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Cytokine Induction of Proliferation and Expression of CDC2 and Cyclin A in FDC-P1 Myeloid Hematopoietic Progenitor Cells: Regulation of Ubiquitous and Cell Cycle-Dependent Histone Gene Transcription Factors

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Abstract To evaluate transcriptional mechanisms during cytokine induction of myeloid progenitor cell proliferation, we examined the expression and activity of transcription factors that control cell cycle-dependent histone genes in interleukin-3 (IL-3)-dependent FDC-P1 cells. Histone genes are transcriptionally upregulated in response to a series of cellular regulatory signals that mediate competency for cell cycle progression at the G1/S-phase transition. We therefore focused on factors that are functionally related to activity of the principal cell cycle regulatory element of the histone H4 promoter: CDC2, cyclin A, as well as RB- and IRF-related proteins. Comparisons were made with activities of ubiquitous transcription factors that influence a broad spectrum of promoters independent of proliferation or expression of tissue-specific phenotypic properties. Northern blot analysis indicates that cellular levels of cyclin A and CDC2 mRNAs increase when DNA synthesis and H4 gene expression are initiated, supporting involvement in cell cycle progression. Using gel-shift assays, incorporating factor-specific antibody and oligonucleotide competition controls, we define three sequential periods following cytokine stimulation of FDC-P1 cells when selective upregulation of a subset of transcription factors is observed. In the initial period, the levels of SP1 and HiNF-P are moderately elevated; ATF, AP-1, and HiNF-M/IRF-2 are maximal during the second period; while E2F and HiNF-D, which contain cyclin A as a component, predominate during the third period, coinciding with maximal H4 gene expression and DNA synthesis. Differential regulation of H4 gene transcription factors following growth stimulation is consistent with a principal role of histone gene promoter elements in integrating cues from multiple signaling pathways that control cell cycle induction and progression. Regulation of transcription factors controlling histone gene promoter activity within the context of a staged cascade of responsiveness to cyclins and other physiological mediators of proliferation in FDC-P1 cells provides a paradigm for experimentally addressing interdependent cell cycle and cell growth parameters that are operative in hematopoietic stem cells.

Key words: IL-3-dependent FDC-P1 cells, histone H4 gene, cell cycle control

Gene therapy through bone marrow transplantation represents a promising strategy for treatment of many diseases. Implementation depends on an in-depth understanding of physiological parameters that support cytokine-stimulated growth of genetically altered hematopoietic progenitor cells, as well as the successful engraftment of these engineered cells in the host bone marrow cavity. Cell growth regulation of cultured bone marrow-derived cells requires the intricate interplay of both autocrine and paracrine, as well as culture condition-dependent processes [Quesenberry et al., 1994]. Cytokine stimulation of cell growth in interleukin-3

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(IL-3)-dependent FDC-P1 myeloid hematopoietic progenitor cells represents an excellent model for dissecting the growth factor-dependent mechanisms that result in commitment to induction of proliferation and cell cycle progression [Carroll and May, 1994; Dexter et al., 1980; Gold et al., 1994; Mayo et al., 1994; McCubrey et al., 1990; Reddy et al., 1992; Redner et al., 1992], including initiation of DNA synthesis at the onset of S-phase.

The progression from G1- into S-phase is regulated in part by tumor suppressor proteins, including p53, pRB, and IRF-1/IRF-2 [Levine, 1993; Hinds and Weinberg, 1994; Harada et al., *1993*], as well as by sequential combinatorial interactions between distinct cyclins, cyclin-dependent kinases, and E2F transcription factors [Farnham et al., 1993; Hunter, 1993; Marraccino et al., 1992; Krek et al., 1994; Pines, 1993; Sherr, 1993; van den Heuvel and Harlow, 1993]. The G1/S-phase transition coincides with the transcriptional activation of genes encoding enzymes necessary for DNA synthesis [Pardee, 1989; Hofbauer and Denhardt, 1991], including thymidine kinase [Dou et al., 1991, 1992, 1994; Kim and Lee, 1992; Li et al., 1993], thymidylate synthase [Horie et al., 1993; Joliff et al., 1991], dihydrofolate reductase [Slansky et al., 1993; Azizkhan et al., 1993], ribonucleotide reductase [Bjorklund et al., 1993], and DNA polymerase-α [Sudo et al., 1992; Verma et al., 1991]. Transcriptional upregulation of the five classes of histone genes (H4, H3, H2B, H2A, and H1) is required to support packaging of newly replicated DNA into chromatin [Stein et al., 1994].

Histone H4 gene expression is tightly coupled with DNA replication, and H4 mRNA levels closely parallel the rate of DNA synthesis [Stein et al., 1984; Osley, 1991]. H4 gene transcription during the cell cycle is controlled by two principal *cis*-regulatory elements: Sites I and II [Pauli et al., 1987]. Site II is required for proliferation-specific transcription and enhancement of H4 gene transcription at the G1/S-phase transition [Kroeger et al., 1987, 1994; Ramsey-Ewing et al., 1994]. Site I interacts with two general transcription factors, SP-1 and ATF-1, that together function to amplify H4 transcription rates during the cell cycle [Birnbaum et al., 1995; van Wijnen et al., 1989]. Site II is a multipartite protein/DNA interaction site for at least three distinct classes of *trans*-acting factors (HiNF-D, -M, and -P) [van Wijnen et al., 1989, 1991, 1992]. HiNF-D is a proliferation-specific multisubunit complex containing CDC2, cyclin A, and an RB-related protein [Holthuis et al., 1990; Stein et al., 1989; van Wijnen et al., 1994]. HiNF-M is identical to transcription factor IRF-2 [Vaughan et al., 1995] and may act in conjunction with IRF-1 [Harada et al., 1993; Tanaka et al., 1993], which can also bind to site II. Because both cyclin A and cyclin-dependent CDC2 kinase, as well as two classes of tumor suppressor proteins (RB and IRF) converge on the cell cycle regulatory sequences of the H4 gene, transcriptional control of this gene is directly linked to multiple phosphorylation-dependent signaling pathways mediating both cell cycle and growth control.

In this study, we have analyzed the induction of cyclin A, cyclin B, and CDC-2 in relation to both general and histone H4 gene-related transcription factors during cytokine-stimulated growth of FDC-P1 hematopoietic progenitor cells. Our results show that cytokine stimulation of FDC-P1 cells results in sequential upregulation of at least three groups of transcription factors. These regulatory events may define distinct periods of the cytokine-stimulated proliferative cycle of PDC-P1 cells that reflect both cell cycle and metabolic regulation of gene expression.

MATERIALS AND METHODS

Cell Lines and Cell Culture

FDC-P1 cells, derived from long-term bone marrow culture [Dexter et al., 1980], were maintained in RPMI 1640 with 25% WEHI-3 conditioned medium (WEHIcm) and 10% fetal calf serum (FCS). For cytokine-response studies, these cells were deprived of growth factors overnight by culture in RPMI plus 10% FCS only, then transferred into fresh medium supplemented with IL-3 or granulocyte–macrophage colony-stimulated factor (GM-CSF) at 100 U/ml each. In each case, maximal cell growth stimulation was predetermined by dose-response titration of the individual growth factor; the concentration of each factor resulting in maximal growth response was selected for use in each of these experiments.

Cell Synchronization

The FDC-P1 cell line was synchronized by isoleucine deprivation, which arrests cells in early G1-phase [Reddy et al., 1992]. Cells grow-

ing actively in early to mid log-phase were precultured for 36 h in isoleucine-deficient medium (i.e., isoleucine-deficient DMEM supplemented with 10% dialyzed WEHIcm and 10% FCS). At time zero, half of the cells were resuspended in growth medium, while the control cells were resuspended in DMEM plus 10% FCS only. Cells were grown for 48 h, during which time samples were withdrawn for RNA preparation [Sambrook et al., 1989] and analysis of ^{3}H-thymidine incorporation [Reddy et al., 1992]. Additionally, cell growth and viability were monitored at selected time points by cell counts and trypan blue exclusion.

Northern Blot Analysis

RNA samples were electrophoretically fractionated in 1% agarose gels [Sambrook et al., 1989]. The integrity and quantity of RNA samples were evaluated by ethidium bromide staining. Samples were transferred to a nylon membrane and the blots subjected to hybridization conditions as described previously [Shalhoub et al., 1989], using probes derived from cloned murine cDNAs homologous to cyclins A2 and B1 (kindly provided by Debra Wolgemuth, Columbia University) [Chapman and Wolgemuth, 1992; S. Ravkin and D.J. Wolgemuth, unpublished data] and CDC2 (generously supplied by Kerstin Nyberg, Imperial Cancer Research Fund), as well as a human H4 gene.

Gel-Shift Assays

Isolation of nuclear proteins by 0.4 M KCl extraction of nuclei, description of sequences for transcription factor oligonucleotides, and preparation of binding reactions with ^{32}P-labeled probes were documented in previous studies [van Wijnen et al., 1991, 1992]. Detection of transcription factors HiNF-M, HiNF-P, and E2F (salmon sperm DNA), HiNF-D (mixture of poly G/C and poly I/C DNA), as well as SP-1, AP-1 and ATF (poly I/C DNA) was performed in the presence of different nonspecific competitor DNAs, as indicated in parentheses. Electrophoretic fractionation of protein–DNA complexes was performed in 4% (80:1) polyacrylamide gels using 0.5 × TBE as buffer [Ausubel et al., 1987]. Gel-shift immunoassays for HiNF-D were performed with antibodies directed against CDC2 (G6) [Draetta and Beach, 1989], CDK2 [Rosenblatt et al., 1992], cyclin A (C160) [Giordano et al., 1989], and cyclin B and RB (XZ104) [Hu et al., 1991].

RESULTS AND DISCUSSION

Induction of Cyclin A, Cyclin B, and Cyclin-Dependent Kinase CDC2 During Cytokine Stimulation of FDC-P1 Cell Proliferation

Cyclins A and B are the regulatory components of multisubunit CDC2 kinase complexes, and cyclin A/CDC2 and cyclin B/CDC2 kinase complexes perform key regulatory functions during cell cycle transitions. In addition, cyclins A and B, as well as CDC2, represent cell cycle stage-specific markers reflecting progression through the S/G2/M-period of the cell cycle. Histone H4 mRNA levels were determined because H4 gene expression is restricted to the S-phase of the cell cycle [Plumb et al., 1983]. To evaluate expression of these proteins during cytokine stimulation of FDC-P1 cells, we induced FDC-P1 cell growth by treating cytokine-deprived FDC-P1 cells with either IL-3 or GM-CSF. Under these conditions, acute stimulation of FDC-P1 cell proliferation is observed in the presence of IL-3, whereas FDC-P1 cells treated with GM-CSF have a delayed proliferative response (Fig. 1).

To analyze effects of IL-3 relative to GM-CSF on cell cycle regulatory mechanisms during cytokine stimulation of FDC-P1 cell proliferation, we determined mRNA levels of cyclin A, cyclin

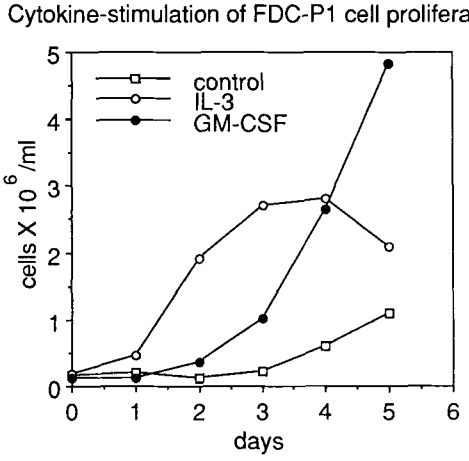

Fig. 1. Determination of FDC-P1 cell growth following cytokine stimulation of growth factor-deprived FDC-P1 cells. Cell counts were corrected for cell viability by trypan blue exclusion. At 0 hr, FDC-P1 cells were incubated in the presence of IL-3 (○) or GM-CSF (●) containing medium, or in medium not supplemented with cytokines (□).

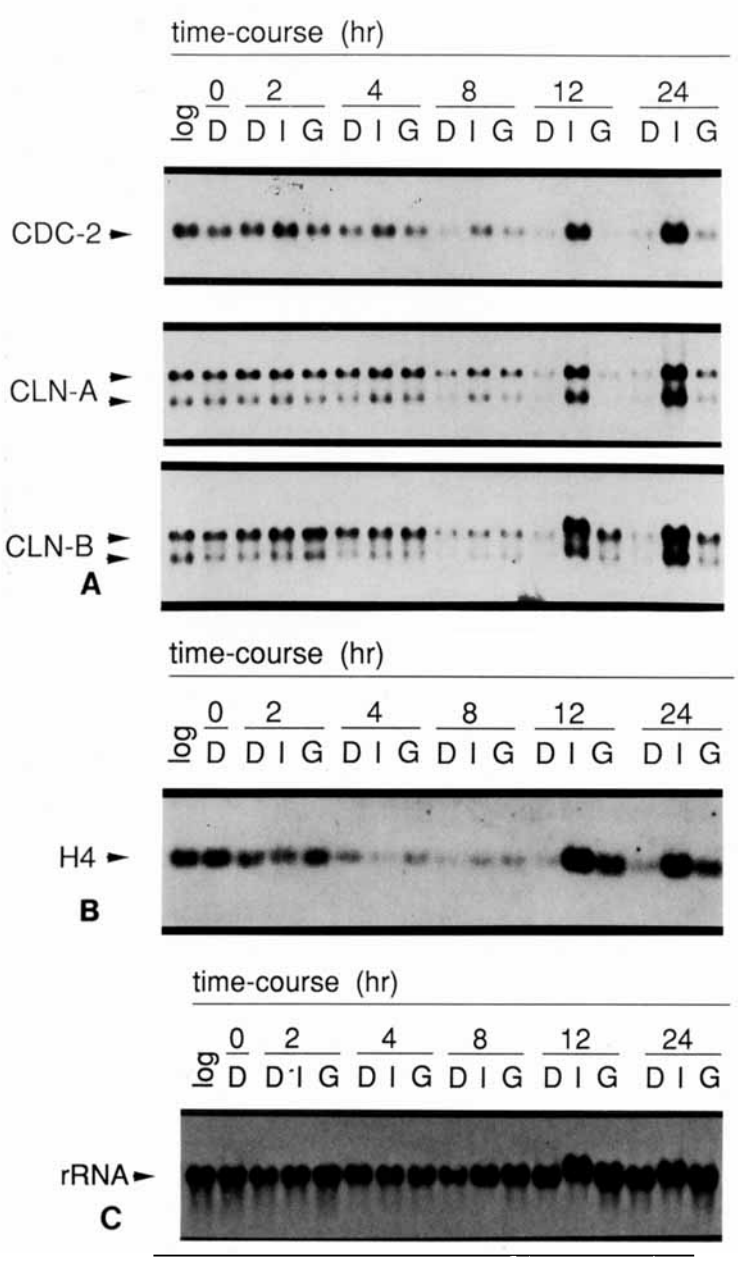

Fig. 2. Northern blot analysis of CDC2, cyclin A, and cyclin B mRNA levels (**A**), as well as H4 mRNA levels (**B**) and 18 S rRNA (**C**) in cytokine-stimulated FDC-P1 cells following release from cytokine deprivation as assessed in Figure 1. Total cellular RNA was isolated at the indicated times after refeeding cells with culture medium containing either IL-3 (I) or GM-CSF (G), or control medium that was not supplemented with cytokines (D). The cyclin A and cyclin B probes each detect two distinct mRNA species (see text for details).

B, CDC2, and histone H4 by Northern blot analysis (Fig. 2). The cyclin A probe detects two mRNAs species (3.0 and 1.7 kB) encoding the A2-type cyclin [S. Ravkin and D.J. Wolgemuth, personal communication]. Similarly, the cyclin B probe also detects two mRNA variants that encode the B1-type cyclin (2.5 and 1.7 kB); these mRNAs differ in the 3′ untranslated region, owing to usage of alternative polyadenylation signals [Chapman and Wolgemuth, 1992]. The results demonstrate that IL-3 stimulation of FDC-P1 cells results in elevation of cyclin A, cyclin B, and CDC2 levels between 12 and 24 h (Fig. 2). By contrast, at this time, no significant stimulation of these cell cycle mediators is observed in cells supplemented with GM-CSF. In IL-3-stimulated cells, induction of histone H4 gene expression, which is tightly coupled to DNA

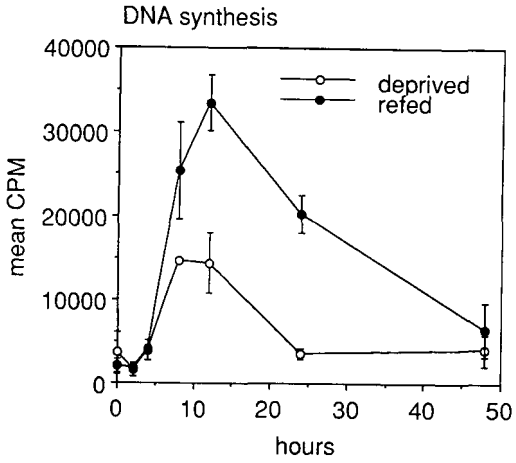

Fig. 3. DNA synthesis time course following cytokine stimulation of FDC-P1 cells after cell cycle inhibition by isoleucine deprivation. DNA synthesis rates were determined by ^{3}H-thymidine incorporation into acid-precipitable material and expressed as cpm. At 0 hr, FDC-P1 cells were supplemented with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

synthesis, occurs at the same time (at 12–24 h) when maximal levels of cyclin A and CDC2 mRNAs are observed. This result is consistent with a requirement for cyclin A and CDC2 in gene regulatory events during S-phase. We conclude that IL-3, but not GM-CSF, induces selective and immediate progression through the cell cycle with concomitant elevation of the principal components for CDC2/cyclin kinase complexes that mediate key cell cycle transitions during the S/G2/M period of the cell cycle.

Upregulation of Cyclins A and B, As Well As CDC2 During Cytokine Stimulation of FDC-P1 Cell Proliferation Following Inhibition of Cell Cycle Progression

Inhibition of cell cycle progression by isoleucine deprivation of FDC-P1 myeloid hematopoietic progenitor cells can be relieved by supplementation with amino acids and the appropriate cytokines. Under our conditions, maximal levels of DNA synthesis for cytokine-stimulated cells are observed 12–24 h after release from inhibition, based on the level of ^{3}H-thymidine uptake (Fig. 3). The timing of the peak of DNA synthesis indicates that the majority of cytokine-

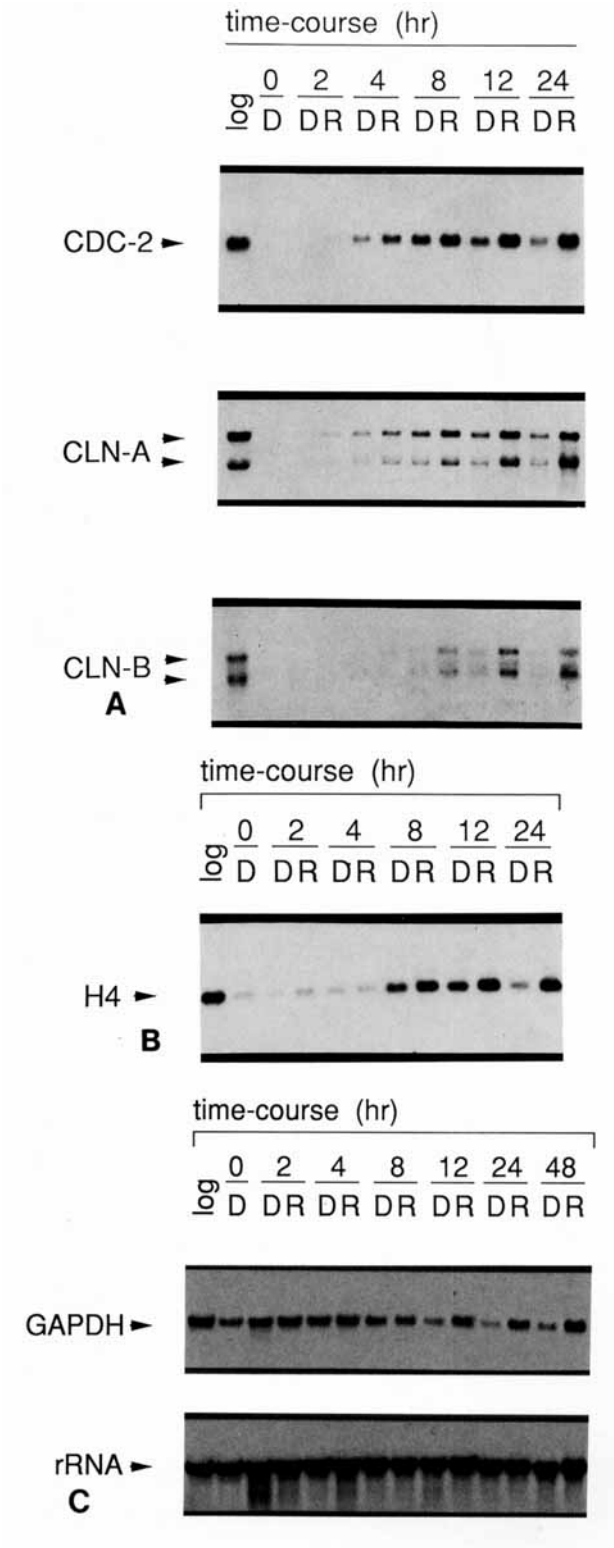

Fig. 4. Northern blot analysis of CDC2, cyclin A, and cyclin B mRNA levels (**A**), as well as histone H4 mRNA levels (**B**), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18 S rRNA (**C**) in cytokine-stimulated FDC-P1 cells following cell growth inhibition by isoleucine deprivation. Total cellular RNA was isolated at the indicated times after refeeding growth inhibited cells in the absence (D) or presence (R) of cytokine-containing conditioned medium (WEHIcm) supplemented with amino acids. The cyclin A and cyclin B probes each detect two distinct mRNA species (see text for details).

stimulated FDC-P1 cells apparently begin cell cycle progression from a period in early G1-phase. The sustained period of elevated ^{3}H-thymidine incorporation reflects growth factor-dependent but parasynchronous growth of the cell population.

To assess induction of cyclins A and B, as well as CDC2 during cell cycle progression in FDC-P1 cells, we determined the cellular representation of mRNAs for these key cell cycle mediators following release from cell growth inhibition by isoleucine deprivation. Figure 4 shows that cyc-

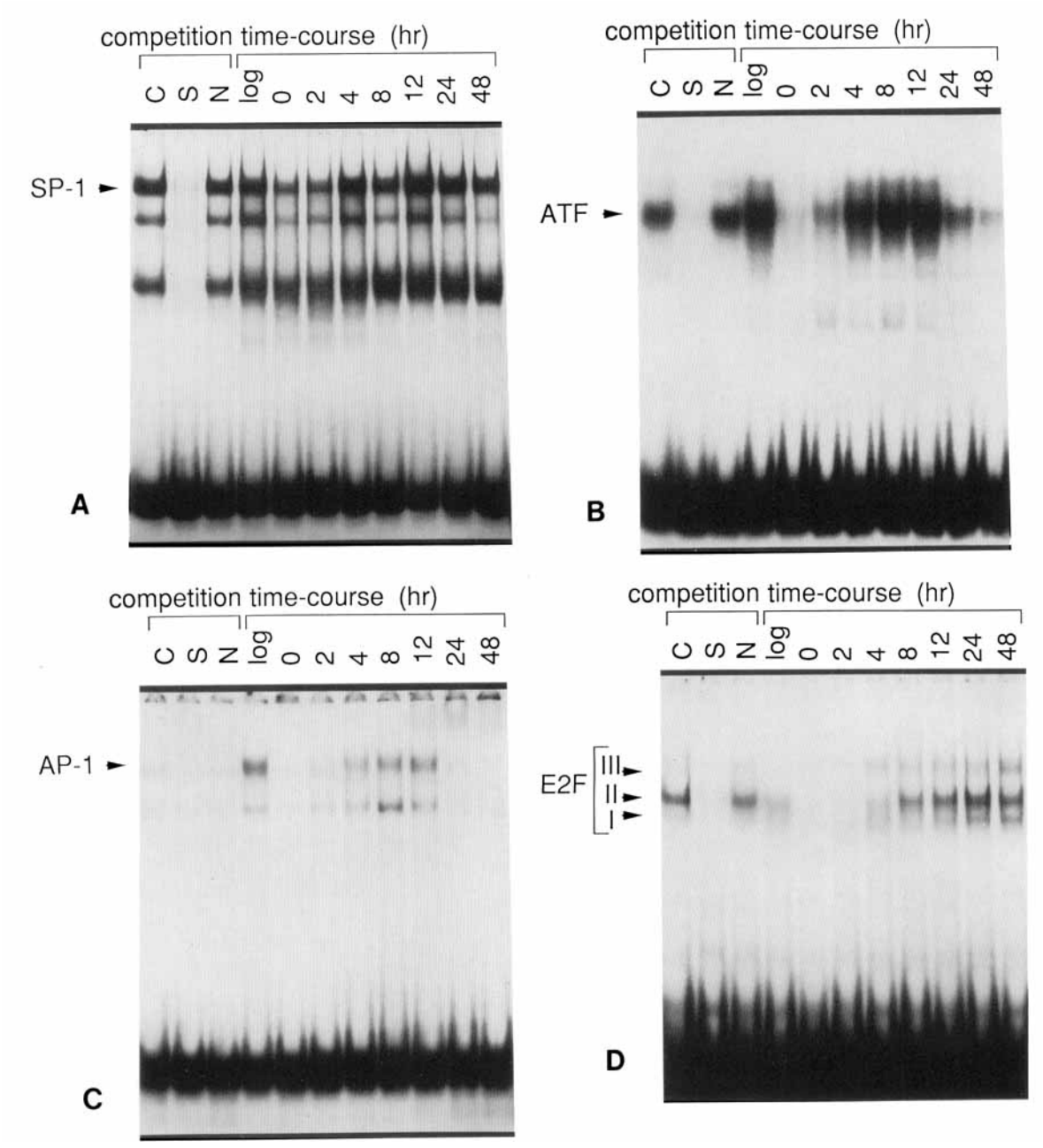

Fig. 5. Gel-shift analysis of ubiquitous SP-1, ATF, AP-1, and E2F DNA-binding activities following release from isoleucine deprivation of FDC-P1 cells. Binding reactions were performed with nuclear proteins from exponentially growing cells (log) or from cells isolated at the indicated times after cell growth stimulation (time course). **A–D:** *Lanes 1–3,* sequence-specific competition assay performed in the absence (C) or presence of either unlabeled specific (S) or nonspecific (N) oligonucleotides. *Arrowheads* identify the major complexes mediated by SP-1, ATF, and AP-1. The E2F probe interacts with three distinct E2F-related complexes, with complex II representing an E2F–cyclin A complex (see Fig. 7).

lin A and CDC2 mRNA levels begin to increase at 4 h after FDC-P1 cell growth induction, reaching maximal levels at 12–24 h. These results are consistent with the initiation of FDC-P1 cell proliferation following cytokine induction, with the concomitant upregulation of cyclin A and CDC2 at the G1/S-phase transition during the cell cycle [Marraccino et al., 1992]. Increased representation of cellular cyclin A and CDC2 mRNAs coincides with induction of H4 gene expression (Fig. 4), which parallels the rate of DNA synthesis during cell cycle progression in FDC-P1 cells (Fig. 3). Upregulation of cyclin B mRNA levels is initiated at 8 h and is maximal at 12–24 hr after cytokine stimulation (Fig. 4). Thus, the increase in the cellular levels of cyclin B mRNA in FDC-P1 cells is delayed relative to those of cyclin A and CDC2, in agreement with maximal representation of cyclin B during the S/G2/M-period of the cell cycle.

Differential Regulation of SP-1, ATF, AP-1, and E2F During Cytokine-Induced Cell Growth of FDCP-1 Cells

To assess cell growth-related modifications in the transcriptional control of gene expression operative in a broad biological context, we monitored the DNA-binding activities of several ubiquitous transcription factors by gel-shift assays. These regulatory proteins ultimately transduce, integrate, and/or amplify cytokine-stimulated signals into cell cycle-related changes in gene expression. Initially, we analyzed the binding activities of the SP-1, ATF, AP-1, and E2F transcription factor families. Each of these factors has established functions in the *trans*-activation of multiple classes of gene promoters.

After cytokine induction of FDC-P1 proliferative activity, and preceding the maximal level of H4 gene expression and DNA synthesis at 24 h (Figs. 3, 4), AP-1- and ATF-binding activities reach maximal levels at 12 h (Fig. 5). AP-1 proteins (encoded by c-FOS, FRA-1, FRA-2, c-JUN, JUN-B, and JUN-D genes) and ATF proteins (encoded by multiple ATF-, CREB-, and CREM- genes) are DNA-binding factors of the bZIP class that form selective homo- and heterodimers with members from both within and between each family. Upregulation of these factors as a consequence of cell cycle progression, but at a time preceding maximal H4 gene expression and DNA synthesis, is consistent with the concept that combinatorial interactions between AP-1 and ATF proteins may *trans*-activate a number of AP-1 and ATF responsive genes with putative functions during G1.

In contrast to AP-1 and ATF proteins, the level of E2F-binding activity reaches maximal levels at 24 h after cytokine stimulation (Fig. 5), and its modulation parallels the level of ^{3}H-thymidine incorporation (Fig. 3), as well as the mRNA levels of cyclin A and CDC-2 (Fig. 4). Transcription factor E2F is a heterodimeric protein encoded by multiple E2F- and DP-1 genes and forms higher-order complexes with RB-, cyclin-, and CDC2-related proteins. Indeed, one of the complexes detected in our gel-shift assays, which is upregulated after IL-3 stimulation, represents a cyclin A/E2F complex (Fig. 6 and data not shown). E2F factors are known to bind to *cis*-acting elements of multiple genes that are transcriptionally activated at the G1/S-phase boundary, including genes encoding thymidine kinase, dihydrofolate reductase, and CDC2. Thus, we observe apparent simultaneous upregulation of E2F binding activity and cyclin A mRNA levels, in conjunction with increased DNA synthesis in FDC-P1 cells. As all these events occur with a temporal pattern distinct of that observed for AP-1 and ATF transcription factors, these results appear to reflect modifications in ubiquitous gene regulatory mechanisms related to the G1/S-phase transition.

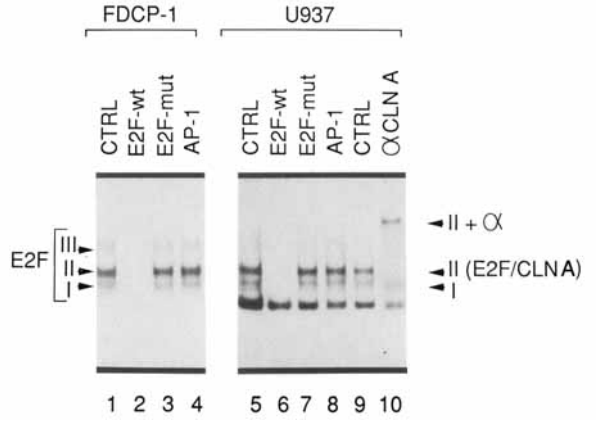

Fig. 6. Gel-shift competition and immunoassay of distinct E2F complexes. Nuclear proteins derived from mouse FDC-P1 (*lanes 1–4*) and human U937 cells (*lanes 5–10*) cells mediate several specific E2F complexes. Sequence specificity of these complexes is established by competition assays using wild-type (lanes 2, 6) and mutant (lanes 3, 7) E2F-binding site oligonucleotides, as well as a nonspecific AP-1 oligonucleotide (lanes 4, 8). Pre-incubation with a human cyclin A antibody results in a selective supershift of the human E2F complex II (lane 10) identifying this as a cyclin A–E2F complex. This antibody also reacts weakly with the murine E2F complex II from FDC-P1 cells, as reflected by partial inhibition of binding (data not shown).

SP-1 *trans*-activates a multiplicity of genes encoding proteins with both housekeeping and/or general metabolic functions. The level of SP-1-binding activity is upregulated early during the temporal sequence of cell growth regulatory processes initiated by cytokine induction of IL-3-dependent FDC-P1 cells (Fig. 5), and appears to be moderately elevated at 4 and 12 h after cytokine stimulation. These results have several implications. First, isoleucine-deprived cells, which most likely have a low metabolic rate, as compared to actively dividing cells, not only contain relatively low levels of SP-1, but also of other DNA-binding activities, including AP-1 and ATF. This observation suggests that the levels of many transcription factors may be coupled at least in part to changes in the overall rate of cell metabolism. Second, two distinct peaks for SP-1 activity during the G1-period leading up to the first entry into S-phase may reflect a biphasic response. This biphasic response may be related to (1) release of cells from a biochemical blockade that relieves inhibition of protein synthesis and most likely occurs concomitant with a general increase in cell metabolism, and (2) cytokine stimulation of cells that directly influences cell cycle regulatory events dictating metabolic rates during late G1.

In conclusion, the observation that neither SP-1 nor other DNA-binding activities remain at constant levels during cell growth stimulation of FDC-P1 cells suggests that modifications in transcription factor-mediated gene control mechanisms cannot be entirely attributed to cell

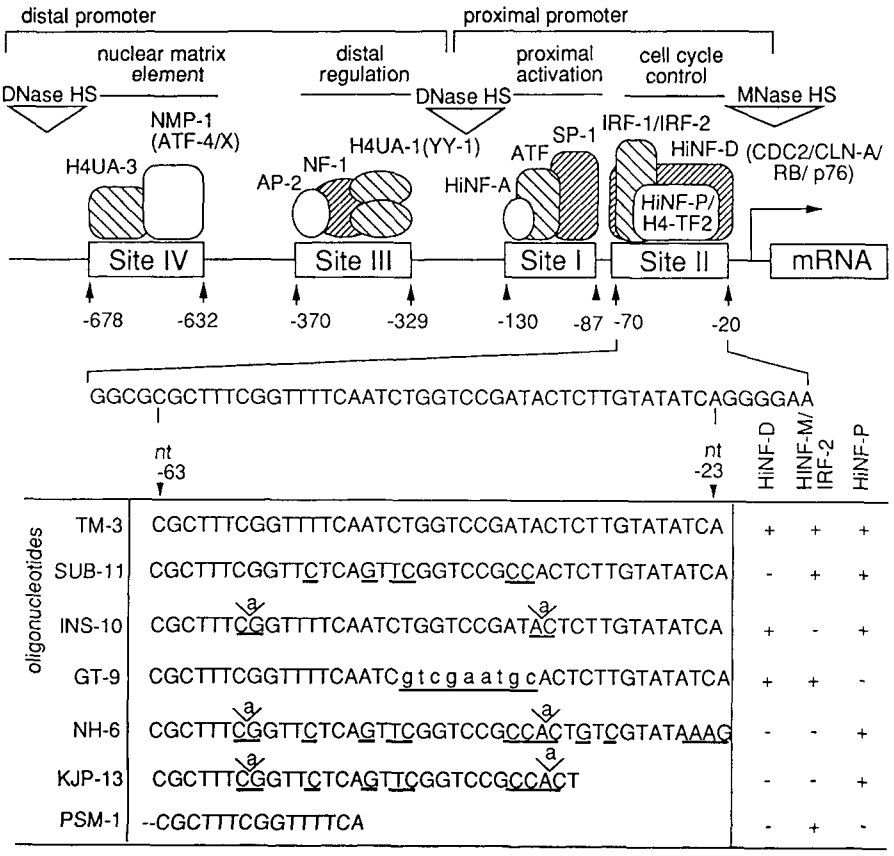

Fig. 7. Schematic representation of protein–interactions at the cell cycle regulatory element site II of the H4 gene (see text for references) [Stein et al., 1994]. Site II was originally defined as an in vivo genomic DNase I footprint, and this element is required for proliferation-specific and cell cycle-regulated transcription. Identification of individual sequence-specific interactions of HiNF-D (containing cyclin A, CDC2, and RB-related proteins), HiNF-M/IRF-2, and IRF-1 (Vaughn et al., 1995), as well as HiNF-P, were established by multiple complementary approaches, including several nuclease and chemical protection assays, nucleotide modification interference techniques, as well as mutation and competition analyses. In vivo DNase I footprints and in vivo DMS fingerprints, as well as in vitro protein–DNA contacts and minimal binding domains were established for each factor. Factor-specific mutant oligonucleotides [van Wijnen et al., 1992] used for competition analysis in this study are indicated at the bottom, with the effect of nucleotide mutations (lowercase and underlined) on the binding of HiNF-D, -M, and -P, indicated by plus (+) and minus (−) signs.

cycle regulatory events under these conditions. However, cytokine stimulation of FDC-P1 cell growth after isoleucine deprivation clearly results in the sequential upregulation of three temporal groups of transcription factor-binding activities: class I, early, SP1; class II, intermediate, AP-1 and ATF; and class III, late, E2F.

Cytokine-Dependent Stimulation of Histone Gene Transcription Factors Controlling the Histone Gene Cell Cycle Regulatory Element

The cell cycle regulatory element, site II, of the DNA replication-dependent histone H4 gene interacts with at least three key nuclear factors, HiNF-D, -M, and -P, that bind in overlapping arrangements to highly conserved promoter sequences (Figs. 7, 8). During FDC-P1 cell growth stimulation, the level of HiNF-P remains relatively constant, with modest upregulation of HiNF-P activity occurring at 4–12 h after cytokine-dependent release from isoleucine deprivation (Fig. 9). Hence, similar to SP-1, HiNF-P represents a class I ("early") binding activity. We have previously shown that HiNF-P is downregulated upon cell density-induced cell growth inhibition of ROS 17/2.8 osteosarcoma cells and during differentiation of rat osteoblasts [van den Ent et al., 1993], suggesting that this DNA-binding activity is regulated with respect to long-term cessation of cell growth. By contrast, detection of HiNF-P in isoleucine-deprived FDC-P1 hematopoietic progenitor cells (Fig. 9) indicates that temporary inhibition of cell growth apparently does not result in complete downregulation of HiNF-P.

Factor HiNF-M recognizes a regulatory element in the distal segment of site II that is critical to G1/S-phase enhancement of H4 transcription. HiNF-M was originally defined in HeLa cervical carcinoma cells [van Wijnen et al., 1991] and after purification to homogeneity shown to be identical to IRF-2 (Vaughan et al., 1995). IRF-2 recognizes the same sequence as tumor suppressor protein IRF-1 [Tanaka et al., 1993]. Nuclear proteins from FDC-P1 cells mediate two types of complexes (M1 and M2) with site II that have competition characteristics indistinguishable from HiNF-M (Fig. 8). Based on the gel-shift results with growth-stimulated FDC-P1 cells (Fig. 9), the HiNF-M1 variant appears to represent a class I protein, whereas HiNF-M2 is a class II protein. The temporal levels of HiNF-M1- and HiNF-M2-binding activities are comparable to modulations in the levels

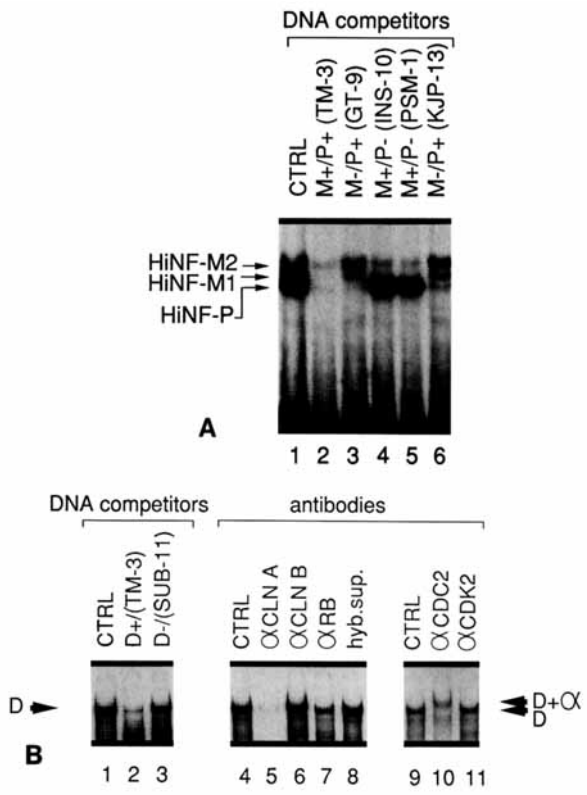

Fig. 8. Identification of protein–DNA complexes formed by HiNF-P and the IRF-related protein HiNF-M, as well as the cyclin A/CDC2/RB-containing multisubunit protein HiNF-D in FDC-P1 cells. Sequence-specific competition assays for identification of HiNF-P, -M, and -D were performed with a diagnostic panel of factor-specific mutant oligonucleotides (refer to Fig. 7), indicated above the lanes in A and B. **A:** Presence of a single HiNF–P complex and two HiNF–M complexes (designated HiNF-M1 and HiNF-M2). HiNF-M1- and HiNF-M2-binding activities are regulated differently (see Fig. 9), and the pattern of regulation is consistent with these factors, representing IRF-2 and IRF-1, respectively. **B:** Gel-shift immunoassay using CDC2 (G6), cyclin A (C160), and RB (XZ104) antibodies to demonstrate presence of these proteins in the multisubunit protein HiNF-D-derived form murine FDC-P1 myeloid progenitor cells. The presence of these key cell cycle mediators in HiNF-D was previously established in human HeLa cervical carcinoma cells, as well as a broad spectrum of mammalian nonhematopoietic cell types [van Wijnen et al., 1994].

of IRF-2 and IRF-1, respectively, that have been observed during cell growth stimulation in other cell culture systems [Harada et al., 1993].

Factor HiNF-D is a cell growth and cell cycle-regulated multisubunit complex containing cyclin A, CDC2, and an RB-related protein (Fig. 8) [van Wijnen et al., 1994]. Maximal levels of HiNF-D are observed at 24 h (Fig. 9) when the level of DNA synthesis (Fig. 3); levels of histone H4, cyclin A, and CDC2 mRNAs (Fig. 4); as well as the binding activity of the cyclin A/E2F complex (Fig. 5) are elevated. Thus, HiNF-D is cat-

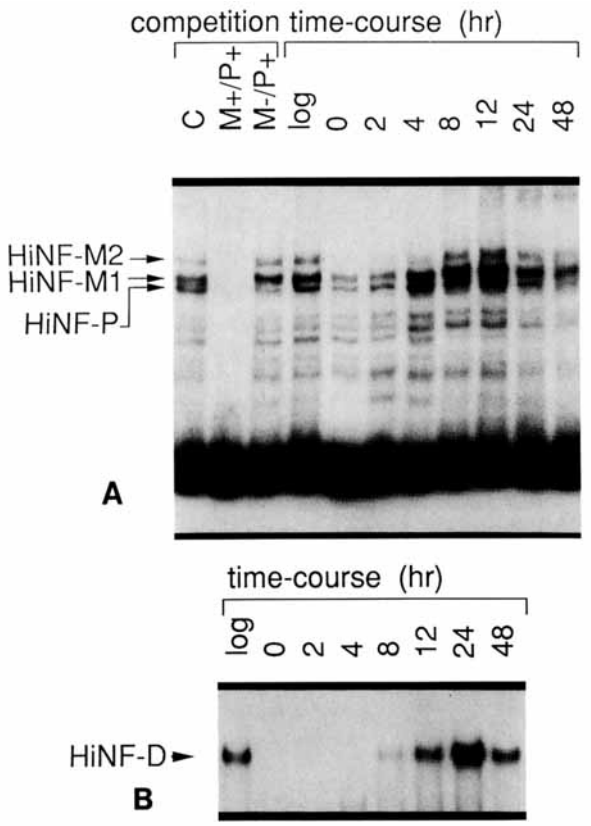

Fig. 9. Gel-shift analysis of histone gene transcription factors HiNF-P, HiNF-M, and HiNF-D following release from isoleucine deprivation of FDC-P1 cells. Binding reactions were performed with nuclear proteins from exponentially growing cells (log) or from cells isolated at the indicated times after cell growth stimulation (time course). **A:** *lanes 1–3,* sequence-specific competition assay performed in the absence (C) or presence of either unlabeled mutant M+/P+ (TM-3) or mutant M−/P+ (INS-10) oligonucleotides as described in Figs. 7 and 8. The complexes mediated by HiNF-M (represented in FDC-P1 cells as complexes HiNF-M1 and HiNF-M2; see Fig. 8) and HiNF-P (**A**), as well as HiNF-D (**B**) are indicated by *arrows.*

egorized as a class III binding activity. Consistent with previous data showing modulation of HiNF-D-binding activity during the cell cycle with respect to S-phase in normal diploid cells, but not in tumor-derived and transformed cell types [Holthuis et al., 1990], the observation that HiNF-D is regulated with respect S-phase in FDC-P1 cells during cytokine-stimulated proliferation suggests that these myeloid hematopoietic progenitor cells have retained growth- and cell cycle-related phenotypic properties of the normal diploid cell.

Interestingly, the temporal regulation of the three factors (HiNF-D, -M, and -P) interacting with the site II cell cycle regulatory sequences of the H4 gene is different in each case. This differential regulation of H4 gene transcription factors during cytokine stimulation of FDC-P1 proliferation suggests that the site II cell cycle element represents a molecular integrator of multiple cell cycle and cell growth-related signaling pathways accommodating cell stage-specific requirements for histone H4 gene transcription.

CONCLUSION

This study shows that cytokine stimulation of cell growth in the IL-3-dependent myeloid line FDC-P1 after isoleucine deprivation results in a sequential series of events that involve temporal modulations in at least three classes of transcription factor-binding activities, as well as the regulation of distinct cyclins and of cyclin-dependent kinases. These findings will provide an experimental framework for studies aimed at understanding the precise growth factor-dependent mechanisms that determine commitment to cell proliferation and support competency for cell cycle progression in hematopoietic cells. In particular, the definition of physiological conditions that permit the proliferation of genetically altered hematopoietic progenitor cells, but that do not preclude engraftment of these cells in the host, would provide an experimental foundation for gene therapy through bone marrow transplantation.

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REFERENCES

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) (1987): "Current Protocols in Molecular Biology." New York: John Wiley & Sons.

Azizkhan JC, Jensen DE, Pierce AJ, Wade M (1993): Transcription from TATA-less promoters: Dihydrofolate reductase as a model. Crit Rev Eukaryot Gene Expr 3:229–254.

Birnbaum MJ, Wright KL, van Wijnen AJ, Ramsey-Ewing AL, Bourke MT, Last TJ, Aziz F, Frenkel B, Aronin N, Stein GS, Stein JL (1995): The proximal promoter element required for maximal transcription of a human cell

cycle regulated H4 histone gene: A dominant role for Sp1. Biochemistry 36:7968–7658.

Bjorklund S, Hjortsberg K, Johansson E, Thelander L (1993): Structure and promoter characterization of the gene encoding the large subunit (R1 protein) of mouse ribonucleotide reductase. Proc Natl Acad Sci USA 90:11322–11326.

Carroll MP, May WS (1994): Protein kinase C mediated serine phosphorylation directly activates Raf-1 in murine hematopoietic cells. J Biol Chem 269:1249–1256.

Chapman DL, Wolgemuth DJ (1992): Identification of a mouse B-type cyclin which exhibits developmentally regulated expression in the germ line. Mol Reprod Devel 33: 259–269.

Dexter TM, Garland J, Scott D, Scolnick E, Metcalf D (1980): Growth factor dependent hemopoietic precursor cell lines. J Exp Med 152:1036–1047.

Dou Q-P, Fridovich-Keil JL, Pardee AB (1991): Inducible proteins binding to the murine thymidine kinase promoter in late G1/S phase. Proc Natl Acad Sci USA 88: 1157–1161.

Dou Q-P, Markell PJ, Pardee AB (1992): Retinoblastoma-like protein and cdc2 kinase are in complexes that regulate a G1/S event. Proc Natl Acad Sci USA 89:3256–3260.

Dou Q-P, Zhao S, Levin AH, Wang J, Helin K, Pardee AB (1994): G1/S regulated E2F containing complexes bind to the mouse thymidine kinase gene. J Biol Chem 269:1306–1313.

Draetta G, Beach D (1988): Activation of cdc2 protein kinase during mitosis in human cells: Cell cycle dependent phosphorylation and subunit rearrangement. Cell 54:17–26.

Farnham P, Slansky, JE, Kollmar R (1993): The role of E2F in the mammalian cell cycle. Biochim Biophys Acta 155: 125–131.

Giordano A, Whyte P, Harlow E, Franza BR Jr, Beach D, Draetta G (1989): A 60 kD cdc2-associated polypeptide complexes with the E1A proteins in adenovirus-infected cells. Cell 58:981–990.

Gold MR, Duronio V, Saxena SP, Schrader JW, Aebersold R (1994): Multiple cytokines activate phosphatidylinositol 3-kinase in hemopoietic cells: Association of the enzyme with various tyrosine phosphorylated proteins. J Biol Chem 269:5403–5412.

Harada H, Kitagawa M, Tanaka N, Yamamoto H, Harada K, Ishihara M, Taniguchi T (1993): Anti-oncogenic and oncogenic potentials of interferon regulatory factors-1 and -2. Science 259:971–974.

Hinds PW, Weinberg RA (1994): Tumor suppressor genes. Curr Opin Genet Dev 4:135–141.

Hofbauer R, Denhardt DT (1991): Cell cycle regulated and proliferation stimulus responsive genes. Crit Rev Eukaryot Gene Expr 1:247–300.

Holthuis J, Owen TA, van Wijnen AJ, Wright KL, Ramsey-Ewing A, Kennedy MB, Carter R, Cosenza SC, Soprano KJ, Lian JB, Stein JL, Stein GS (1990): Tumor cells exhibit deregulation to the cell cycle human histone gene promoter factor HiNF-D. Science 247:1454–1457.

Horie N, Chimoto M, Nozawa R, Takeishi K (1993): Characterization of regulatory sequences and nuclear factors that function in cooperation with the promoter of the human thymidylate synthase gene. Biochim Biophys Acta 1216:409–416.

Hu Q, Bautista C, Edwards G, Defeo-Jones D, Jones R, Harlow E (1991): Antibodies specific for the human retinoblastoma protein identify a family of related polypeptides. Mol Cell Biol 11:5792–5799.

Joliff K, Li Y, Johnson LF (1991): Multiple protein/DNA interactions in the TATAA less mouse thymidylate synthase promoter. Nucleic Acids Res 19:2267–2274.

Kim YK, Lee AS (1992): Identification of a protein binding site in the promoter of the human thymidine kinase gene required for the G1-S-regulated transcription. J Biol Chem 267:2723–2727.

Krek W, Ewen ME, Shirodkar S, Arany Z, Kaelin WG Jr, Livingston DM (1994): Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell 78:161–172.

Kroeger P, Stewart C, Schaap T, van Wijnen A, Hirshman J, Helms S, Stein G, Stein J (1987): Proximal and distal regulatory elements that influence in vivo expression of a cell cycle-dependent human H4 histone gene. Proc Natl Acad Sci USA 84:3982–3986.

Kroeger PE, van Wijnen AJ, Pauli U, Wright KL, Stein GS, Stein JL (1994): In vivo occupancy of histone gene proximal promoter elements reflects copy-number dependent titratable transactivation factors and cross-species compatibility of regulatory sequences. J Cell Biochem 57:191–207.

Levine AJ (1993): The tumor suppressor genes. Annu Rev Biochem 62:623–651.

Li L, Naeve GS, Lee AS (1993): Temporal regulation of cyclin A-p107 and p33^{cdk2} complexes binding to a human thymidine kinase promoter element for G_1-S phase transcriptional regulation. Proc Natl Acad Sci USA 90:3554–3558.

Marraccino RL, Firpo EJ, Roberts JM (1992): Activation of the p34 CDC2 protein kinase at the start of S phase in the human cell cycle. Mol Biol Cell 3:389–401.

Mayo MW, Steelman LS, McCubrey JA (1994): Phorbol esters support the proliferation of a hematopoietic cell line by upregulating c-jun expression. Oncogene 9:1999–2008.

McCubrey JA, Steelman LS, Sandlin G, Riddle RS, Ways DK (1990): Effects of phorbol esters on an interleukin-3-dependent cell line. Blood 76:63–72.

Osley MA (1991): The regulation of histone synthesis in the cell cycle. Annu Rev Biochem 60:827–861.

Pardee A (1989): G1 events and regulation of cell proliferation. Science 246:603–608.

Pauli U, Chrysogelos S, Stein G, Stein J, Nick H (1987): Protein-DNA interactions in vivo upstream of a cell cycle-regulated human H4 histone gene. Science 236:1308–1311.

Pines J (1993): Cyclins and cyclin-dependent kinases: Take your partners. Trends Biochem Sci 18:195–197.

Plumb M, Stein J, Stein G (1983): Coordinate regulation of multiple histone mRNAs during the cell cycle in HeLa cells. Nucleic Acids Res 11:2391–2410.

Quesenberry PJ, Ramshaw H, Peters S, Rao S, Tiarks C, Lowry P, Stewart M, Becker P, Stein G (1994). In Nance ST (ed): "Blood Supply: Risks, Perceptions and Prospects for the Future." Bethesda, MD: American Association of Blood Banks, pp 79–94.

Ramsey-Ewing AL, van Wijnen AJ, Stein GS, Stein JL (1994): Delineation of the cell cycle element of human H4 histone gene transcription. Proc Natl Acad Sci USA 91: 4475–4479.

Reddy GPV, Reed WC, Deacon DH, Quesenberry PJ (1992): Growth factor-dependent proliferative stimulation of hematopoietic cells is associated with modulation of cytoplasmic and nuclear 68 kD calmodulin-binding protein. Blood 79:1946–1955.

Redner RL, Lee AW-M, Osawa GA, Nienhuis AW (1992): Variable patterns of jun and fos gene expression in different hematopoietic cell lines during interleukin 3-induced entry into the cell cycle. Oncogene 7:43–50.

Rosenblatt J, Gu Y, Morgan DO (1992): Human cyclin-dependent kinase 2 is activated during the S and G2 phases of the cell cycle and associates with cyclin A. Proc Natl Acad Sci USA 89:2824–2828.

Sambrook J, Fritsch EF, Maniatis T (eds) (1989): "Molecular Cloning: A Laboratory Manual." 2nd Ed., Vol. 1. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 7.15–7.15.

Shalhoub V, Gerstenfeld L, Collart D, Lian JB, Stein GS (1989): Downregulation of cell growth and cell cycle regulated genes during chick osteoblast differentiation with the reciprocal expression of histone gene variants. Biochemistry 28:5318–5322.

Slansky JE, Li Y, Kaelin G, Farnham PJ (1993): A protein synthesis dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. Mol Cell Biol 13:1610–1618.

Stein GS, Stein JL, Marzluff WF (eds) (1984): "Histone Genes." New York: John Wiley & Sons.

Stein G, Lian J, Stein J, Briggs R, Shalhoub V, Wright K, Pauli U, van Wijnen A (1989): Altered binding of human histone gene transcription factors during the shutdown of proliferation and onset of differentiation in HL-60 cells. Proc Natl Acad Sci USA 86:1865–1869.

Stein GS, Stein JL, van Wijnen AJ, Lian JB (1994): Histone gene transcription: A model for responsiveness to an integrated series of regulatory signals mediating cell cycle control and proliferation/differentiation. J Cell Biochem 54:393–404.

Sudo T, Miyazawa H, Hanaoke F, Ishii S (1992): The c-myb proto-oncogene product binds to but does not activate the promoter of the DNA polymerase alpha gene. Oncogene 7:1999–2006.

Tanaka N, Kawakami T, Taniguchi T (1993): Recognition DNA sequences of interferon regulatory factor 1, regulators of cell growth and the interferon system. Mol Cell Biol 13:4531–4538.

van den Ent FMI, van Wijnen AJ, Lian JB, Stein JL, Stein GS (1993): Concerted control of multiple histone promoter factors during cell density inhibition of proliferation in osteosarcoma cells: Reciprocal regulation of cell cycle controlled and bone-related genes. Cancer Res 53: 2399–2409.

van den Heuvel S, Harlow E (1993): Distinct roles for cyclin dependent kinases in cell cycle control. Science 262:2050–2054.

van Wijnen AJ, Wright KL, Lian JB, Stein JL, Stein GS (1989): Human H4 histone gene transcription requires the proliferation-specific nuclear factor HiNF-D: Auxiliary roles for HiNF-C (Sp1-like) and HiNF-A (high mobility group-like). J Biol Chem, 264:15034–15042.

van Wijnen AJ, Ramsey-Ewing AL, Bortell R, Owen TA, Lian JB, Stein JL, Stein GS (1991): Transcriptional element H4-Site II of cell cycle regulated human H4 histone gene transcription is a multipartite protein/DNA interaction site for factors HiNF-D, HiNF-M and HiNF-P: Involvement of phosphorylation. J Cell Biochem 46:174–189.

van Wijnen AJ, van den Ent FMI, Lian JB, Stein JL, Stein GS (1992): Overlapping and CpG methylation-sensitive protein–DNA interactions at the histone H4 transcriptional cell cycle domain: Distinctions between two human H4 gene promoters. Mol Cell Biol 12:3273–3287.

van Wijnen AJ, Aziz F, Grana X, 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 RB related proteins. Proc Natl Acad Sci USA 91:12882–12886.

Vaughn PS, Aziz F, van Wijnen AJ, Wu S, Harada H, Tauiguchi T, Soprano KJ, Stein GS, Stein JL (1995) Activation of a cell cycle regulated histone gene by the oncogenic transcription factor IRF2. Nature, in press.

Verma R, Patapoutian A, Gordon CB, Campbell JL (1991): Identification and purification of a factor that binds to the MluI cell cycle box of yeast DNA replication genes. Proc Natl Acad Sci USA 88:7155–7159.