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Multiple Mechanisms Regulate the Proliferation-Specific Histone Gene Transcription Factor HiNF-D in Normal Human Diploid Fibroblasts†

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ABSTRACT: The proliferation-specific transcription factor complex HiNF-D interacts with sequence specificity in a proximal promoter element of the human H4 histone gene FO108, designated Site II. The occupancy of Site II by HiNF-D has been implicated in proper transcription initiation and as a component of the cell cycle regulation of this gene. In the present study we have investigated the role of the HiNF-D/Site II interaction in controlling the level of H4 histone gene transcription during modifications of normal cellular growth. HiNF-D binding activity is present at high levels in rapidly proliferating cultures of human diploid fibroblasts and is reduced to less than 2% upon the cessation of proliferation induced by serum deprivation of sparsely populated fibroblast cultures. Density-dependent quiescence also abolishes HiNF-D binding activity. Downregulation of transcription from the H4 gene occurs concomitant with the loss of the HiNF-D/Site II interaction, further suggesting a functional relationship between Site II occupancy and the capacity for transcription. Serum stimulation of quiescent preconfluent cells results in an increase in HiNF-D binding activity as the cells are resuming DNA synthesis and H4 histone gene transcription. Density-inhibited quiescent cells respond to serum stimulation with only a minimal increase in the HiNF-D binding activity, 30% of maximal levels. However, H4 histone gene transcription is stimulated to a level equal to that detected in extracts of the sparsely populated serum-stimulated cultures. These results suggest that there is a threshold level of HiNF-D binding activity necessary for the activation of H4 histone gene transcription. Additionally, these findings suggest that there may be a mechanism repressing HiNF-D binding activity in the density-inhibited cultures which is not operative in the sparsely populated, serum-deprived cultures. Density-inhibited cultures may have reached a state analogous to the initial steps of differentiation and have invoked a series of mechanisms to decrease expression of proliferation-specific factors. Serum stimulation is able to overcome the one mechanism downregulating HiNF-D in both sparsely populated and density-inhibited quiescent cultures but is unable to reverse the repression of proliferation-specific factors that occurs in density-inhibited cultures. These results are consistent with the presence of at least two levels of control over the HiNF-D/Site II interaction which are responsive to and reflect the proliferative state of the cell and the extent to which the cell exhibits properties associated with differentiation.

The control of growth-related genes is central to maintaining stringent cell cycle control and regulating the transition between proliferation and a commitment to differentiate. Understanding molecular mechanisms operative in regulating expression of cell-cycle- and cell-growth-related genes should provide insights into the loss of growth control in transformed and tumor cells. The human H4 histone gene FO108 (Sierra et al., 1982) is a cell-cycle-dependent, growth-related gene for which expression is temporally and functionally coupled to DNA synthesis (Plumb et al., 1983). Regulation of this coupling is mediated at both the transcriptional and post-transcriptional levels (Baumbach et al., 1987). This H4 histone gene exhibits a basal level of transcription throughout the cell cycle, with a 2–3-fold enhanced level of transcription during the early stages of S phase (Baumbach et al., 1987; Ramsey-Ewing et al., 1992).

Two in vivo protein–DNA interactions, Sites I and II, have been delineated within the first 200 nucleotides upstream of the H4-FO108 transcription initiation site (Pauli et al., 1987, 1988). At least three independent protein factors have been detected that bind to these domains in vitro (van Wijnen et al., 1989, 1991; Wright et al., 1992): HiNF-C, an Sp1-like protein, and HiNF-E, an ATF-related transcription factor, bind adjacent to each other in the Site I domain. The interactions at Site I do not change during the cell cycle nor during the complete shutdown of H4 histone gene transcription that occurs during differentiation (Pauli et al., 1987; Stein et

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al., 1989; Owen et al., 1990). However, deletion of Site I significantly reduces H4 gene transcription in actively proliferating cells, implying that Site I is key in regulating the level of transcription but is not a rate-limiting step in the process of initiation (Kroeger et al., 1987; van Wijnen et al., 1989, Wright et al., 1992).

Factor HiNF-D interacts specifically with Site II sequences at −70 to −20 nucleotides upstream from the transcription initiation site. The region of interaction includes the histone-specific element (5′-GGTCC-3′) and a CAAT box and lies adjacent to the TATA box (van Wijnen et al., 1989). Deletion of this protein–DNA interaction site abolishes transcription in vivo and indicates a critical role for Site II in the regulation of this gene (Kroeger et al., 1987; Ramsey-Ewing et al., 1992). Furthermore, in normal diploid cells HiNF-D binding activity is substantially decreased outside of S phase, implying an involvement in the cell cycle control of histone gene expression. In tumor cells the level of HiNF-D binding activity is uncoupled from the cell cycle and constitutively high binding activity is detected (Holthuis et al., 1990). In addition, in vivo Site II interactions and HiNF-D binding activity are specifically lost during differentiation of HL60 cells into monocytes or of primary cultures of rat calvarial osteoblasts, concomitant with the shutdown of H4 histone gene transcription (Stein et al., 1989; Owen et al., 1990).

These observations prompted us to address other aspects of the relationship between histone gene expression and cell growth control. Human diploid fibroblasts provide a system in which proliferation can be reversibly shut down without inducing differentiation (Pardee et al., 1978; Baserga, 1985; Prescott, 1987; Duncun et al., 1982). Normal fibroblasts cease to proliferate and enter a quiescent state if placed in medium containing very low serum (0–0.5%) or if allowed to become density-inhibited. Sparsely populated cultures maintained in serum-free medium become arrested in the G_1 phase of the cell cycle due to a lack of nutrients. Density-inhibited fibroblasts become arrested in the G_1 phase and subsequently enter G_0 after being maintained at reduced serum concentrations (Duncun et al., 1982). Serum-deprived quiescent cultures of either preconfluent or confluent cells can be stimulated to reenter the proliferative phase upon replenishment of the medium with 10% serum.

In these studies we have manipulated the proliferative state of the cells and examined the extent to which the level of HiNF-D binding at Site II is coupled to H4 histone gene transcription. A strict correlation between proliferation, the presence of HiNF-D binding activity, and H4-FO108 histone gene transcription has been established. However, stimulation of density-inhibited cultures induced a significantly lower level of HiNF-D binding activity than stimulation of sparsely populated cultures despite approximately equivalent induction of H4 histone gene transcription. These results suggest that density-inhibited human fibroblasts have a mechanism restricting the accumulation of HiNF-D. This mechanism may be related to the limited proliferative activity of serum-stimulated, density-inhibited fibroblasts and may reflect a similarity between the density-inhibited state and the initial stages of differentiation.

Experimental Procedures

Cell Culture. Human diploid fibroblasts used in these experiments were originally derived from newborn foreskin and designated CF-3 (Dell'Orco, 1975). These cells have an in vitro life span of 65 ± 10 population doublings and were used before 50% of this life span had been completed. Cells were subcultured into 150-cm^2 plastic culture vessels (Costar) with McCoy's medium 5a (Gibco) containing 10% fetal bovine serum (Hyclone) and antibiotics as previously described (Duncun et al., 1982). Cells were in exponential growth 3 days after subculture and formed confluent monolayers after 7 days. Stock cultures were determined to be free of mycoplasma contamination by the method of Barile (1973). In some experiments, cells were induced to enter a quiescent state by replacing the medium with serum-free medium 24 h after subculture. These sparsely populated cultures were maintained with serum-free medium for 72 h, at which time the cells were stimulated to enter the division cycle by refeeding with medium containing 10% serum. In other experiments, a quiescent state was induced in confluent populations by maintaining the cells for 16 days with medium containing 0.1% serum which was replaced twice weekly. These cells were also stimulated to enter the division cycle by refeeding with medium containing 10% serum.

DNA Synthesis. DNA synthesis was estimated by the incorporation of ^{3}H-thymidine into acid-precipitable material. Cells were pulse-labeled for 30 min with 0.5 μCi/mL ^{3}H-thymidine (NEN, 82.7 Ci/mmol). Labeled cells were scraped into cold phosphate-buffered saline, pelleted by low-speed centrifugation, and resuspended in high-salt buffer (50 mM Na_2HPO_4 and 2.0 M NaCl, pH 7.4). An aliquot of the cell suspension was used for the determination of DNA by the method of Labarca and Paigen (1980), and acid-precipitable radioactivity was determined in a second aliquot as previously described (Zambetti et al., 1987).

Nuclear Extract Isolation. For the isolation of nuclear extracts, cells were scraped into buffer A1 [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethanesulfonyl fluoride (PMSF), and 20% glycerol] and pelleted by centrifugation at 800*g* for 15 min. The pellet was resuspended in buffer A1, and the cells were lysed by expelling the suspension through a 22-gauge needle 10 times. The nuclei were pelleted by centrifugation at 800*g* for 10 min. The pellet was resuspended in 500 μL of buffer B [20 mM Hepes (pH 7.9), 0.6 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 20% glycerol] and placed on ice with constant stirring for 30 min. Nuclear debris was removed by centrifugation at 25000*g* for 30 min, and the supernatant was transferred to a dialysis bag (10000 MW cutoff). Dialysis was at 4 °C against 500 mL of buffer C [20 mM Hepes (pH 7.9), 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 20% glycerol] for 2 h with one change of dialysis buffer. The dialyzed extract was centrifuged at 12000*g* for 10 min, and small aliquots of the supernatant were immediately frozen in a dry ice/ethanol bath and stored at −80 °C. All buffers were autoclaved before use, and the DTT and PMSF were added immediately prior to use.

Gel Mobility Shift Assay. Gel retardation assays (Fried & Crothers, 1981) were performed essentially as described previously (van Wijnen et al., 1989). Each binding reaction contained 2 ng of a 173 base pair single-end-labeled probe spanning the entire proximal promoter of the human FO108 H4 histone gene promoter (−155 to +18, numbered relative to the initiation site). Crude nuclear extract from CF-3 normal diploid fibroblasts (0.5–10 μg, as indicated) was incubated with the probe and 2 μg of poly(dI-dC)·poly(dI-dC) in a final volume of 20 μL containing 12 mM Hepes (pH 7.9), 12% glycerol, 60 mM KCl, and 0.37 mM DTT for 30 min at 22 °C. The samples were immediately loaded onto a high ionic strength 4% native polyacrylamide gel [0.1% *N*,*N*′-

methylenebis(acrylamide), 50 mM Tris-HCl, 380 mM glycine, and 2 mM EDTA, pH 8.5]. Electrophoresis was at 200 V constant and the gels were maintained at <10 °C throughout the run. The relative levels of HiNF-D binding activity compared to the proliferating controls were determined by densitometric scanning and analysis (Pharmacia-LKB).

mRNA Isolation. Total cellular mRNA was isolated essentially as described previously (Zambetti et al., 1987).

S1 Nuclease Analysis. The S1 nuclease analysis method was modified from the procedure developed by Berk and Sharp (1978) and Haegeman et al. (1979). Total cellular mRNA (15 μg) was precipitated with 10 ng of single-end-labeled FO108 probe (−420 to +279 bp, derived from FO002), 2 μg of glycogen, 0.25 M NaCl, and 2.5 volumes of 95% ethanol at −70 °C for 30 min. The coprecipitate was recovered by centrifugation and resuspended in 2.5 μL of hybridization buffer [2 M NaCl, 0.2 M Pipes (pH 6.4), and 5 mM EDTA) and 10 μL of recrystallized formamide. The nucleic acids were denatured by incubation at 100 °C for 10 min, immediately placed into a 55 °C water bath, and incubated overnight. The hybridized mixture was briefly centrifuged and then kept on ice while 100 μL of S1 buffer [0.25 M NaCl, 30 mM NaAc (pH 4.6), and 4 mM $ZnSO_4$] and 10 μg of salmon sperm DNA were added. The samples were incubated at 21 °C for 1 h in the presence of 4 units/μL S1 nuclease (Boehringer Mannheim) and then an additional 15 min on ice; digestions were stopped by the addition of 0.8% SDS and 16 mM EDTA. Samples were extracted with phenol and chloroform and precipitated with 20 μg of glycogen and 2.5 volumes of 95% ethanol at −20 °C overnight. The protected probe was recovered by centrifugation, resuspended in 5 μL of loading dye (99.8% formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol), heated to 100 °C for 3 min, and resolved in a 6% polyacrylamide/7.75 M urea gel. The relative levels of accurately initiated FO108 H4 mRNA compared to the proliferating control were determined by densitometric scanning and analysis.

In Vitro Transcription. In vitro transcription (Dignam et al., 1983; Hirose et al., 1985) was performed with 500 ng of supercoiled H4 histone gene DNA template (FO002) containing the first 1000 nucleotides of the promoter and the entire coding region. Template DNA was incubated for 15 min at 4 °C with 25–75 μg of nuclear extract in a 21-μL binding reaction containing 14 mM Hepes (pH 7.9), 140 μM EDTA, 70 mM KCl, 9.5 mM $MgCl_2$, 0.35 mM DTT, and 14% glycerol. Transcription was initiated by the addition of 4 μL of NTP stock (1.75 mM ATP, CTP, GTP, and UTP) and allowed to proceed at 30 °C for 30 min. The reaction was terminated by making the solution 50 mM NaAc (pH 5.2), 0.5% SDS, and 100 ng/μL yeast tRNA in a final volume of 100 μL. The sample was extracted with phenol and chloroform and precipitated with 20 μg of glycogen, 0.3 M NaAc, and 2.5 volumes of 95% ethanol at −70 °C for 30 min. The nucleic acids were recovered by centrifugation and resuspended in 49 μL of DNase I digestion buffer [40 mM Tris (pH 7.9), 10 mM NaCl, and 6 mM $MgCl_2$). The sample was incubated for 15 min at 37 °C in the presence of 1 unit of DNase I (RQ1, Promega). The digestion was stopped by making the solution 0.4% SDS and extracting with phenol and chloroform. The RNA transcribed in vitro was then assayed by the S1 analysis method described above.

Results

Cessation of Proliferation Is Associated with the Down-regulation of HiNF-D Binding Activity. Human diploid fibroblasts were examined during exponential growth and two modes of induced quiescence: (i) serum deprivation and (ii) density-dependent quiescence. The proliferative state of the cultures was monitored by the level of DNA synthesis detected by ^{3}H-thymidine incorporation. Sparsely populated, proliferating cultures were incubated for 3 days in serum-free medium. DNA synthesis in these cultures was reduced to 2.5% of the proliferating rate, indicating that the cells had entered a quiescent state (Table I). Human diploid fibroblasts will naturally cease to proliferate and enter an extended quiescent state when the cultures are allowed to become confluent. Density-dependent, quiescent populations were prepared by maintaining cultures in normal growth medium without subcultivation. Eight days after initial seeding the cultures had reached confluence and were quiescent as reflected by a 94% reduction in the rate of DNA synthesis; an additional 1 or 2 days at confluence did not further reduce the rate of ^{3}H-thymidine incorporation into the DNA (Table I, day 9 and day 10).

Table I: DNA Synthesis in Normal Diploid Human Fibroblasts[a]

sample	^{3}H-dThd (dpm/μg of DNA)
exponentially growing	2250
preconfluent, serum-deprived	60
preconfluent, serum-stimulated, 5 h	90
preconfluent, serum-stimulated, 24 h	2850
confluent, 8 days	140
confluent, 9 days	160
confluent, 10 days	120
confluent, serum-deprived	100
confluent, serum-stimulated, 5 h	30
confluent, serum-stimulated, 24 h	3050

[a] Cells were pulse-labeled with ^{3}H-thymidine for 30 min, and incorporation into acid-precipitable material was used to estimate DNA synthesis as described in Experimental Procedures.

HiNF-D binding activity to Site II of the H4-FO108 promoter was measured in the quiescent fibroblasts. High-salt nuclear extracts were prepared from proliferating cells and from quiescent cells induced by serum deprivation or density-dependent inhibition. Gel mobility shift assays were utilized to detect the level of HiNF-D binding in each of the nuclear extracts. Previously, the band indicated as HiNF-D in Figure 1 has been shown by competition analysis to be specifically related to binding at Site II (data not shown; van Wijnen et al., 1989). The rapidly proliferating cells contained a high level of HiNF-D binding activity (Figure 1). However, cells which had ceased to proliferate due to serum deprivation lost >95% of the HiNF-D activity, and had nearly undetectable levels relative to the proliferating control [Figure 1 (left)]. Similarly, density-dependent quiescent fibroblasts contained at most 15% of the HiNF-D binding activity found in rapidly proliferating cultures [Figure 1 (right), days 8, 9, and 10].

Reentry of Quiescent Fibroblasts into the Proliferative Cycle Results in a Stimulation of HiNF-D/Site II Binding Activity. Quiescent fibroblasts, in contrast to terminally differentiated cells, can be stimulated to reenter the proliferative cycle. This system provides the opportunity to address the molecular mechanisms operative during the reactivation of histone gene expression and the role of the HiNF-D/Site II interaction in this process.

Sparsely populated, quiescent cultures were stimulated by replacing the serum-free medium with fresh medium containing 10% serum. The cells required approximately 14 h to reenter the DNA replication period (data not shown). The maximal rate of DNA synthesis occurred 24 h poststimulation (Table I). High-salt nuclear extracts were prepared at 5 and

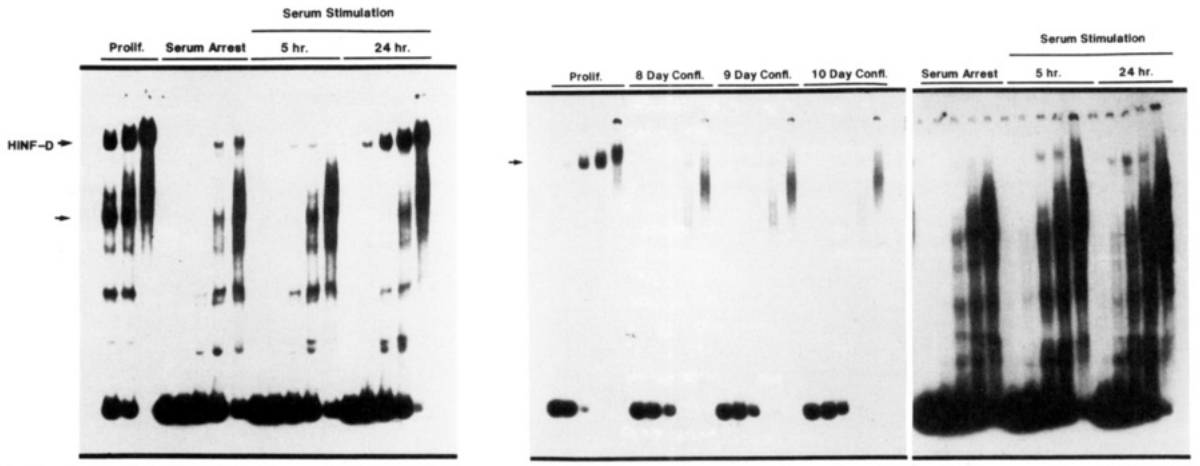

FIGURE 1: Analysis of HiNF-D binding activity in CF-3 normal diploid human fibroblasts by gel mobility shift assay. A radiolabeled DNA fragment (−155 to +18 bp) containing the Site II domain of the human H4-FO108 histone gene was used in a gel mobility shift assay to probe for HiNF-D binding activity in nuclear extracts. Each group of five lanes represents increasing concentrations of nuclear extract (0.5, 1, 2, 5, and 10 μg) added to the binding reactions. The arrow indicates the position of the HiNF-D/Site II complex. (Left panel) HiNF-D activity in extracts from proliferating normal diploid fibroblasts, cultures arrested for 72 h in serum-free medium, and cultures 5 and 24 h after stimulation with medium containing 10% serum. The small arrow indicates the nonoptimized binding of Sp1 to Site I of this probe. The additional bands are nonspecific. (Right panel) Analysis of HiNF-D binding activity in extracts from density-inhibited CF-3 normal diploid fibroblasts. Nuclear extracts were prepared from proliferating cultures, quiescent cultures 8, 9, and 10 days after initial plating, confluent cultures arrested for 16 days in medium containing 0.1% serum, and confluent cultures 5 and 24 h after stimulation with 10% serum-containing medium. A longer autoradiographic exposure of the right half of this panel is shown to reveal the HiNF-D binding activity after serum stimulation. Thus the lower region becomes relatively intensified. The reduction in HiNF-D binding activity seen at high concentrations of nuclear extracts from confluent 24-h serum-stimulated cells (lanes 4 and 5) was not reproducible.

24 h after serum stimulation and assayed for the presence of HiNF-D/Site II binding activity. Prior to entry of these sparsely populated fibroblasts into S phase, the HiNF-D/Site II binding activity was barely detectable [Figure 1 (left), 5 h]. However, when the fibroblasts resumed DNA replication, HiNF-D binding activity increased to levels approximating those detected in the initial proliferating cultures [Figure 1 (left), 24 h].

The onset of DNA synthesis requires histone protein synthesis to package the newly replicated DNA, and the initiation of S phase is marked by a large increase in histone mRNA levels (Plumb et al., 1983) and elevated histone transcription in cycling cells (Baumbach et al., 1987). Therefore, these results suggest that not only is HiNF-D involved in the downregulation of H4 histone gene transcription occurring at quiescence but also it is a critical element in the reactivation of H4 histone gene expression during serum stimulation.

Density-Inhibited Fibroblasts Increase the Level of HiNF-D/Site II Binding Activity upon Stimulation but Do Not Accumulate the Large Amounts of HiNF-D Detected in Sparsely Populated Cultures. Density-inhibited fibroblasts can be induced to undergo limited proliferation by serum stimulation before returning to the quiescent state (Baserga, 1985). In this sense, density-dependent quiescence may be more analogous to conditions present in normal tissues. Thus, it is important to determine whether the same mechanisms regulating HiNF-D binding activity in preconfluent cells are operational in density-inhibited cultures.

Density-inhibited, quiescent CF-3 fibroblasts were maintained in medium containing 0.1% serum for 16 days. At this stage the quiescent cells were still >95% viable as measured by trypan blue exclusion. The fibroblasts were then stimulated to proliferate by replacing the medium with fresh medium containing 10% serum; they exhibited the same time course of stimulation as the sparsely populated, serum-deprived cultures, with maximal DNA synthesis occurring 24 h poststimulation (Table I).

Interestingly, the stimulation of HiNF-D binding activity in the density-inhibited fibroblasts was not the same as in the sparsely populated cultures. At 24 h after stimulation, when DNA replication had reached maximal levels, HiNF-D binding activity had increased to no more than 30% of the level present in rapidly proliferating cells (Figure 1 (right), long exposure). This small increase in HiNF-D activity was in contrast to the nearly 100% increase induced in the sparsely populated fibroblast cultures 24 h after serum stimulation. It should be noted that the reduction in HiNF-D binding activity seen at high nuclear extract concentrations in the confluent 24-h serum-stimulated extract (lanes 4 and 5) was not reproducible.

Serum Stimulation of either Sparsely Populated or Density-Inhibited Fibroblasts Upregulates H4 Histone mRNA Levels. The differences in HiNF-D/Site II binding activity observed upon stimulation of quiescent, sparsely populated cultures compared to density-inhibited cultures prompted us to investigate the levels of histone gene expression in both types of fibroblast cultures. Initially, we examined H4-FO108 histone mRNA levels in proliferating, quiescent, and serum-stimulated populations of CF-3 cells. H4 mRNA levels were measured by S1 nuclease protection analysis of total cellular RNA in order to resolve H4-FO108 mRNAs from transcripts of the other members of the H4 multigene family. The mRNA levels as determined from the amount of ^{32}P-labeled probe protected from S1 nuclease digestion (Figure 2), paralleled HiNF-D binding activity and DNA synthesis, as would be expected for a cell-cycle-regulated histone gene. The high levels of H4 mRNA present in proliferating fibroblasts were completely downregulated in quiescent, sparsely populated cells and density-inhibited cells. Serum stimulation of either quiescent population resulted in the accumulation of H4 histone mRNA in conjunction with maximal DNA synthesis at 24 h poststimulation. Thus, although density-inhibited fibroblasts contained only minimal levels of HiNF-D binding activity after stimulation (30%), these cells were able to accumulate high levels of H4 histone mRNA.

H4 Transcription Is Upregulated to High Levels upon Stimulation of Density-Inhibited Fibroblasts despite Only a 30% Increase in HiNF-D Binding Activity. Because accu-

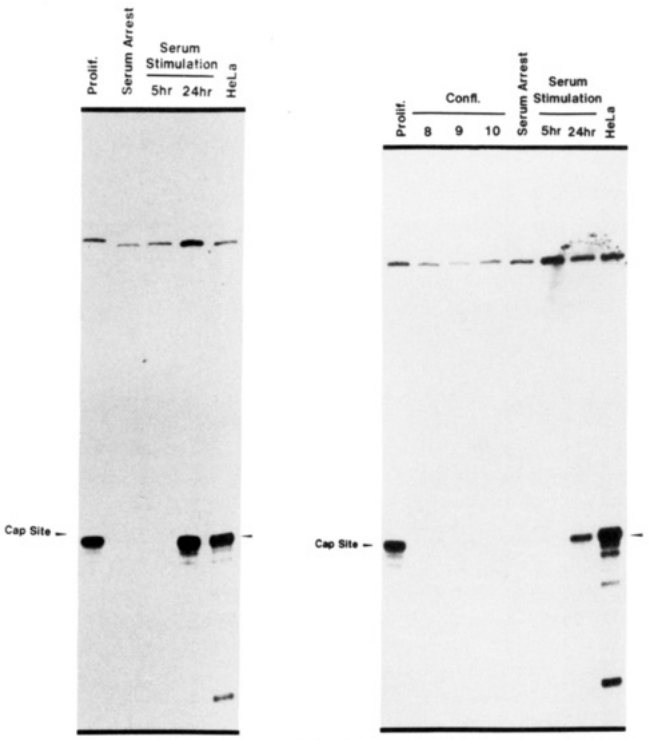

FIGURE 2: Expression of human H4 histone mRNA in normal diploid fibroblasts as a function of serum arrest or density inhibition followed by serum stimulation. Cultures were maintained as described for Figure 1. (Left panel) Total cellular RNA was isolated from proliferating, serum-arrested, and serum-stimulated sparse cultures as described in Experimental Procedures. The H4-FO108 histone mRNA was specifically assayed by S1 nuclease protection using a single end-labeled probe spanning the 5′ terminus of the mRNA. Accurately initiated H4-FO108 mRNA should protect a 279-nt fragment as indicated by the arrowhead. The band at the top of the gel represents the undigested full-length probe, and bands appearing below the 279-nt band are derived from other H4 histone mRNAs with partial homology to the probe. The HeLa lane represents positive control RNA known to contain accurately initiated H4-FO108 mRNA. (Right panel) Total cellular RNA was isolated from proliferating cultures, confluent cultures 8, 9, and 10 days after plating, confluent cultures arrested in 0.1% serum for 16 days, and confluent cultures 5 and 24 h after serum stimulation. The arrowhead indicates the position of the 279-nt protected fragment representing the accurately initiated human H4-FO108 mRNA.

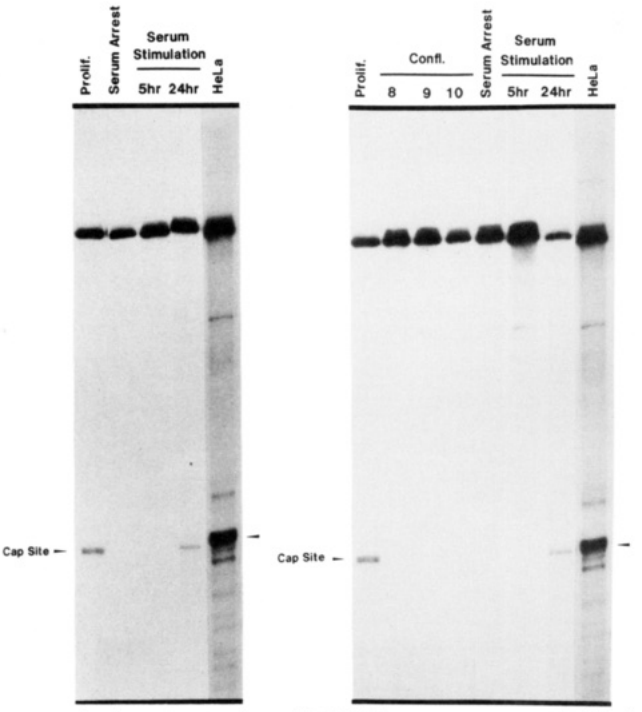

FIGURE 3: Assessment of specific H4 histone gene transcriptional activity in human diploid fibroblasts. Cell-free transcription analysis was performed as described in Experimental Procedures, and resultant RNA transcripts were assayed by S1 nuclease protection as described for Figure 2 and in Experimental Procedures. (Left panel) Nuclear extracts from preconfluent cells were the same as used in the determination of HiNF-D binding activity described for Figure 1 (left panel). The arrowhead indicates the 279-nt protected fragment corresponding to the accurately initiated human H4-FO108 mRNA. The dark band at the top of the gel represents the undigested, full-length probe. The HeLa lane represents nuclear extracts isolated from proliferating HeLa cells which had been shown previously to promote in vitro transcription of this H4 histone gene. (Right panel) Specific H4 histone gene transcriptional activity during density inhibition, serum arrest, and stimulation of confluent normal diploid fibroblasts. Nuclear extracts were the same as used in the determination of HiNF-D binding activity described for Figure 1 (right panel). The arrowhead indicates the accurately initiated human H4 histone mRNA.

mulation of histone mRNA is modulated at both the transcriptional and posttranscriptional levels, a direct measurement of FO108 H4 histone gene transcription is required to accurately determine the effect of changes in HiNF-D binding activity on H4 histone gene expression. The same high-salt nuclear extracts used to monitor HiNF-D binding activity were used to promote in vitro transcription from the H4-FO108 histone gene promoter. The resultant mRNA was then assayed by the same S1 analysis technique used to detect the in vivo mRNA levels. As shown in Figure 3, in vitro analysis of this gene revealed that transcription paralleled HiNF-D/Site II binding activity, H4 histone mRNA levels, and DNA synthesis. Nuclear extracts from proliferating cells were able to initiate transcription from the H4 histone gene promoter. However, nuclear extracts from serum-deprived or density-dependent, quiescent cells, which contained very low levels of HiNF-D binding activity, were unable to support detectable levels of in vitro transcription of the H4 histone gene.

The relationship between HiNF-D binding activity and H4 histone gene transcription was then examined during the stimulation of both types of quiescent fibroblasts (Figure 3). In each case only nuclear extracts prepared from fibroblasts 24 h after stimulation were able to support detectable levels of in vitro transcription (>70% of proliferating control) (Figure 3). These results paralleled the levels of in vivo H4 histone mRNA and the rate of DNA synthesis. Both density-inhibited and sparsely populated quiescent cells exhibited approximately the same rate of H4 histone gene transcription during the peak of DNA synthesis after serum stimulation. This finding is in contrast to the observation that HiNF-D/Site II binding activity is only minimally increased in the serum-stimulated, density-inhibited cultures and suggests an alternative or additional mechanism may be controlling the levels of HiNF-D binding activity in the tissuelike, density-inhibited fibroblasts.

Because quiescent cells may exhibit a reduced level of general RNA polymerase II activity, each of the nuclear extracts was also assayed for in vitro transcription activity on the adenovirus 2 major late promoter (Figure 4). While prolonged quiescence resulted in a gradual decay of the general polymerase II activity, the quiescent cell extracts which were devoid of H4 histone gene transcription activity still exhibited a significant level of adenovirus transcription (Figure 4). These results suggest that the quiescent cells have selectively downregulated transcription of the histone gene.

Discussion

Interaction between the proliferation-specific transcription factor HiNF-D and the H4 histone gene cis-acting DNA element Site II is required for initiation of H4 histone gene

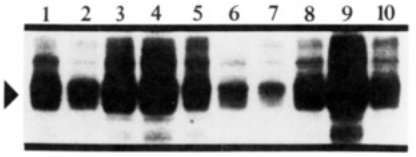

FIGURE 4: Estimation of the general RNA polymerase II activity present in the nuclear extracts. The level of cell-free transcription from the adenovirus 2 major late promoter (plasmid construct pMLH-1) (Hawley & Roeder, 1987) was used to demonstrate general RNA polymerase II activity in nuclear extracts which have specifically downregulated human H4 histone gene transcription. Proliferating normal diploid fibroblasts (lane 1), preconfluent serum-arrested cultures (lane 2), preconfluent serum-stimulated cultures 5 h (lane 3) and 24 h (lane 4) after stimulation, density-inhibited cultures 8 (lane 5), 9 (lane 6), and 10 days (lane 7) after initial plating, density-inhibited, serum-arrested cultures (lane 8), and density-inhibited, serum-stimulated cultures 5 h (lane 9) and 24 h (lane 10) after stimulation are shown. RNA transcripts were assayed by S1 nuclease protection as described in Experimental Procedures. The broad arrowhead indicates the accurately initiated adenovirus transcripts.

transcription and has been demonstrated to play a critical role in the downregulation of transcription during differentiation. In the present study we have investigated the role of this interaction in regulating H4 histone gene transcription within the context of fibroblast quiescence and serum stimulation of proliferation.

Proliferating normal diploid CF-3 fibroblasts, derived from newborn human foreskin, have high levels of HiNF-D binding activity and H4 histone gene transcription. When sparsely populated cultures become quiescent due to serum deprivation or density inhibition, H4 histone gene transcription is downregulated simultaneously with a loss of HiNF-D binding activity. This relationship between HiNF-D binding activity and H4 histone gene transcription during quiescence is the same as demonstrated for the cessation of proliferation occurring at the onset of differentiation of HL60 cells and rat osteoblasts (Stein et al., 1989; Owen et al., 1990). These results suggest that regulation of the HiNF-D/Site II interaction is an important event in the downregulation of H4 histone gene transcription. Moreover, regulation of this interaction may impinge on a common mechanism associated with the downregulation of cell growth that accompanies differentiation, serum-deprived quiescence, and density-dependent quiescence.

Sparsely populated cultures of quiescent fibroblasts can be serum-stimulated to reenter the proliferative cycle (Zetterberg & Larson, 1985), and thus they provide the opportunity to observe the reactivation of H4 histone gene transcription and the role of the HiNF-D/Site II interaction in this process. HiNF-D binding activity is undetectable in the quiescent populations but is upregulated in parallel with the activation of H4 histone gene transcription as the cultures resume proliferation. This suggests that HiNF-D is not only functionally related to the downregulation of H4 histone gene transcription during the onset of quiescence or differentiation but, additionally, is a key element in the upregulation of transcription occurring during serum stimulation of normal quiescent cells.

Normal human fibroblasts, when allowed to become density-inhibited in culture, downregulate proliferation and enter a G_0 resting state. This may be analogous to the conditions present in tissues. Serum stimulation of such density-dependent, quiescent fibroblasts initiates proliferative activity and the required H4 histone gene transcription. However, HiNF-D binding activity is upregulated to only 30% of maximal levels, while H4 histone gene transcription is activated to near normal levels. Thus, the 30% of HiNF-D binding activity present may represent a threshold level of HiNF-D required by the fibroblast. Furthermore, the difference in activation of HiNF-D binding activity between serum stimulation of sparsely populated quiescent fibroblasts and of density-dependent quiescent fibroblasts indicates two different mechanisms may be operational in the regulation of HiNF-D binding activity.

One possible model for the regulation of HiNF-D binding activity incorporates a primary mechanism controlling HiNF-D in actively proliferating fibroblasts and fibroblasts which are serum-arrested but capable of proliferation. This may be the same mechanism controlling the cell cycle regulation of HiNF-D in normal diploid cells (Holthuis et al., 1990). Upon density-dependent quiescence and entry into the G_0 state, a second mechanism may be activated to repress HiNF-D binding activity. Serum stimulation is not able to overcome completely this second level of repression. One can speculate that the function of a second mechanism downregulating HiNF-D binding activity would be to control replication-dependent H4 histone gene transcription in cells which are programmed to remain quiescent or to undergo only a limited amount of proliferation before reentering a quiescent state. The appearance of an extracellular matrix and cell to cell communication analogous to that found in an intact tissue may be the means by which the cell is signaled to enter a deeper quiescent state and to activate a second mechanism for regulating proliferation-specific factors such as HiNF-D. A process akin to the serum stimulation of density-inhibited cultures may be the limited proliferative activity observed in dermal fibroblasts during wound healing (Tucker & Boone, 1981).

In conclusion, the HiNF-D/Site II interaction plays a critical role in the regulation of H4 histone gene transcription. It is important during both the downregulation of proliferation at the onset of differentiation and quiescent and the upregulation of proliferation due to serum stimulation of quiescent fibroblasts. HiNF-D binding activity is regulated minimally at two levels, dependent on the proliferative state of the cell and the programmed future requirements for H4 histone gene transcription.

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The Iron Regulatory Region of Ferritin mRNA Is Also a Positive Control Element for Iron-Independent Translation†

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ABSTRACT: The iron regulatory element (IRE) in the 5′-untranslated region of ferritin mRNA interacts with a specific regulator protein (P-90, IRE-BP, or FRP) to block translation. High cellular iron changes the IRE/P-90 interaction to relax the translational block and allow polyribosome formation. We now show that the IRE and base-paired flanking regions also enhance translation in the absence of P-90, explaining the high translational efficiency of deregulated ferritin mRNA observed previously. The effect of the IRE on translational efficiency was examined by comparing four sets of mRNAs: (1) ±IRE in animal (frog) ferritin, regulated translationally by iron in vivo; (2) ±animal IRE fused with plant (soybean) ferritin, regulated transcriptionally by iron in vivo; (3) repositioned IRE in animal ferritin; (4) mutated IRE in animal ferritin with G16A substitution, which decreases P-90 binding (negative control). The IRE region increased translational efficiency of both the animal ferritin and the heterologous IRE/soybean ferritin fusion mRNAs; the effect was observed in cell-free translation systems from either plants (wheat germ) or animals (rabbit reticulocyte). Repositioning the IRE further from the 5′ cap eliminated positive control of translation. The single base mutation had no effect, indicating that positive and negative translational control involves different sections of the IRE region. Thus, the IRE region in ferritin mRNA encodes both positive translational control and, when combined with the regulator protein P-90, negative translational control.

A highly conserved, 28-nt, complex stem–loop structure (IRE/iron regulatory element) is present in the 5′-untranslated region (UTR) of all known vertebrate ferritin mRNAs (Theil, 1990) and in the 3′ UTR of transferrin receptor (TfR) mRNAs (Klausner & Harford, 1989). The IRE is the first example of an RNA element which mediates coordinate regulation of two mRNAs encoding metabolically related proteins (ferritin for iron storage, TfR for iron uptake) by the same signal and may be a model for coordinate regulation of mRNAs encoding other metabolically related proteins.

In ferritin mRNAs, the IRE is structurally integrated with base-paired flanking regions close to the 5′ cap. The flanking sequences are conformationally altered (increased base-stacking) when the mRNA regulator protein P-90 binds the IRE (Harrell et al., 1991); P-90 specifically blocks translation of ferritin mRNA at low levels of intracellular iron (Walden et al., 1988, 1989; Brown et al., 1989). Increased intracellular iron leads to increased ferritin synthesis, apparently through changes in the IRE/P-90 interaction which relax structure in the flanking regions (Harrell et al., 1991); under such conditions, ferritin mRNA forms polyribosomes either in vivo (Zahringer et al., 1976) or in vitro in cell-free extracts lacking

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