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Modifications of protein–DNA interactions in the proximal promoter of a cell-growth-regulated histone gene during onset and progression of osteoblast differentiation

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ABSTRACT **A temporal sequence of interrelated cellular, biochemical, and molecular events which occurs during the progressive expression of the differentiated osteoblast phenotype in primary cultures of fetal rat calvarial cells results in the development of a bone-tissue-like organization. This ordered developmental sequence encompasses three periods: proliferation, matrix maturation, and mineralization. Initially, the cells actively proliferate and synthesize type I collagen. This is followed by a period of matrix organization and maturation and then by a period of extracellular matrix mineralization. At the completion of proliferation, when expression of osteoblast phenotype markers such as alkaline phosphatase is observed, the cell-cycle-related histone genes are down-regulated transcriptionally, suggesting that a key signaling mechanism at this transition point involves modifications of protein–DNA interactions in the regulatory elements of these growth-regulated genes. Our results demonstrate that there is a selective loss of interaction of the promoter binding factor HiNF-D with the site II region of an H4 histone gene proximal promoter that regulates the specificity and level of transcription only when the down-regulation of proliferation is accompanied by modifications in the extracellular matrix that contribute to progression of osteoblast differentiation. Thus, this specific loss of protein–DNA interaction serves as a marker for a key transition point in the osteoblast developmental sequence, where the down-regulation of proliferation is functionally coupled to the appearance of osteoblast phenotypic properties associated with the organization and maturation of an extracellular matrix that becomes competent to mineralize.**

Defining the cellular, biochemical, and molecular events associated with osteoblast development and differentiation can provide valuable understanding of bone formation and a broad spectrum of bone-related diseases. Primary cultures of osteoblasts isolated from the calvaria of fetal rats undergo during a 35-day period an ordered developmental sequence in which the cells progressively acquire the phenotypic properties of mature osteoblasts in a mineralized extracellular matrix having a bone-tissue-like organization (1–4). The temporal expression of genes observed in cultured osteoblasts (3, 5, 6) is similar to that during fetal formation of the calvarium in intact animals (7), supporting the biological relevance of the culture system. This series of changes may be used to define three periods during the development of the osteoblast phenotype where, in this normal diploid cell culture system, the regulatory mechanisms operative in the relationship between proliferation and differentiation can be expected to be retained. The osteoblasts undergo proliferation during the initial period following isolation of primary cultures, as reflected by [^{3}H]thymidine incorporation and H4 histone gene expression, which parallel DNA synthesis. Expression of type I collagen genes during the proliferative period results in production of the characteristic osteoblast extracellular matrix. The decline in proliferative activity is accompanied by a reciprocal increase in expression of genes such as alkaline phosphatase, marking a transition point between proliferation and the initial events related to extracellular matrix maturation and specialization. A second transition point is associated with extracellular matrix mineralization and induction of the bone-specific protein osteocalcin (8). This second transition point represents a restriction point in the osteoblast developmental sequence that was experimentally defined by culture conditions in which the onset of extracellular matrix mineralization and mineralization-related gene expression were coordinately inhibited (3). Understanding molecular mechanisms that are operative during these periods of the developmental sequence and, in particular, at the transition points, can provide insight into the relationship of cell growth to expression of the bone cell phenotype.

Regulation of histone gene expression is fundamental to the control of cell proliferation because of the stringent requirement for histone proteins to package newly replicated DNA into nucleosomes (9, 10). This relationship is supported by the coordinate expression of the cell-cycle-regulated core (H2A, H2B, H3, H4) and H1 histone genes in a manner that is tightly coupled with DNA replication (11, 12). Equally important, the down-regulation of the cell-growth-related histone genes at the completion of the proliferative period when expression of genes associated with the organization and maturation of the extracellular matrix is initiated may reflect an important regulatory event at this transition point in the developmental sequence. We have previously shown in cultured osteoblasts that the down-regulation of histone gene expression which occurs with the cessation of proliferation is transcriptionally mediated, since the decreased cellular levels of histone mRNA parallel the decline in histone gene mRNA synthesis (4). Thus, a key signaling mechanism associated with the down-regulation of cell proliferation genes at the transition point early during the osteoblast developmental sequence when expression of genes related to extracellular matrix maturation and specialization is up-regulated is one that impinges on regulatory sequences that influence the interactions of transcription factors with cis-acting promoter elements. To better understand the molecular mechanisms by which the expression of cell-growth-regulated genes is down-regulated at this transition point, we examined modifications in protein–DNA interactions that occur in the proximal promoter region of a cell-cycle-regulated H4 histone gene. Here, we report that with the onset of osteoblast extracellular matrix maturation and specialization, there is a selective loss of interaction of a

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Abbreviation: nt, nucleotides.

specific promoter binding factor, HiNF-D, with a region of the proximal promoter of this cell-growth-controlled histone gene that regulates its expression.

MATERIALS AND METHODS

Cell Culture and Growth Conditions. Osteoblasts were derived from the calvaria of 21-day fetal rats and maintained in culture as previously described (3, 6). In cell cycle synchronization studies, osteoblasts were synchronized with respect to the cell cycle by double thymidine block 2 days after plating and their growth was arrested in S phase by addition of 1 mM hydroxyurea (13, 14).

Biochemical Assays. DNA synthesis was monitored after a 1-hr pulse-labeling with [^{3}H]thymidine (10 μCi/ml, 20 Ci/mmol; Amersham; 1 Ci = 37 GBq) by analyzing either trichloroacetic acid-precipitable radioactive material or labeled nuclei after *in situ* autoradiography (15). Total DNA, alkaline phosphatase activity, media osteocalcin, and total calcium accumulation were analyzed as previously described (3, 6, 16, 17). For DNA, alkaline phosphatase, osteocalcin, and total calcium determinations on each indicated day after seeding, the value represents the mean of three independent samples.

RNA Isolation and Analysis. Total cellular RNA was generally prepared by the SDS/proteinase K method (11). However, in all cases in which nuclear protein preparations were made, total cytoplasmic RNA was precipitated from the supernatant of the first cell lysis after addition of 10 mM vanadyl ribonucleoside complex by addition of LiCl/urea as described (18). RNA was quantitated by absorbance at 260 nm and its integrity was confirmed by ethidium bromide staining after electrophoresis in a 6.6% formaldehyde/1% agarose gel. RNA fractionated on such gels was transferred to Zeta-Probe membrane (Bio-Rad) in 20× SSC (1× SSC is 150 mM NaCl/15 mM sodium citrate, pH 7) by the capillary method of Thomas (19). In other cases, RNA samples were bound to Zeta-Probe blotting membrane by slot blot under conditions described by the apparatus manufacturer (Schleicher & Schuell). All filters were hybridized under high-stringency conditions to a rat H4 histone gene [a 440-base-pair (bp) *Hin*dIII fragment (pPS2) spanning the entire coding region (20)] labeled with [^{32}P]dCTP by the random primer method (21). The hybridization and washing conditions were as previously described (4). The resulting autoradiographs were quantitated by scanning laser densitometry.

Isolation of Nuclear Proteins and Gel Mobility Shift Assay. Nuclear proteins were prepared from primary fetal rat osteoblasts or exponentially growing HeLa cells (24 hr after seeding) and analyzed as previously described (14, 22, 23). HiNF-D binding activity was analyzed by using either a probe spanning the site II region of the human FO108 gene proximal promoter [*Hin*dIII/*Taq* I fragment of pFP-1 spanning nucleotides (nt) −130 to −40] (22) or a 211-bp DNA probe (nt +7 to −188) from the rat H4 histone gene analogous to the FO108 site II region (24). Competition analysis of DNA–protein complexes in the human H4 histone gene promoter FO108 was performed by using an 80-fold excess of an oligonucleotide spanning the HiNF-D binding domain (22) in the H4 histone gene promoter (nt −91 to −64) in the presence of either human or rat nuclear proteins. Binding of nuclear proteins to the proximal promoter regions of the human H1 and H3 histone genes was analyzed by using the *Hin*dIII/*Rsa* I fragment of pϕX001 (25) spanning nt −213 to −78 of the H1 histone gene FNC16 and the *Hin*dIII/*Hpa* I fragment of ST519δH (26) spanning nt −200 to −21 of the H3 histone gene ST519. Competition analysis of protein–DNA complexes in the human H1 and H3 histone gene promoters was performed by using an 80-fold excess of an oligonucleotide spanning the HiNF-D binding domain (22) in the H4 histone gene promoter (nt −91 to −64) in the presence of either human (HeLa) or rat (osteoblast) nuclear proteins.

RESULTS AND DISCUSSION

Results from several laboratories have established that sequences residing in the 200 nucleotides immediately upstream from the transcription start site are functionally related to the fidelity and extent of mammalian H4 histone gene transcription (27–31). Analysis of the *in vitro* and *in vivo* transcriptional properties of 5′ deletion mutants, site-directed mutants, and chimeric constructs of histone gene promoter segments fused to the chloramphenicol acetyltransferase (CAT) gene have demonstrated a modular organization of the H4 histone gene promoters (28, 30). The primary regulatory element in these promoters is a highly conserved histone-specific sequence [5′-d(GGTTTTCAATCTGGTCCG)] located immediately upstream of the TATA motif that has been shown to regulate both the specificity and level of transcription *in vivo* (22, 28).

In the FO108 human H4 histone gene, two primary sites of protein–DNA interactions in the proximal promoter, desig-

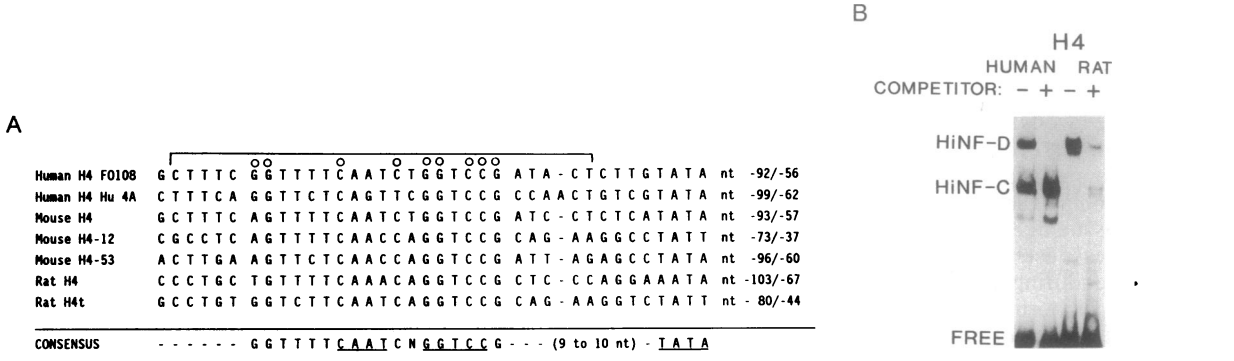

FIG. 1. (*A*) The DNA sequences immediately upstream of the TATA boxes in the proximal promoters of published mammalian H4 histone genes show a high degree of sequence similarity. This region coincides with the binding site of HiNF-D in the distal portion of site II in the human H4 histone gene FO108 (top sequence); the bracket indicates the domain protected from DNase I by HiNF-D (22) and open circles represent specific guanine–protein contacts determined *in vivo* (32). Histone gene DNA sequences (34) are numbered from the translational start codon: human FO108 (32); human Hu4A (30); mouse H4 (35); mouse H4-12 (36); mouse H4-53 (36); rat H4t (20); and rat H4 (24). (*B*) Competition analysis of protein–DNA complexes in the human H4 histone gene promoter. Competition analysis of protein–DNA complexes in the FO108 gene promoter (nt −130 to −40) was performed by using an oligonucleotide spanning the HiNF-D binding domain (nt −91 to −64) (22) in the presence of nuclear proteins from either HeLa cells (human; 4 μg; left lanes) or proliferating rat osteoblasts (day 7 after plating; 4 μg; right lanes). + and − signs indicate the presence or absence of an 80-fold molar excess of the HiNF-D binding site oligonucleotide. All designations of protein–DNA complexes are according to van Wijnen *et al.* (22).

nated site I (−110 to −150 bp) and site II (−50 to −90 bp), have been defined in the intact cell at single-nucleotide resolution by dimethyl sulfate protection analysis with confirmation of protein–DNA contacts by DNase I protection and native genomic blotting (32, 33). Two nuclear factors, HiNF-A and HiNF-C, bind to site I, possibly in conjunction with other proteins, while factor HiNF-D binds to site II (22, 26). The occupancy of site II by HiNF-D is required for *in vivo* expression of this gene (22, 28, 32). The sequence and organization of the proximal promoters of several mammalian growth-regulated H4 histone genes are compared in Fig. 1*A*. It can be seen that there is extensive sequence similarity between the site II promoter elements of the FO108 human H4 histone gene and the analogous regions of the rat H4 histone genes as well as with other mammalian histone genes, immediately upstream of the TATA box (20, 24, 30, 32, 34–36). Most importantly, the nucleotides that make specific DNA–protein contacts *in vivo* are conserved in these sequences (32). The ability of rat sequences to compete with human sequences for protein binding to site II has established the compatibility of rat and human site II regulatory elements and promoter binding factors (Fig. 1*B*). This is consistent with the previous demonstration that transfection of this human H4 histone gene into rodent cells results in its regulated expression (28). Therefore in the present studies we initially assayed rat osteoblast nuclear factors by using human histone gene probes and then confirmed the results by examining the binding of rat osteoblast factors to rat histone gene promoter sequences.

Some of the principal events associated with the developmental sequence of osteoblast proliferation, maturation, and differentiation monitored during the time course of this experiment are shown in Fig. 2. Nuclear proteins were prepared from proliferating osteoblasts (7 days after plating), from osteoblasts immediately following the completion of proliferation, when genes associated with organization and maturation of the extracellular matrix are expressed (15 days after plating), and from osteoblasts that had developed a mineralized extracellular matrix (30 days after plating). Electrophoretic mobility shift assays revealed a selective loss of factor HiNF-D binding (to the site II region of the H4 histone gene proximal promoter) in cells that had completed proliferation and displayed markers of the differentiated osteoblast phenotype that characterize the extracellular matrix maturation and specialization period or the extracellular matrix mineralization period. In contrast, factor HiNF-C (which binds to the site I region) did not exhibit a lower binding activity in cells that had progressed past the proliferation period (Fig. 3). The small amount of HiNF-D–site II interaction remaining in the postproliferation osteoblasts reflects the small number of cells (<2%) proliferating at this point in the cultures (see Fig. 2*A*). The HiNF-D–site II interactions in the proximal promoter of the H4 histone gene reflect the level of DNA synthesis throughout the osteoblast developmental sequence. These data suggest that the transcriptionally mediated down-regulation of this cell-growth-regulated H4 histone gene, as proliferation declines during the progressive expression of the osteoblast phenotype in culture, is accompanied by and is functionally related to specific modifications in protein–DNA interactions in the proximal promoter of the gene. The persistence of HiNF-C binding to regulatory elements of the H4 histone gene proximal promoter is consistent with a rate-limiting role for HiNF-D–site II interactions in rendering this gene transcribable. The loss of HiNF-D binding activity occurs at the time during the osteoblast

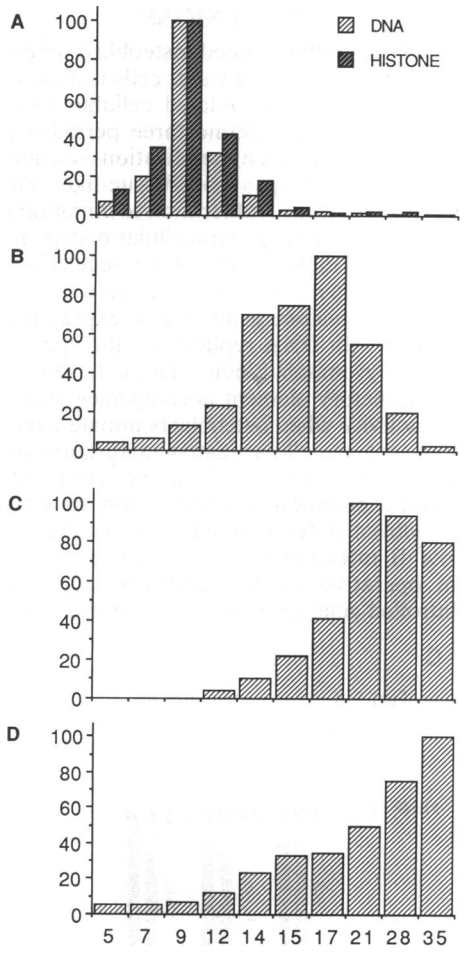

FIG. 2. Principal events associated with the osteoblast developmental sequence of proliferation, maturation, and differentiation. Abscissa, days *in vitro*; ordinates, relative levels (expressed as percent of maximum). (*A*) H4 histone mRNA (determined by Northern blot analysis) and DNA synthesis (total trichloroacetic acid-precipitable radioactivity per 35-mm culture dish after a 1-hr pulse-labeling with [^{3}H]thymidine). (*B*) Alkaline phosphatase enzyme activity expressed as nmol of *p*-nitrophenol per μg of DNA. (*C*) Media osteocalcin per μg of DNA as determined by radioimmunoassay. (*D*) Total mineral (calcium) accumulation per μg of DNA determined by atomic absorption spectroscopy in samples hydrolyzed in 6 M HCl under reduced pressure at 110°C for 24 hr.

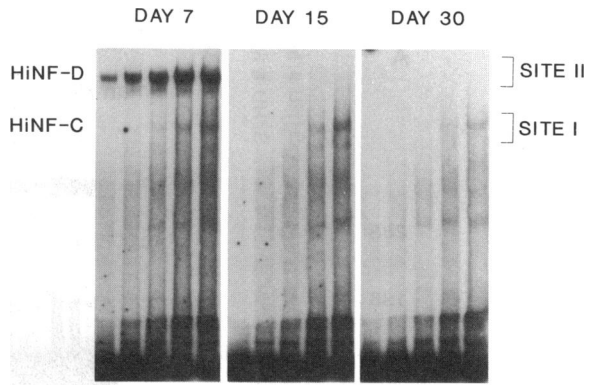

FIG. 3. Selective loss of factor HiNF-D–site II interactions only when proliferation is down-regulated during the progression of the osteoblast developmental sequence. Nuclear protein extracts were prepared from primary fetal rat osteoblasts at day 7 (proliferation period), day 15 (extracellular matrix maturation period), or day 30 (mineralization period) after plating and analyzed for HiNF-D DNA binding activity, using the *Hin*dIII/*Taq* I fragment (nt −130 to −40) of the human H4 histone gene FO108 (22) as a probe. For each of the three samples, the lanes, from left to right, contained 2, 4, 6, 8, or 10 μg of nuclear protein in the binding reaction mixture.

developmental sequence when proliferation is completed and the initial appearance of osteoblast phenotypic markers related to extracellular matrix maturation and specialization is observed. Therefore, to better understand how protein–DNA interactions in the H4 histone gene promoter relate to regulatory mechanisms operative at this transition point during the sequential expression of the osteoblast phenotype, we determined whether the observed changes in HiNF-D–site II interactions in the H4 histone gene promoter are functionally coupled to the shut-down of proliferation in general or to the cessation of proliferation only when it is associated with the progression of osteoblast differentiation.

To establish that the loss of interaction of HiNF-D with site II is functionally related to events associated with osteoblast differentiation that occur following completion of the proliferative period, we synchronized exponentially growing primary rat osteoblast cells with respect to the cell cycle. This permitted us to examine the effect of DNA synthesis inhibition on HiNF-D binding activity during the S phase of the cell cycle, when the H4 histone gene is maximally expressed, without inducing expression of postproliferation osteoblast phenotype markers (e.g., alkaline phosphatase or osteocalcin). Actively proliferating osteoblasts were blocked at the G_1/S boundary by two cycles of 2 mM thymidine block. Several hours after release from the second thymidine block, the cells synchronously entered DNA synthesis, which was maximal between 4 and 6 hr, at which time nearly 100% of the cells were replicating DNA. Mitotic activity was first observed 9 hr after release from the second thymidine block, and by 12 hr after release the number of cells per culture dish had doubled and the cells were in the G_1 phase, on the basis of the lack of [^{3}H]thymidine labeling.

Synchronized cultures were treated with 1 mM hydroxyurea for either 1 hr or 8 hr, beginning at mid-S phase, 4 hr after release from the second thymidine block, resulting in a >90% inhibition of DNA synthesis. Fig. 4*A* shows that, as expected, the cells actively engaged in DNA synthesis had high levels of H4 histone mRNA, while those cells that were treated in S phase with hydroxyurea for 1 or 8 hr exhibited a >10-fold decrease in cellular H4 histone mRNA, which paralleled the decreased [^{3}H]thymidine incorporation. As seen in Fig. 4*B*, the HiNF-D promoter binding activity persists following either short-term (1-hr) or long-term (8-hr) inhibition of DNA synthesis, consistent with the continued presence of HiNF-D binding in cells that do not initiate extracellular matrix maturation, specialization, or mineralization despite the inhibition of proliferation.

We confirmed that nuclear factors from rat osteoblasts bound specifically to the site II proximal promoter element in both the human and rat cell-growth-regulated H4 histone genes by showing that electrophoretic mobility shift assays carried out with rat and human probes provided indistinguishable results (Fig. 4*C*). The conservation of these protein–DNA interactions was additionally demonstrated by showing that nuclear factors similar or identical to factor HiNF-D were also detectable in the rat osteoblast nuclear protein extracts and exhibit sequence-specific binding activity not only to the human H4 histone proximal promoter but also to the promoters of the human cell-growth-regulated H3 and H1 histone genes. The binding of these factors to the H3 and H1 histone gene promoters persisted, as observed for HiNF-D, after the inhibition of proliferation by hydroxyurea in synchronized S-phase cells, where extracellular matrix maturation was not induced (Fig. 5), suggesting that a common mechanism may exist for the coordinate transcriptional regulation of both core (H3 and H4) and H1 histone genes during the osteoblast developmental sequence.

CONCLUSIONS

Expression of the differentiated osteoblast phenotype in cultures of isolated fetal rat calvarial cells is associated with a temporal sequence of interrelated cellular, biochemical, and molecular events that define three periods—proliferation, matrix maturation, and mineralization—during a period in which the cells develop a bone-tissue-like organization (3–6). The transition from proliferation to initiation of events that accompany the onset of extracellular matrix maturation and mineralization includes the down-regulation of cell-growth-regulated gene expression. In the case of the cell-cycle-regulated H4 histone gene, whose expression is functionally coupled with DNA replication, the specific loss of interaction of a promoter binding factor (HiNF-D) with a 5′-proximal regulatory element accompanies completion of proliferative activity when osteoblasts initiate expression of genes related to extracellular matrix maturation and mineralization. Persistence of HiNF-D binding activity under conditions in which inhibition of proliferation does not further promote osteoblast differentiation suggests that the loss of HiNF-D–site II interactions is a component of a specific mechanism operative in the down-regulation of a cell-growth-controlled gene early during the osteoblast develop-

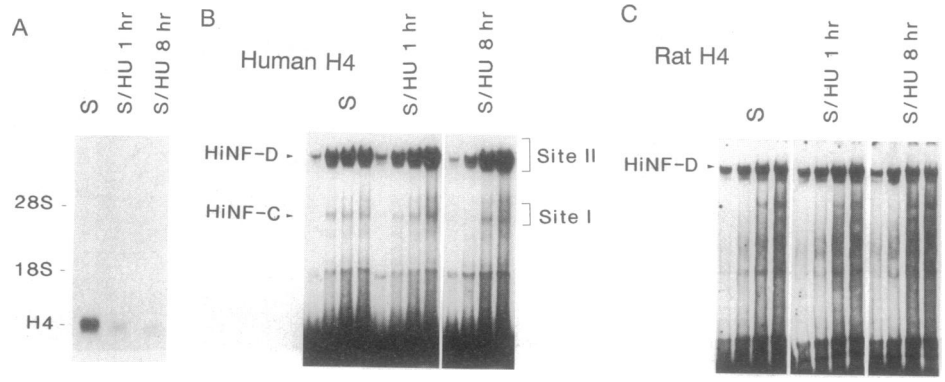

FIG. 4. Analysis of H4 histone mRNA and HiNF-D binding activity after cell cycle synchronization of primary rat osteoblasts. Rat osteoblasts were synchronized by double thymidine block. Beginning at 4 hr after release from the last thymidine block, S-phase cells (S) were treated for 1 or 8 hr with 1 mM hydroxyurea (S/HU 1 hr or S/HU 8 hr) and harvested, and nuclear protein extracts and cytoplasmic RNA were prepared. (*A*) For each sample, 20 μg of RNA was fractionated in an agarose/formaldehyde gel, transferred to Zeta-Probe membrane, and hybridized with the rat H4 histone gene fragment. (*B*) Nuclear protein extracts prepared from S-phase control and hydroxyurea-treated cells were analyzed for HiNF-D DNA binding activity by gel retardation, with the *Hin*dIII/*Taq* I fragment (nt −130 to −40) of the human H4 histone gene FO108 (22) used as a probe. For each cell sample, the binding reaction mixtures in the lanes from left to right contained 3, 6, 9, or 12 μg of nuclear protein. (*C*) The same amounts of these nuclear extracts were analyzed for HiNF-D binding activity using a 193-bp DNA probe (nt +7 to −188) from the rat H4 histone gene analogous to the human H4 histone gene FO108 site II region (24).

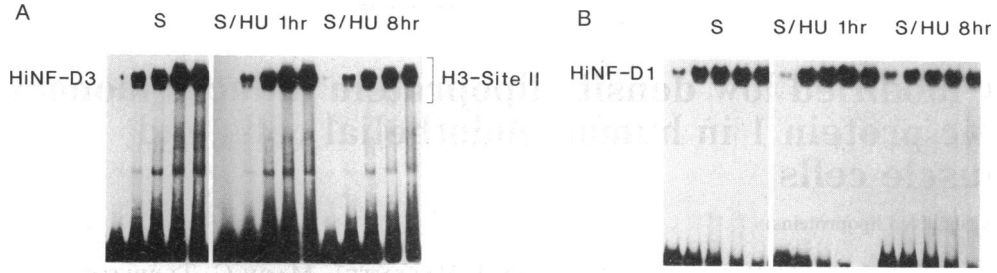

FIG. 5. The nuclear extracts prepared from rat osteoblasts synchronized with respect to the cell cycle by double thymidine block were analyzed for HiNF-D binding activity to the proximal promoters of the H3 and H1 human histone genes. For H3 histone (*A*), binding reactions were performed with the *Hin*dIII/*Hpa* I fragment of ST519δH spanning nt −200 to −21 of the H3 histone gene ST519 (26) and for H1 histone (*B*), the *Hin*dIII/*Rsa* I fragment of pφX001 spanning nt −213 to −78 of the H1 histone gene FNC16 (25). For both H3 and H1 histone and for each cell sample, the lanes from left to right represent binding reaction mixtures that contained 2, 4, 6, 8, or 10 μg of nuclear protein.

mental sequence. As such, this serves as a molecular marker for the transition point where proliferation is down-regulated and events associated with the organization and maturation of the osteoblast extracellular matrix are initiated.

A selective loss of HiNF-D interaction with the site II region of the H4 histone gene promoter has similarly been observed both *in vitro* and *in vivo* in HL-60 promyellocytic leukemia cells induced to differentiate into monocytes (37). It appears, therefore, that this modification in protein–DNA interaction is a general mechanism invoked when proliferation is terminated and specific events associated with the progression of differentiation are initiated. While this may not be the rate-limiting step in the termination of proliferation, the loss of the HiNF-D–site II interaction in the proximal promoters of several histone genes suggests that it is a key event in the coordinate down-regulation of the multigene family of cell-cycle-expressed histone genes that accompanies initiation of principal processes associated with cell- and tissue-specific gene expression. Whether the down-regulation of other cell-cycle and cell-growth-regulated genes that occurs during the progression of differentiation is mediated by similar mechanisms remains to be resolved. Here it should be noted that not all cell-growth-regulated genes are initially down-regulated at the transcriptional level. For example, the down-regulation of c-*myc* and c-*fos* gene expression at the completion of the proliferative period in the osteoblast developmental sequence is, in contrast to that of the histone genes, initially mediated by mRNA destabilization and subsequently by decreased transcription (4). In a broader context, our results suggest that a question which must be addressed is the extent to which the progression of osteoblast differentiation is dependent on the shut-down of proliferation and on molecular mechanisms that may mediate this relationship. Other results suggest that early events associated with development of the osteoblast phenotype, including alkaline phosphatase and osteopontin gene expression, are indeed functionally coupled to the down-regulation of proliferation (3).

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