcription at the G_1/S phase transition in FDC-P1 cells is functionally linked to key cell cycle regulators that may mediate competency for cell cycle progression and differentiation in hematopoietic progenitor cells. Using several different experimental approaches, we have systematically examined the mechanisms by which histone gene regulators are recruited to Site II cell cycle control elements to modulate histone H4 gene transcription rates. The data presented in this study, as well as previous studies [Shakoori et al., 1995; Quesenberry et al., 1996] have defined the molecular events leading up to the initiation of S phase in FDC-P1 cells in response to cytokines. Through understanding of the molecular events that regulate G_1 progression, we will also gain insight into the potential targets for therapeutic manipulation of the hematopoietic system. This knowledge could ultimately lead to the development of improved transplantation strategies, effective methods for engraftment of genetically modified PHSCs, and successful treatment of hematopoietic malignancies and other dysfunctions.

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