Journal of Cellular Biochemistry 50:62–72 (1992)

Differential Regulation of H4 Histone Gene Expression in 3T3-L1 Pre-Adipocytes During Arrest of Proliferation Following Contact Inhibition or Differentiation and Its Modulation by TGFβ_1

Rita Bortell, Andre J. van Wijnen, Anna L. Ramsey-Ewing, Gary S. Stein, and Janet L. Stein

Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655

Abstract The aim of this study was to address whether there is a fundamental difference in regulation of histone gene expression in cells that have become quiescent but retain the ability to proliferate, compared wtih those cells that have differentiated. We compared multiple levels of regulation of histone gene expression during 3T3-L1 pre-adipocyte differentiation. Confluent cells induced to differentiate by treatment with insulin, dexamethasone, and isobutylmethylxanthine initially exhibited an increased proliferative response compared with cells given serum alone. This initial differentiation response was associated with a twofold increase in both histone gene transcription and cellular histone mRNA levels, as well as with enhanced sequence-specific binding of nuclear factors to the proximal cell-cycle-regulatory element of the H4 histone promoter. Transforming growth factor β_1, an inhibitor of 3T3-L1 differentiation, increased both the percentage of proliferating cells and the cellular levels of histone mRNA when given in addition to serum stimulation, but no enhancement of these parameters was observed upon addition of TGFβ_1 to the differentiation treatment. Interestingly, although TGFβ_1 enhanced binding of nuclear factors to the proximal cell cycle regulatory element of the histone promoter, these protein/DNA interactions were not associated with an increase in histone transcription. Our results are consistent with the down-regulation of histone gene expression at confluency being controlled primarily at the post-transcriptional level, in contrast to an increased involvement of transcriptional down-regulation at the onset of differentiation. © 1992 Wiley-Liss, Inc.

Key words: adipogenesis, quiescence, transcription, mRNA, nuclear factors

The regulation of histone gene expression is tightly controlled during the cell cycle, as it is necessary to ensure the timely synthesis of core histone proteins for the packaging of newly synthesized DNA into nucleosomes, the primary unit of chromatin structure [McGhee and Felsenfeld, 1980; Weisbrod, 1982]. Our laboratory and others have studied histone expression during the cell cycle and found both transcriptional and post-transcriptional (via mRNA stability) regulation to play a role [Artishevsky et al., 1987; Baumbach et al., 1987; Heintz et al., 1983; Morris et al., 1990; Osley, 1991; Plumb et al., 1983]. In addition, a further mechanism to regulate histone gene expression at the level of mRNA 3′ end processing appears to be involved [Harris et al., 1991; Stauber et al., 1986]. Although the control of histone expression during the cell cycle has been extensively studied, to date limited information is available concerning the relative contribution of these gene regulatory mechanisms when cells exit the cell cycle and enter a phase of arrested proliferation. A fundamental question is whether this regulation differs depending on the type of proliferation arrest, as in the case of contact inhibition (G_0), as opposed to the arrest of proliferation that occurs during differentiation (G_D).

We have addressed these questions in the 3T3-L1 pre-adipocyte cell line because these cells are strongly contact-inhibited and additionally can be induced to differentiate into adipocytes [Elks and Manganiello, 1985; Green and Kehinde, 1974, 1975; Hauner, 1990]. More importantly, unlike many other cells that exhibit decreased proliferative activity immediately upon induction of differentiation, 3T3-L1 pre-adipo-

Received February 17, 1992; accepted April 30, 1992.

Address reprint requests to Gary S. Stein, Department of Cell Biology, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655.

© 1992 Wiley-Liss, Inc.

cytes undergo one or more rounds of cell division upon stimulation to differentiate. Experimental evidence indicates that this stimulated proliferative activity is required for the complete development and expression of the adipocyte phenotype [Green and Meuth, 1974b]. Taken together, these properties permit a comparison of the regulation of histone gene expression under two distinguishable growth regulatory conditions: during proliferative subconfluent growth followed by contact inhibition at confluency, and following induction of differentiation where stimulated proliferative activity is followed by the arrest of proliferation. Because this second round of post-confluent proliferation is necessary for differentiation, we investigated whether transforming growth factor β_1 (TGFβ_1), an inhibitor of 3T3-L1 differentiation [Ignotz and Massague, 1985], would alter the regulation of histone gene expression when the cells are prevented from differentiating.

MATERIALS AND METHODS

Cell Culture

The 3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained at 37°C with 95% air/5% CO_2. The cells were plated at a density of 7×10^4/35 mm diameter well or 5×10^5/100 mm diameter plate. The cultures were fed twice weekly with Dulbecco's modified minimal medium supplemented with 5% calf serum. Cells used for differentiation experiments were maintained at confluency for 3–4 days and re-fed on days 0 and 2 with medium supplemented with 10% fetal calf serum and no additions (control group), 100 pM TGFβ_1 (TGFβ_1 group), induction "cocktail" (0.2 μM insulin, 0.4 μM dexamethasone, and 0.5 mM methyl isobutylxanthine) (differentiation group), or induction "cocktail" plus 100 pM TGFβ_1 (differentiation + TGFβ_1 group).

In Situ Thymidine Incorporation

Cells grown on plastic cover slips were incubated for 24 h with 1 μCi/ml [^{3}H]-thymidine. The cover slips were then air dried, mounted on glass slides, covered with emulsion, and allowed to develop for 6–7 days before processing. The percentage of cells with labeled nuclei was determined under phase microscopy.

RNA Analysis

At appropriate time points, cells were scraped in phosphate buffered saline with 10 μg/ml diethylpolyvinylcarbonate. The cells were pelleted by centrifugation for 5 min at 3,000*g* and total cellular RNA was isolated [Baumbach et al., 1987]. Isolated RNA was bound to Zetaprobe nylon (BioRad Laboratory, Richmond, CA) in a slot blot apparatus and hybridized with the insert from a human H4 histone gene cloned in construct pFO108 [Sierra et al., 1983] which was [^{32}P]-labeled by random priming [Feinberg and Vogelstein, 1983].

Transcription Run-On Analysis

Nuclei were isolated [Greenberg and Ziff, 1984], frozen in liquid nitrogen, and stored at −70°C. Run-on analysis was done as described by Baumbach et al. [1987]. Labeled RNA transcripts ($2.5–5 \times 10^5$ cpm/ml) were hybridized to Zetaprobe nylon containing H4 histone gene plasmid pFO108 with 2 μg of insert and a 28S ribosomal gene plasmid containing 2 μg of insert to normalize the data.

Gel Retardation Assays

Nuclei were isolated and nuclear extracts prepared as described [Holthuis et al., 1990], with the exception that the Triton X-100 in the lysis step was replaced with NP-40. Also, Mg^{2+} was replaced in all buffers with a mixture of spermine/spermidine/EGTA [van Wijnen et al., 1991a]. The DNA fragment used for gel retardation assays was the −97 to −38 nt fragment (relative to the ATG translational start site) of the FO108 H4 histone gene [van Wijnen et al., 1991b]. Binding reactions contained the single-end radiolabeled probe (0.5 ng), 3–9 μg of nuclear extract proteins, nonspecific competitor DNA [2 μg poly(dG-dC) × poly(dG-dC) + 0.2 μg poly(dI-dC) × poly(dI-dC)], and were done in buffer containing 50 mM KCl, 12.5 mM Hepes, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 0.005% NP-40, and 10% glycerol; incubations were for 15 min at 25°C. Protein/DNA interactions were fractionated on 4% polyacrylamide gels (80:1 acrylamide:bis-acylamide) in low ionic strength buffer (6.6 mM Tris/HCl, pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA) maintained at 4°C and continuously recirculated between the anode and cathode chambers. Electrophoresis was for 2.5 h at 200 V. Gels were vacuum dried and exposed to XAR-5 autoradiography film.

RESULTS

The primary objective of these experiments was to study systematically the regulation of H4

histone gene expression during proliferation and the subsequent cessation of proliferation in two different biological situations: during proliferation of sub-confluent 3T3-L1 pre-adipocytes followed by contact inhibition at confluency, and during the stimulation of proliferative activity of confluent 3T3-L1 pre-adipocytes associated with inducation of differentiation and the subsequent cessation of proliferation with expression of the mature adipocyte phenotype. We studied several levels at which H4 histone gene expression may be regulated, including cellular H4 mRNA amount, transcriptional activity of the H4 gene, and binding activity of the histone nuclear factor D (HiNF-D) to the cell-cycle-regulatory Site II element of the H4 promoter [van Wijnen et al., 1989, 1991a; Ramsey-Ewing et al., manuscript submitted].

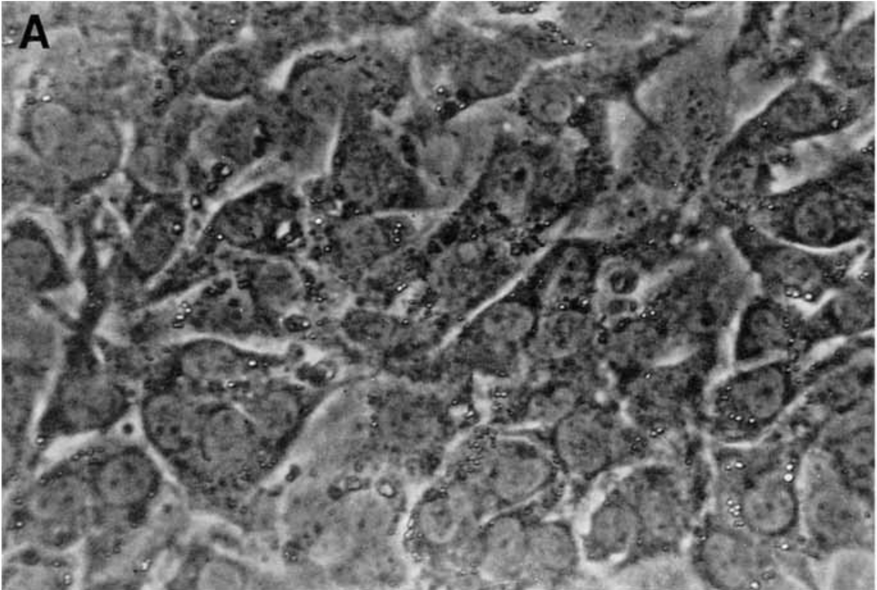
To monitor morphological characteristics of control and differentiated 3T3-L1 cultures, we examined each plate of cells by phase microscopy. As seen in Figure 1A, control cells exhibited no phenotypic characteristics of spontaneous adipocyte differentiation, but maintained the fibroblast-like morphology of pre-adipocytes. Cells in the TGFβ_1 group (Fig. 1B) showed a more elongated, needle-like fibroblast morphology compared with cells from the other groups. The 3T3-L1 cells treated with differentiation cocktail attained the larger, more rounded shape of the mature adipocyte, with numerous lipid vacuoles in the cytosol (Fig. 1C). Cells given the differentiation cocktail + TGFβ_1 (the inhibitor of 3T3-L1 differentiation) showed a fibroblast-like morphology (Fig. 1D) similar to that of control 3T3-L1 cells. These micrographs indicate that each treatment had distinct morphological effects on the 3T3-L1 cells.

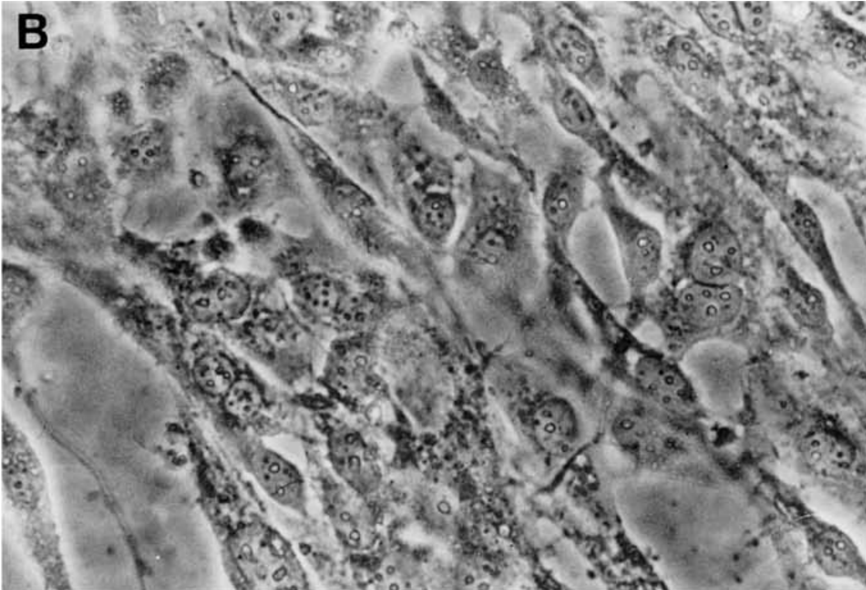
To determine the proliferative activity of the 3T3-L1 pre-adipocytes, cell counts were done over the time course of these experiments. Subconfluent 3T3-L1 cells exhibited exponential growth for a period of 2 days, at which time the cells approached confluency and cell numbers reached a plateau (Fig. 2). This corresponded to the first arrest of proliferation. After 3–4 days at confluency these cells were stimulated (day 0) with 10% fetal calf serum in the presence or absence of induction cocktail. We observed that the induction of differentiation resulted in a greater increase in cell counts than serum stimulation alone (Fig. 2). The addition of TGFβ_1 to serum also increased cell growth, although less than for the differentiation treatment. The addition of TGFβ_1 to the differentiation treatment, however, did not further increase cell growth. By day 3–4 the cell counts approached a plateau in all treatment groups. This corresponded to the second arrest of proliferation. Hence, in our experimental time course there were two distinct proliferative periods, and the amount of proliferative activity during the second period varied among treatments.

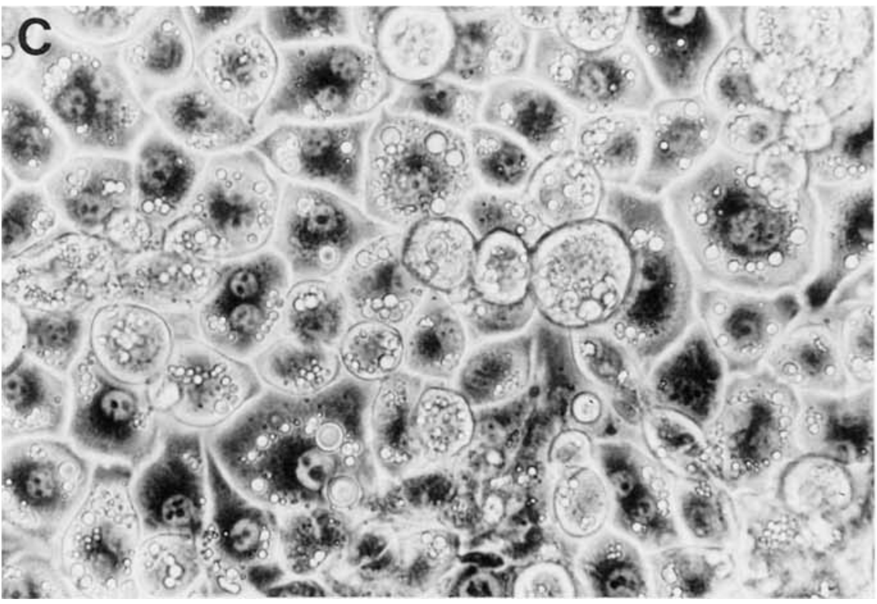
Because cell counts may not accurately reflect the percentage of cells in the population undergoing proliferation, we also measured in situ [^{3}H]-thymidine incorporation. The data in Table I indicate that > 95% of the subconfluent cells (day −4) were proliferating as reflected by [^{3}H]-thymidine–labeled nuclei. When the cells reached confluency (day 0), thymidine incorporation decreased to < 10%. When these confluent cells were stimulated at day 0 with 10% fetal calf serum (control), only 30% of the cells incorporated thymidine after 1 day, whereas > 90% of the cells in each of the 3 treatment groups were stimulated to proliferate. The percentage of cells incorporating thymidine following 2 and 3 days of treatment was generally the same as for day 1; that is, cells from the TGFβ_1, differentiation, or differentiation + TGFβ_1 groups were maximally proliferating, whereas only 30–45% of cells in the control group were proliferating. At day 4 the percent of cells incorporating thymidine in all but the control group began to decline, and by days 5 and 6 thymidine incorporation in all groups was < 5%. These results demonstrate that in situ thymidine labeling was consistent with cell counts; for example, treated cells exhibited > 90% thymidine incorporation and higher cell counts (Fig. 2) than control cells, whereas control cells exhibited both decreased thymidine incorporation (30–45%) and lower cell counts (Fig. 2) than treated cells.

We next measured the cellular levels of H4 histone mRNA in the 3T3-L1 cells during the experimental time course. The H4 histone mRNA levels were twofold higher in proliferating, subconfluent cells compared to those that

Fig. 1. Morphological changes in 3T3-L1 pre-adipocytes following control, TGFβ_1, differentiation, or differentiation + TGFβ_1 treatments. Subconfluent 3T3-L1 cells were allowed to become confluent for 3–4 days and then treated on day 0. Cells from the 4 different groups were examined by phase microscopy 6 days following treatment. Cells shown are from the following groups: (**A**) control; (**B**) TGFβ_1; (**C**) differentiation; and (**D**) differentiation + TGFβ_1. ×100.







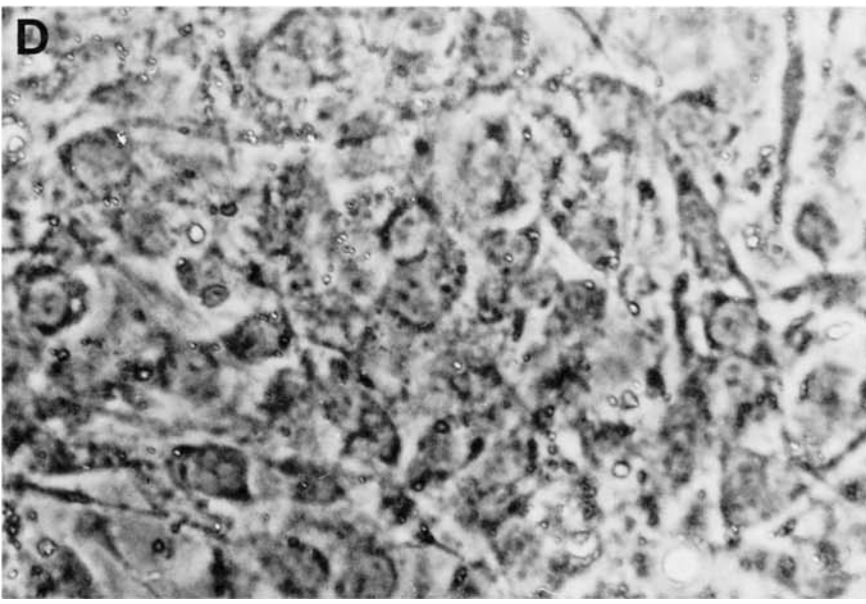


Figure 1.

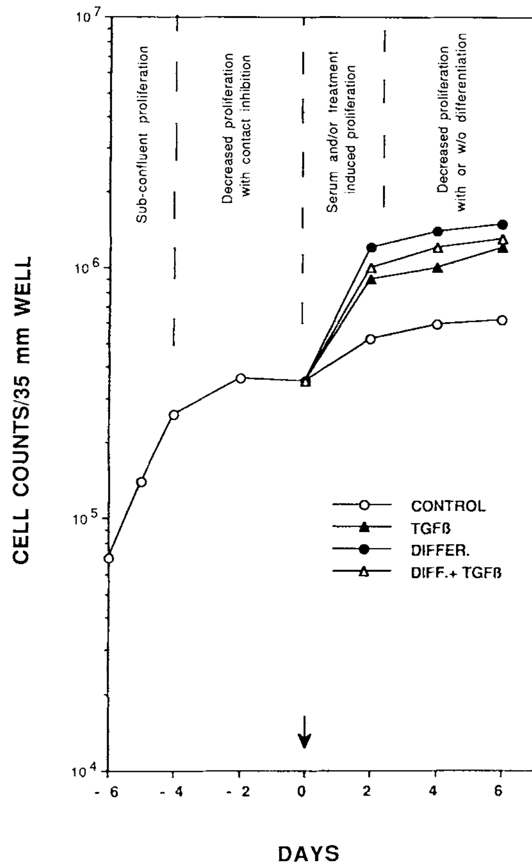


Fig. 2. Two distinct proliferative periods occur in the time course of 3T3-L1 development. Cell counts were determined for subconfluent 3T3-L1 pre-adipocytes beginning at day −6 (6 days prior to treatment) and continuing for 6 days after treatment. Arrest of cell proliferation at day −3 to −2 coincided with the attainment of confluency. Following initiation of treatments on day 0 (as shown by arrow), a second proliferative period was induced for 3 days followed by arrested cell proliferation in all groups. Data are the mean of 2 independent experiments.

TABLE I. Percent Nuclei Incorporating [^{3}H]-Thymidine in Control or Treated 3T3-L1 Cells Over a Time Course Spanning −4 to +6 Days (Treatments Began on Day 0)

Treatment	Day	Thymidine incorporation (%)
None (subconfluent)	−4	98
None (confluent)	0	6
Control	1	30
TGFβ$_1$		94
Differentiation		90
Differentiation + TGFβ$_1$		93
Control	2	45
TGFβ$_1$		96
Differentiation		91
Differentiation + TGFβ$_1$		90
Control	3	40
TGFβ$_1$		85
Differentiation		95
Differentiation + TGFβ$_1$		98
Control	4	45
TGFβ$_1$		75
Differentiation		40
Differentiation + TGFβ$_1$		50
Control	5	2
TGFβ$_1$		5
Differentiation		1
Differentiation + TGFβ$_1$		1
Control	6	5
TGFβ$_1$		2
Differentiation		1
Differentiation + TGFβ$_1$		1

were confluent (Fig. 3). The second proliferative phase was initiated by treatment of contact-inhibited cells with 10% fetal calf serum ± the 3 different treatments. The mRNA levels of H4 histone increased twofold in cells serum-stimulated for 1 day, restoring cellular H4 histone mRNA levels to those of subconfluent cells. Following induction of differentiation, however, there was an additional 30% increase in mRNA above that for serum stimulation alone. The addition of TGFβ$_1$ either to confluent control cells or to cells induced to differentiate did not affect the level of H4 histone mRNA at day 1 (Fig. 3). However, at day 2 the H4 histone mRNA levels in the TGFβ$_1$-treated cells remained elevated, whereas the values for all other groups decreased to the level observed during contact inhibition. By day 4, the H4 histone mRNA levels of the TGFβ$_1$-treated cells had decreased to contact-inhibited levels. At the end of the time course (day 6), with the exception of untreated controls, all cells exhibited an additional 20–30% decrease in H4 histone mRNA content. These results show a correlation between H4 histone mRNA levels and the proliferative activity of the 3T3-L1 cells; that is, H4 mRNA levels were highest (Fig. 3) when the rate of cell proliferation was greatest (Fig. 2).

To determine the relative contribution of transcription to the cellular levels of H4 histone mRNA, we assayed the transcriptional activity of the H4 histone gene by nuclear run-on analysis. Proliferating, subconfluent cells exhibited no change (< 10%) in H4 histone gene transcription compared with that observed at confluency (Fig. 4), while cellular H4 histone mRNA content was twofold greater in subconfluent compared with confluent cells (Fig. 3). This suggests

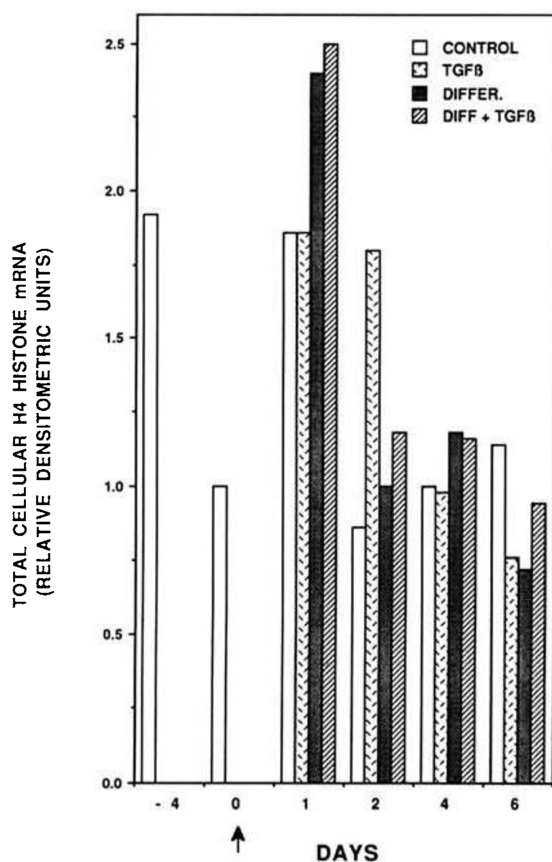


Fig. 3. Cellular levels of histone H4 mRNA during the two proliferative periods of 3T3-L1 development. By cell counts and in situ thymidine incorporation, days −4 and 0 correspond to rapid proliferation of subconfluent cells and contact inhibition at confluency, respectively. Histone H4 mRNA levels declined twofold at the end of this first proliferative period in the 3T3-L1 time course. The arrow at day 0 indicates the initiation of control, $TGF\beta_1$, differentiation, or differentiation + $TGF\beta_1$ treatments; this corresponds to the beginning of the second proliferative period. Histone mRNA levels increased to subconfluent (or higher) levels, but decreased again with the decline in proliferative activity beginning at day 3–4. Data shown are the mean of 4 independent time course experiments.

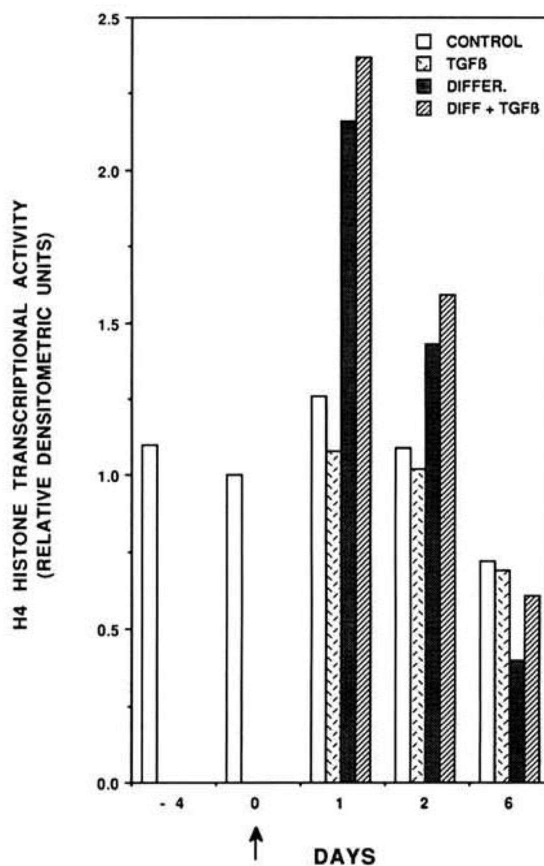


Fig. 4. Transcriptional activity of the H4 histone gene during the two proliferative periods of 3T3-L1 development. By cell counts and in situ thymidine incorporation, days −4 and 0 correspond to rapid proliferation of subconfluent cells and contact inhibition at confluency, respectively; this represents the first proliferative period in the 3T3-L1 time course. Virtually no change in the transcriptional activity of histone H4 was seen during this period. The arrow at day 0 indicates the initiation of control, $TGF\beta_1$, differentiation, or differentiation + $TGF\beta_1$ treatments. This corresponds to the beginning of the second proliferative period and was marked by a twofold increase in transcription for cells given the differentiation cocktail (± $TGF\beta_1$), but only minor changes in transcription for cells given serum alone (± $TGF\beta_1$). At day 6 the adipocytes in the differentiated group exhibited 50% less H4 transcriptional activity than the 3T3-L1 cells in the control group which have not differentiated. Data shown are the mean of 3 independent time course experiments.

that post-transcriptional mechanisms predominate in the down-regulation of histone gene expression at the end of the first proliferative period (contact inhibition). During the second proliferative period, stimulation of cells for 1 day with 10% fetal calf serum (control) increased H4 histone gene transcription slightly (Fig. 4). The addition of $TGF\beta_1$ did not change the rate of H4 histone gene transcription relative to control. Following induction of differentiation both in the presence or absence of $TGF\beta_1$, however, there was a twofold increase in H4 histone gene transcription by day 1 (Fig. 4), which was almost sufficient to account for the 2.5-fold increase in cellular H4 histone mRNA content relative to the confluent cells (day 0) (Fig. 3). Thus, increased transcriptional activity plays a greater role in the accumulation of H4 histone mRNA in those cells induced to differentiate, irrespective of the presence or absence of $TGF\beta_1$, compared with those which were serum stimulated (control). Moreover, these data indicate a fundamental difference in the down-regulation of histone gene expression with contact inhibition (primarily post-transcriptional)

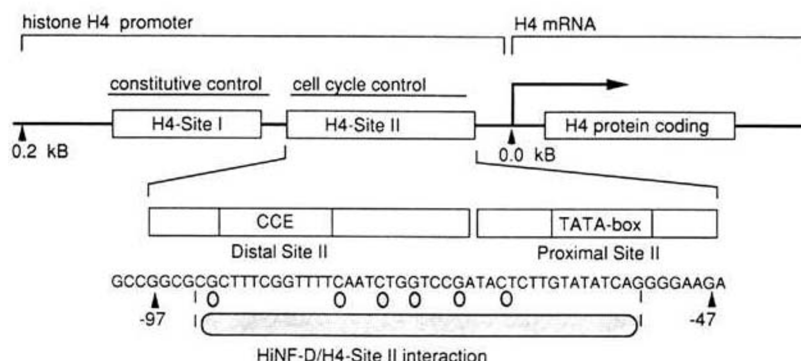


Fig. 5. Schematic diagram of the H4 histone gene (pFO108A). The elements labeled Site I and Site II have been identified by DMS protection in vivo [Pauli et al., 1987]. Site I is involved in constitutive control of H4 transcription, whereas Site II is involved in the cell cycle regulation of the H4 gene [Ramsey-Ewing et al., manuscript submitted; Holthuis et al., 1990]. The cell cycle element (CCE) is contained within the distal region of Site II and the TATA element is found within the proximal region. The region in which histone nuclear factor D (HiNF-D) binds Site II was determined by DNase I footprinting; methylation interference contacts are depicted by closed circles below the nucleotides [van Wijnen et al., 1989; 1991a].

compared with that observed with differentiation (both transcriptional and post-transcriptional).

To further address transcriptional control of H4 histone gene expression in the 3T3-L1 adipocyte system, we assayed protein/DNA interactions in the Site II proximal promoter element of the H4 histone gene responsible for S phase-specific up-regulation of transcription [Ramsey-Ewing et al., manuscript submitted]. A diagram of this Site II promoter element and its in vivo DMS protection contacts is shown in Figure 5. Specific binding to the Site II promoter element by HiNF-D (histone nuclear factor D) derived from 3T3-L1 cells is demonstrated in Figure 6. Here a hundredfold molar excess of unlabeled Site II oligomer completely eliminates HiNF-D binding, whereas the same molar excess of an unrelated AP-1 oligomer has no effect.

To correlate HiNF-D binding activity with the transcriptional activity of the H4 gene, nuclear extracts were prepared from 3T3-L1 cells throughout the experimental time course. Binding of HiNF-D to the Site II promoter element was prominent in rapidly proliferating subconfluent cells (day −4) (Fig. 7A). After 3–4 days of confluency (day 0), however, the presence of HiNF-D binding activity in nuclear extracts had decreased dramatically (Fig. 7A), even though the amount of H4 histone gene transcription (Fig. 4) did not change at the end of this first proliferative period. When the second proliferative period was initiated by stimulating cells with 10% fetal calf serum alone (control) or with the induction cocktail and/or TGFβ_1, there was a pronounced difference in HiNF-D binding activity one day after treatment (Fig. 7B); that is, control extracts at day 1 exhibited binding activity similar to day 0 confluent extracts, whereas differentiation or TGFβ_1 extracts showed enhanced binding at day 1. Also, there was an additive effect on HiNF-D binding activity of simultaneous TGFβ_1 and differentiation treatments. By 2 days of treatment the HiNF-D/Site II protein/DNA complex was enhanced in all groups (Fig. 7C). However, by day 6 the HiNF-D binding activity in all groups had declined dramatically (Fig. 7D) in parallel with a decrease in the amount of H4 histone gene transcription (Fig. 4). These results show a down-regulation of both HiNF-D binding activity and H4 gene transcriptional activity during the cessation of the second proliferative period, whereas this correlation was not observed during the first proliferative period.

DISCUSSION

These studies were directed towards addressing molecular mechanisms operative in the down-regulation of H4 histone gene expression associated with exit from the cell cycle into a nonproliferative G_0 state and into a post-proliferative state accompanied by the onset and progression of differentiation. We examined the regulation of a cell-cycle-controlled H4 histone gene in quiescent, contact-inhibited 3T3-L1 preadipocytes and following induction of adipocyte differentiation by insulin, dexamethasone, and methylisobutylxanthine.

We observed a twofold higher level of H4 histone mRNA but little or no change in transcription (as measured by nuclear run-on analy-

Fig. 6. Protein/DNA interactions of HiNF-D at the Site II proximal promoter region are sequence specific. Nuclear protein extracts (5 μg) from rapidly proliferating subconfluent 3T3-L1 cells were incubated with radiolabeled Site II H4 histone promoter alone or in the presence of hundredfold molar excess of the specific unlabeled Site II oligomer or hundredfold molar excess of the non-specific AP-1 binding site from the metallothionein promoter [Rauscher et al., 1990]. The unlabeled Site II oligomer, but not the non-specific oligomer, was able to compete for binding.

sis) between proliferating, subconfluent cells and those which were contact inhibited. We and others have previously reported a ten- to twenty-fold increase in histone mRNA and a two- to fivefold increase in transcription in the S phase compared with the G_1 phase of the cell cycle in synchronized HeLa cells [Baumbach et al., 1987; La Bella et al., 1989; Sive et al., 1984]. This apparent difference between subconfluent and confluent 3T3-L1 cells vs. S and G_1 phase HeLa cells may in part be explained by the fact that the exponentially growing, subconfluent 3T3-L1 cells were not synchronized; consequently, differences in H4 histone gene expression between these cells and confluent cells were muted. On the basis of the ratio of H4 histone mRNA levels to the rate of H4 histone gene transcription, however, our results are consistent with findings from the cell cycle studies which indicate that post-transcriptional regulation of histone mRNA makes a major contribution to the accumulation of cellular H4 histone mRNA during S phase [Baumbach et al., 1987; La Bella et al., 1989; Morris et al., 1990; Sive et al., 1984]. The twofold increase in H4 histone mRNA levels and only minor changes in transcription in subconfluent cells compared to confluent 3T3-L1 cells indicate the significant contribution of post-transcriptional control.

When confluent 3T3-L1 cells were stimulated with serum or the differentiation cocktail, there were major differences in the regulation of histone gene expression. For example, 3T3-L1 cells stimulated for 1 day with serum alone (control) showed a twofold increase in H4 histone mRNA levels but only a minor increase in H4 histone gene transcription. Induction of differentiation resulted in a 2.5-fold increase in H4 histone mRNA levels, but unlike serum-stimulation of cells, this increase was almost accounted for by the twofold increase in H4 histone gene transcription. Furthermore, this stimulation in transcription was not restricted to the H4 histone gene. A similar relationship between mRNA levels and rates of transcription was observed for H3 and H1 histone genes (data not shown). A coordinate regulation of histone genes during induction of differentiation, in addition to that seen during the cell cycle [Plumb et al., 1983], is therefore suggested.

Equally important, our results provide a direct indication that regulatory mechanisms operative in control of histone gene expression are different when quiescent cells are stimulated to proliferate by serum alone, compared with the stimulation of proliferation leading to tissue-specific phenotype expression. Indeed, the H4 histone promoter element in the distal portion of Site II, which is responsible for cell cycle regulation, is not necessary for transcriptional regulation during 3T3-L1 differentiation. Rather, a minimal H4 promoter (nt −76 to −41) mediates the differentiation-induced response of histone gene expression [Ramsey-Ewing et al., manuscript submitted]. These differences in regulation of a cell cycle gene may provide insight into regulatory mechanisms mediating the proliferation/differentiation relationship in a broad biological context.

Several lines of evidence suggest that changes in "programming" occur within the first 36–40

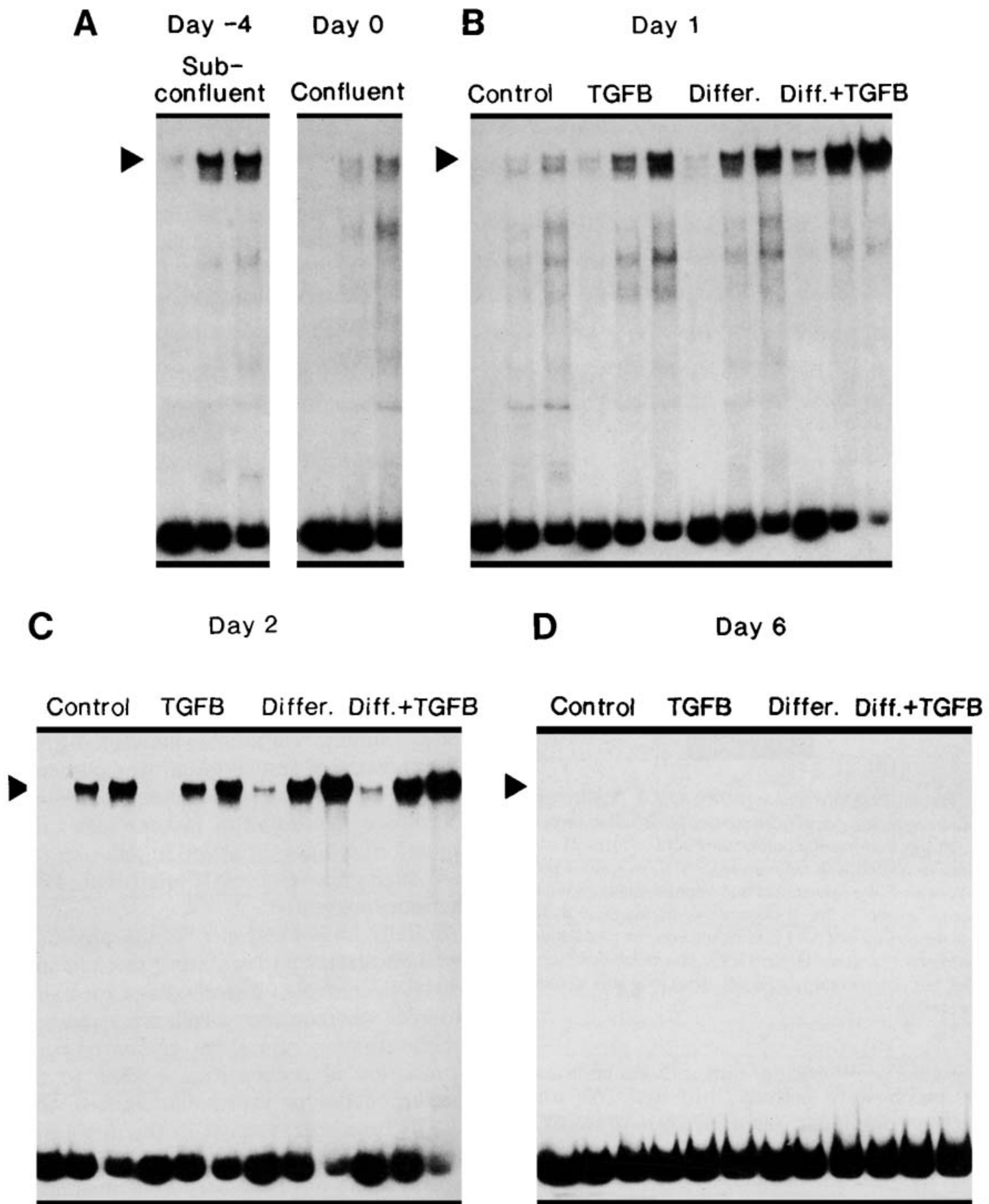


Fig. 7. HiNF-D/Site II interactions during the two proliferative periods of 3T3-L1 development. Nuclear protein extracts were prepared from 3T3-L1 cells over a time course encompassing the two proliferative periods. Nuclear proteins (3, 6, and 9 μg) were incubated with radiolabeled Site II H4 histone promoter and the resulting protein/DNA interactions were fractionated by electrophoresis. The protein/DNA interaction representing binding of HiNF-D is designated by an arrow. **A:** HiNF-D/Site II interactions in subconfluent (proliferating) extracts were prominent as opposed to the decreased binding activity seen at confluency (non-proliferating). **B:** Following 1 day of treatment with fetal bovine serum there was little change in HiNF-D binding activity, but this activity was enhanced with $TGF\beta_1$, differentiation, or differentiation + $TGF\beta_1$ treatments. **C:** Two days following treatment all groups exhibited marked HiNF-D binding activity. **D:** Six days following treatment proliferation had ceased in all groups and HiNF-D binding activity was not evident or was barely detectable.

h of pre-adipocyte differentiation, since TGFβ_1 is able to block differentiation of these cells only within, but not after this period has passed [Ignotz and Massague, 1985]. Our results clearly demonstrate that in confluent 3T3-L1 cultures more cells are stimulated to enter DNA synthesis by TGFβ_1 treatment than by serum alone. TGFβ_1 is known to have variable effects on proliferation in many cell culture systems, depending on the type and developmental stage of the cell [Bortell et al., 1990; Fine and Goldstein, 1987; McMahon et al., 1986; Robey et al., 1987; Sporn et al., 1987]. Although the absence of a TGFβ_1 effect on [^{3}H]-thymidine incorporation into 3T3-L1 cells has been observed [Ignotz and Massague, 1985], this may in part reflect a TGFβ_1 effect on the size and/or specific activity of nucleotide precursor pools which relate to the level of TCA-precipitable radioactivity. We have directly determined the effect of TGFβ_1 on DNA synthesis by autoradiographically assaying the percent of cells undergoing DNA synthesis, permitting [^{3}H]-thymidine incorporation to be monitored at the single cell level. However, we cannot entirely dismiss the possibility that the presence or absence of a TGFβ_1 effect on DNA synthesis may reflect a difference in the growth factor content of the sera, or variations in the phenotypic properties of the 3T3-L1 cells. In addition, although TGFβ_1 had no significant effect directly on H4 histone transcription, it is interesting to note that 3T3-L1 cells exhibited a twofold increase (compared to control) in cellular levels of H4 histone mRNA 2 days following treatment with TGFβ_1. These data suggest an action of TGFβ_1 to increase histone mRNA stability. Taken together, our results indicate that multiple mechanisms mediate cellular responses during the early events of differentiation, preceding the appearance of many biochemical or cellular markers of the adipocyte phenotype [Bernlohr et al., 1985; Pairault and Green, 1979; Spiegelman et al., 1983].

This study also demonstrates that TGFβ_1 treatment of 3T3-L1 cells enhances the binding of a nuclear factor (HiNF-D) to the cell-cycle-regulated Site II element of the histone H4 promoter. A recent report showed that an early event in TGFβ_1 action is the induction of nuclear protein phosphorylation [Kramer et al., 1991a]; more specifically, TGFβ_1 induced phosphorylation of the cyclic AMP responsive binding protein (CREB) in mink lung CC164 cells, with a concomitant increase in binding to the collagenase TPA responsive element [Kramer et al., 1991b]. We have previously demonstrated that HiNF-D binding activity is phosphorylation dependent and that the slow-mobility HiNF-D/Site II complex can be converted to faster-mobility protein/DNA complex(es) by phosphatase treatment [van Wijnen et al., 1991a]. Our data therefore suggest that TGFβ_1 may enhance the HiNF-D/Site II interaction through increased phosphorylation of nuclear proteins. This increase in binding is a very early event in TGFβ_1 action, as we have found enhancement of the HiNF-D/Site II interaction by 3.5 hours of TGFβ_1 treatment (data not shown).

Although we observe a TGFβ_1-mediated increase in binding of HiNF-D to the H4 histone promoter at day 1, this is not accompanied by an increase in H4 histone gene transcription. These findings suggest that, in 3T3-L1 cells, the occupancy of Site II by HiNF-D may be necessary but not sufficient to support upregulated transcription of the H4 histone gene by TGFβ_1. This implies that additional modifications in protein/DNA interactions at the H4 Site II, or at other cis-acting elements in the H4 promoter, are required to accommodate alterations in the H4 histone gene transcription rates. Thus, further understanding of the TGFβ_1 influence on the HiNF-D/Site II complex necessitates defining TGFβ_1 effects on the interaction of an interrelated series of transcription factors with the multiple regulatory elements present in the H4 histone gene promoter [van Wijnen et al., 1989, 1991a]. The multiplicity and combinatorial possibilities of these interactions may account for variations in the manner by which specific transcriptional levels are established and maintained under different biological conditions.

In a broader context our results are consistent with a fundamental difference in the regulation of histone gene expression in two different biological states of the 3T3-L1 cell, during proliferation arrest following contact inhibition compared with proliferation arrest that accompanies the onset of adipocyte differentiation. During subconfluent growth H4 histone mRNA levels were higher than at confluency, but the rate of transcription was not different. In contrast, following induction of differentiation in 3T3-L1 cells, there was an initial twofold increase in H4 histone gene transcription followed by a down-regulation of transcription to a level that was only 50% of that in control cells. Similarly, the decline in cellular levels of histone mRNA is

accompanied by decreased histone transcription in several other differentiation systems [Bird et al., 1985; Collart et al., 1988; Owen et al., 1990; Shalhoub et al., 1989], which suggests that the down-regulation of cell-cycle-regulated histone gene expression is at least partially transcriptionally mediated. In addition, the loss of HiNF-D binding activity 6 days after 3T3-L1 cells were induced to differentiate (at a time when histone gene expression and proliferative activity are at very low levels) may imply a mechanism whereby H4 histone gene transcription factors are turned over at terminal differentiation; this observation has been made previously for the differentiation of HL-60 promyelocytic cells [Stein et al., 1989]. However, in 3T3-L1 cells, as well as in human diploid fibroblasts [Wright et al., 1992], these transcription factors are retained, at least to some extent, when cells are reversibly quiescent and competent to reinitiate proliferative activity. Taken together, these data suggest that distinct combinations of regulatory mechanisms occur during the up-regulation and down-regulation of H4 histone gene expression. The versatility of these control mechanisms may facilitate stringent regulation of proliferation and tissue-specific gene expression during development of the adipocyte phenotype in the 3T3-L1 cell.

ACKNOWLEDGMENTS

We thank Rosa Mastrototaro for excellent technical assistance and Chris Dunshee for photographic assistance. We thank Dr. Thomas Owen for helpful discussions. This work was supported by grants from the NIH (P01CA39240 and GM32010) and the March of Dimes Birth Defects Foundation (1-1091).

REFERENCES

Artishevsky A, Wooden S, Sharma A, Resendez E Jr, Lee AS: Nature 328:823–827, 1987.
Baumbach LL, Stein GS, Stein JL: Biochemistry 26:6178–6187, 1987.
Bernlohr DA, Bolanowski MA, Kelly TJ Jr, Lane MD: J Biol Chem 260:5563–5567, 1985.
Bird RC, Jacobs FA, Stein GS, Stein JL, Sells BH: Biochim Biophys Acta 824:209–217, 1985.
Bortell R, Barone LM, Tassinari MS, Lian JB, Stein GS: J Cell Biochem 44:81–91, 1990.
Collart DG, Wright KL, van Wijnen AJ, Ramsey AL, Lian J, Stein JL, Stein GS: J Biol Chem 263:15860–15863, 1988.
Elks ML, Manganiello VC: J Cell Physiol 124:191–198, 1985.
Feinberg AP, Vogelstein B: Anal Biochem 132:6–13, 1983.
Fine A, Goldstein RH: J Biol Chem 262:3897–3902, 1987.
Green H, Kehinde O: Cell 1:113–116, 1974.
Green H, Kehinde O: Cell 5:19–27, 1975.
Green H, Meuth M: Cell 3:127–133, 1974.
Greenberg ME, Ziff EB: Nature 311:433–438, 1984.
Harris ME, Bohni R, Schneiderman ML, Ramamurthy L, Schumperli D, Marzluff WF: Mol Cell Biol 11:2416–2424, 1991.
Hauner H: Endocrinology 127:865–872, 1990.
Heintz N, Sive HL, Roeder RG: Mol Cell Biol 3:539–550, 1983.
Holthuis J, Owen TA, van Wijnen AJ, Wright K, Ramsey-Ewing A, Kennedy MB, Carter R, Cosenze SC, Soprano KJ, Lian JB, Stein JL, Stein GS: Science 247:1454–1457, 1990.
Ignotz RA, Massague J: Proc Natl Acad Sci USA 82:8530–8534, 1985.
Kramer IM, Koornneef I, de Vries C, de Groot RP, de Laat SW, van den Eijnden-van Raaij AJM, Kruijer W: Biochem Biophys Res Comm 175:816–822, 1991a.
Kramer IM, Koornneef I, de Laat SW, van den Eijnden-van Raaij AJM: EMBO J 10:1083–1089, 1991b.
LaBella F, Gallinari P, McKinney J, Heintz N: Genes Dev 3:1982–1990, 1989.
McGhee JD, Felsenfeld G: Annu Rev Biochem 48:159, 1980.
McMahon JB, Richards WL, del Campo AA, Song M-KH, Thorgeirsson SS: Cancer Res 46:4665–4671, 1986.
Morris TD, Weber LA, Hickey E, Stein GS, Stein JL: Mol Cell Biol 11:544–553, 1990.
Osley MA: Annu Rev Biochem 60:827–861, 1991.
Owen TA, Aronow M, Shalhoub V, Barone L, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS: J Cell Phys 143:420–430, 1990.
Pauli U, Chrysogelos S, Stein J, Stein G, Nick H: Science 236:1308–1311, 1987.
Pairault J, Green H: Proc Natl Acad Sci USA 76:5138–5142, 1979.
Plumb M, Stein J, Stein GS: Nucleic Acids Res 11:2391–2410, 1983.
Rauscher FJ III, Voulalas PJ, Franza BR Jr, Curran T: Genes Dev 2:1687–1699, 1990.
Robey PG, Young MF, Flanders KC, Roche NS, Kondaiah P, Reddi AH, Termine JD, Sporn MB, Roberts AB: J Cell Biol 105:457–463, 1987.
Shalhoub V, Gerstenfeld LC, Collart D, Lian JB, Stein GS: Biochemistry 28:5318–5322, 1989.
Sierra F, Stein G, Stein J: Nuc Acids Res 11:7069–7086, 1986.
Sive H, Heintz N, Roeder R: Mol Cell Biol 4:2723–2734, 1984.
Spiegelman BM, Frank M, Green H: J Biol Chem 258:10083–10089, 1983.
Sporn MB, Roberts AB, Wakefield LM, de Crombrugghe B: J Cell Biol 105:1039–1045, 1987.
Stauber C, Luscher B, Eckner R, Lotscher E, Schumperli D: EMBO J 5:3297–3303, 1986.
Stein G, Lian J, Stein J, Briggs R, Shalhoub V, Wright K, Pauli U, van Wijnen A: Proc Natl Acad Sci USA 86:1865–1869, 1989.
van Wijnen AJ, Wright KL, Lian JB, Stein JL, Stein GS: J Biol Chem 264:15034–15042, 1989.
van Wijnen AJ, Ramsey-Ewing AL, Bortell R, Owen TA, Lian JB, Stein JL, and Stein GS: J Cell Biochem 46:174–189, 1991a.
van Wijnen AJ, Owen TA, Holthuis J, Lian JB, Stein JL, Stein GS: J Cell Physiol 148:174–189, 1991b.
Weisbrod S: Nature 297:289–295, 1982.
Wright KL, Dell'Orco RT, van Wijnen AJ, Stein JL, Stein GS: Biochemistry (in press) 1992.