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Delineation of a human histone H4 cell cycle element *in vivo*: The master switch for H4 gene transcription

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ABSTRACT **Histone gene expression is cell cycle regulated at the transcriptional and the post-transcriptional levels. Upon entry into S phase, histone gene transcription is stimulated 2- to 5-fold and peaks within 1–3 hr of the initiation of DNA synthesis. We have delineated the proximal promoter element responsible for cell cycle-dependent transcription of a human histone H4 gene *in vivo*. Our results indicate that H4 cell cycle-dependent transcriptional regulation is mediated by an 11-base-pair element, the cell cycle element (5′-CTTTCG-GTTTT-3′), that resides in the *in vivo* protein–DNA interaction site, site II (nucleotides −64 to −24). The H4 cell cycle element functions as a master switch for expression of the FO108 human histone H4 gene *in vivo*; mutations within the H4 cell cycle element drastically reduce the level of expression as well as abrogate cell cycle-regulated transcription. Furthermore, these mutations result in a loss of binding *in vitro* of the cognate nuclear factor HiNF-M. *In vivo* competition analysis indicates that the cell cycle element mediates specific competition for a DNA-binding factor, presumably HiNF-M, that is a rate-limiting step in transcription of this H4 gene.**

A predetermined sequence of requisite events ensures that a cell divides only after it has completely replicated the genome, duplicated all subcellular organelles, and reached a critical mass. Consequently there is an ordered hierarchy of events that results from differential gene expression in response to extracellular and intracellular signals. Replication of DNA occurs during the S phase of the cell cycle and involves DNA synthesis and packaging into chromatin. This process requires stringent coupling of histone gene expression to DNA synthesis and is regulated at the transcriptional and the post-transcriptional levels (reviewed in refs. 1 and 2). As cells enter S phase, histone gene transcription is stimulated 2- to 5-fold and peaks within 1–3 hr of the initiation of DNA synthesis (3–6).

Studies of the promoter sequences involved in regulation of histone gene transcription have implicated several discrete proximal promoter elements in cell cycle-regulated control (7–10). It has been directly demonstrated that the distal H1 subtype-specific element, the AC-box (5′-AAACACA-3′), mediates cell cycle-dependent transcription of a chicken H1 gene in HeLa cells (7). The analogous element in a human H1 gene has been implicated in preferential transcription *in vitro* in S-phase extracts from HeLa cells (10). Growth-regulated transcription of an H3 gene in a temperature-sensitive Chinese hamster fibroblast line requires a 32-nucleotide region located ≈150 bp upstream of the TATA box (8). The H2b subtype-specific consensus element, which contains the core octanucleotide 5′-ATTTGCAT-3′, has been implicated in cell cycle-dependent transcription of a human H2b gene (9). Although much work has been done on the sequences involved in transcriptional regulation of human H4 genes *in vitro* (11–13) and *in vivo* (14–16), definitive identification of the H4 cell cycle regulatory element has not previously been obtained.

In this study we demonstrate that an 11-bp proximal promoter element mediates cell cycle-regulated transcription of the FO108 human H4 histone gene *in vivo*. The cell cycle element (CCE) is located in the distal region of site II, one of two protein–DNA interaction domains identified by *in vivo* genomic footprinting (17). The CCE functions as a master switch for transcription of the H4 gene, as demonstrated by transient expression assays and *in vivo* competition analysis. We suggest that the H4 CCE functions via binding of its cognate factor, HiNF-M, and that this DNA–protein interaction is a rate-limiting step in S-phase activation of this gene *in vivo*.

MATERIALS AND METHODS

Cell Culture and Synchronization. HeLa S3 cells were grown and maintained in suspension at 3–6 × 10^5 cells per ml in Joklik-modified minimum essential medium supplemented with 7% calf serum, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. Exponentially growing cells were synchronized by a double thymidine block procedure (18) and synchrony was monitored by [^{3}H]thymidine incorporation.

Transfection Experiments. Calcium phosphate-DNA coprecipitation (19) was used to transfect HeLa S3 cells growing on plastic culture dishes. To determine the amount of plasmid DNA taken up by transfected cells, episomal DNA was isolated (20) from cells harvested 48 hr after transfection. For selection of stable transformants, monolayer cultures were cotransfected with the DNA of interest and pSV2-neo at 20:1 ratio, respectively, and refed 36–48 hr after glycerol shock with medium containing G418 (Geneticin) at 500 μg of active antibiotic per ml of medium. Expression of fusion genes containing the bacterial chloramphenicol acetyltransferase (CAT) gene was monitored essentially as described by Gorman *et al.* (19). Total protein was quantitated spectrophotometrically using Coomassie blue (Pierce or Bio-Rad).

Site-Directed Mutagenesis. Mutation of the FO108 H4 site II was performed using a modification of the polymerase chain reaction (PCR) method of Higuchi *et al.* (21). Mutagenesis was carried out using the NEN Repliprime kit. After restriction enzyme digestion, the DNA fragments of interest were isolated from polyacrylamide gels and subcloned into pUC19. Mutations were confirmed by sequence analysis.

Gene Expression Assays. Nuclear run-on transcription was performed essentially as described in Baumbach *et al.* (3) using 1 × 10^7 nuclei per reaction. DNA slot blots on either nitrocellulose or Zeta-probe nylon membranes contained denatured DNA fragments complementary to the radiola-

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Abbreviations: CCE, cell cycle element; CAT, chloramphenicol acetyltransferase.

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beled RNA transcripts of interest and various control DNA fragments. After hybridization and washing, the blots were air dried and exposed to preflashed XAR-5 film with a Lightning Plus intensifying screen at −70°C. Gel retardation analysis was performed as described in van Wijnen *et al.* (22).

RESULTS

The H4 Histone Proximal Promoter Is Sufficient for Cell Cycle-Regulated Transcription. To determine if the 5′ flanking region of the FO108 H4 histone gene supports cell cycle-dependent transcription, we analyzed expression of PH4CAT (construct A3 in ref. 16), which contains ≈1 kb of sequences (nucleotides −1039 to −11 relative to the mRNA initiation site) fused to the bacterial CAT gene (Fig. 1*A*). Synthesis of properly initiated CAT mRNA was confirmed by primer extension analysis of poly(A)$^+$ RNA from two stably transfected HeLa cell lines (data not shown). Nuclear run-on analysis revealed that the rates of transcription of the PH4CAT fusion gene and the endogenous H4 genes were elevated 2- to 3-fold during early S phase (Table 1 and data not shown). These results are consistent with previous studies showing that transcription of the endogenous H4 gene peaks during early S phase, preceding the maximal rate of DNA synthesis (3). Thus, the H4 promoter confers cell cycle regulation on CAT gene transcription, independent of chromosomal position.

We then prepared a series of 5′ promoter deletions of the PH4CAT gene to determine the minimal promoter necessary for cell cycle-dependent transcription (Fig. 1*A*). Each deletion construct was assayed at various points during the cell cycle in transient, synchronized HeLa cell cultures and in synchronized stably transfected HeLa cell lines. CAT expression was directly measured by run-on transcription in nuclei isolated from the synchronized cells.

Deletion of nucleotides −1039 to −216 does not alter cell cycle-dependent transcription from the FO108 promoter (Fig. 1*C*, Table 1). Nevertheless, we observed a significant

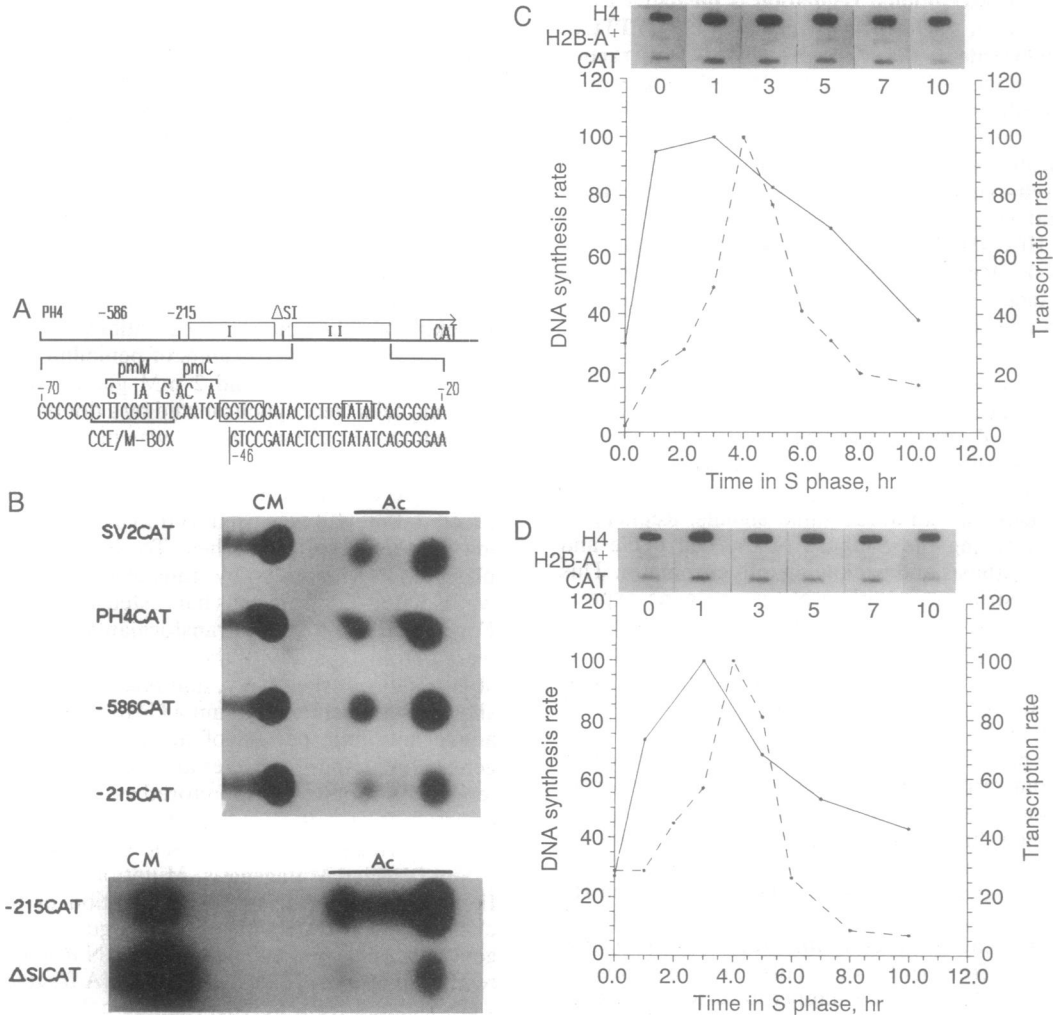

FIG. 1. Analysis of the level of expression and cell cycle transcription of FO108 histone H4CAT promoter deletion mutants. (*A*) Schematic diagram (not drawn to scale) showing deletions and point mutations of the PH4CAT parental construct. PH4CAT was made by fusing ≈1 kb of FO108 H4 5′ flanking sequences to the bacterial CAT gene transcription unit derived from pSV2CAT (16). (*B*) Analysis of transient expression in HeLa cells. Each sample represents CAT activity in lysates from a pooled cell population from 10 independent transfections. Shown are autoradiographs of thin-layer chromatography plates on which [^{14}C]chloramphenicol (CM) and its acetylated products (Ac) were resolved. (*C* and *D*) Transcription of deletion mutants during the cell cycle. Stable cell lines containing the deletion mutants were synchronized and the rates of DNA synthesis and nuclear run-on transcription were measured at the indicated times. Shown are autoradiographs of slot blots containing the indicated plasmids: H4, detects endogenous H4 transcripts; CAT, detects H4CAT fusion gene transcripts; H2B-A$^+$, detects transcripts encoded by a partially replication-dependent polyadenylylated H2B variant (23). Each graph represents transcription data from a typical experiment and is the result of densitometric analysis of several autoradiographs. The relative rates of transcription (——) were determined by comparison to 18S ribosomal gene transcription (24) and the percentage of the maximal rate was plotted. The rates of DNA synthesis were plotted as a percentage of the maximal rate (- - - -). (*C*) −215CAT. (*D*) ΔSICAT.

Table 1. Summary of expression and cell cycle regulation of H4CAT constructs *in vivo*

Construct*	Level of expression: Transient,† %	Level of expression: Stable‡	Transcription§: Cell cycle	Transcription§: T3/T10
PH4CAT	100	9.5	+	2.8 ± 0.8 (3)
−586CAT	95	2.2	+	2.4 ± 0.1 (3)
−215CAT	28	3.8	+	2.6 ± 0.6 (6)
ΔSICAT	4	0.7	+	3.3 ± 0.5 (7)
−46CAT	2	ND	−	0.5 ± 0.1 (3)
−215CATpmM	1	0.8	−	0.7 ± 0.1 (4)
−215CATpmC	28	2.3	+	3.0 ± 0.4 (3)

*Histone H4CAT fusion genes.
†Transient expression data were derived from densitometric analysis of autoradiograms of CAT assays and were corrected for the amount of plasmid DNA taken up by the cells as determined by analysis of Hirt episomal DNA preparations (n = 3–12; SD < 8%).
‡Expression per copy in stable cell lines, calculated from CAT activity data and copy number for two cell lines per construct. ND, not determined.
§Relative rate of transcription determined by nuclear run-on transcription analysis; +, 2- to 3-fold S-phase stimulation; −, no detectable S-phase stimulation. T3/T10, ratio (mean ± SD) of the rate of fusion gene transcription relative to the rate of transcription of the 18S ribosomal gene at 3 hr (early S phase) compared to 10 hr (G2/M phase); n values are in parentheses.

(3-fold) decrease in the level of expression when nucleotides −587 through −216 were deleted (Fig. 1*B*, Table 1), in agreement with previous studies in which an upstream positive regulatory element (nucleotides −410 to −210) was identified by transfection of mouse cells (16) and by *in vitro* transcription analysis (13). The intermediate deletion −586CAT exhibited cell cycle regulation in cells synchronized by two different methods, double thymidine block or thymidine/aphidicolin block (data not shown), and during transient expression, thus demonstrating that our results are not dependent upon the method of synchrony used.

The deletion mutant −215CAT is particularly interesting because it indicates that the proximal promoter of the H4 gene is sufficient for cell cycle-regulated transcription. This region contains cell cycle-regulated nuclease-hypersensitive sites (14, 25) and two *in vivo* protein–DNA interaction domains (17), sites I and II, that interact with several DNA-binding proteins *in vitro* (13, 22, 26–28).

The CCE Resides in Proximal Promoter Site II. Further deletions within the proximal promoter had drastic effects on the overall level of fusion gene expression. Deletion of nucleotides −205 to −71 (ΔSICAT), which include site I, had no effect on cell cycle regulation (Fig. 1 *C* and *D*, Table 1) but reduced expression ≈7-fold (Fig. 1*B*, Table 1). This result suggests that the factors interacting at this site, HiNF-E (13) and HiNF-C (13, 28), act as amplifiers of expression but do not mediate the S-phase-specific stimulation of H4 gene transcription. The observation that the ΔSICAT deletion retains cell cycle-regulated transcription suggests that the CCE resides in site II sequences.

To demonstrate that site II sequences are necessary, as well as sufficient, for cell cycle-regulated transcription, we analyzed another mutant in which the distal site II sequences were deleted. Removal of nucleotides −70 to −47 resulted in loss of cell cycle regulation (Table 1), indicating that these sequences are required for regulated transcription from the FO108 H4 promoter. We also observed a 2-fold decrease in the level of expression compared to an intact site II construct (Table 1).

The CCE Is the Master Switch for H4 Transcription. To determine precisely which sequence elements within distal site II regulate cell cycle-dependent transcription, we introduced two clusters of point mutations into this region (Fig. 1*A*). We mutated the CAAT box (nucleotides −53 to −49), which interacts *in vitro* with nuclear factors HiNF-D and HiNF-P, and the M-box (nucleotides −64 to −54), which interacts with factors HiNF-M and HiNF-P (22, 27). To facilitate analysis of cell cycle transcription, the mutations were introduced in the context of the −215CAT construct, which retains site I and supports a level of transcription sufficient for quantitative assessment of promoter activity (Fig. 2*A*). Within the M box, which includes the minimal recognition sequence for HiNF-M, we mutated the central CGG → CTA and introduced a single T → G mutation in each of two short flanking T stretches (Fig. 1*A*). We therefore disrupted both guanine contacts detected *in vivo* and several nucleotides involved in methylation interference or enhancement *in vitro* on both the upper and lower strands (17, 22). The second cluster of mutations was introduced into the CAAT box element (Fig. 1*A*) such that all *in vitro* and *in vivo* contacts in this region implicated in protein–DNA interactions were disrupted.

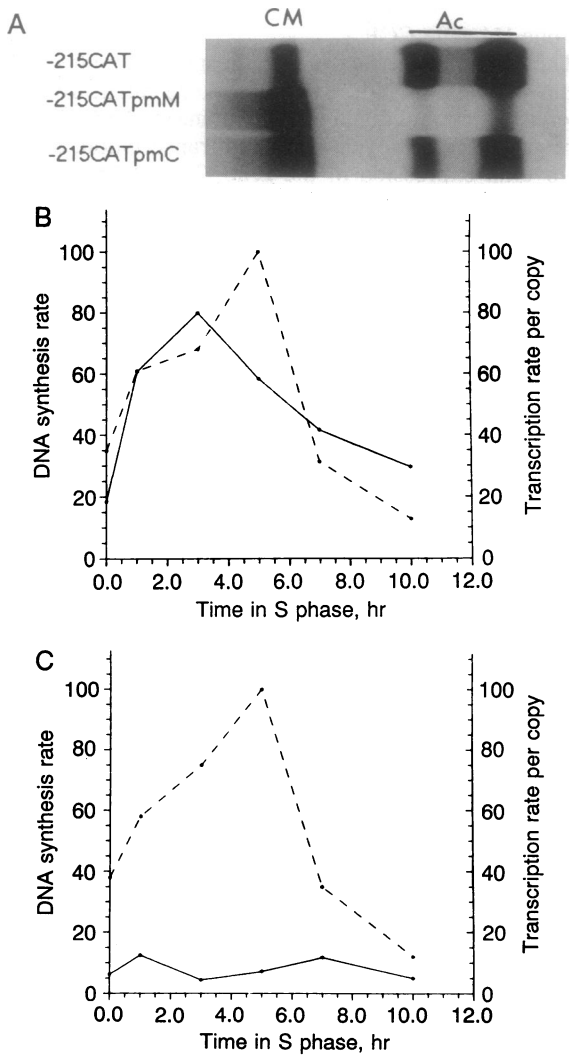

FIG. 2. Expression and cell cycle transcription of FO108 proximal promoter point mutants in HeLa cells. (*A*) Effect of point mutations within distal site II on the level of transient expression. CAT assays were performed as described in the legend to Fig. 1. (*B* and *C*) Transcription of point mutants during the cell cycle in stable cell lines. Nuclear run-on transcription analysis was performed with nuclei isolated at the indicated times after cells had been released into S phase. The rate of transcription of point mutants was calculated relative to the fusion gene copy number in each cell line. (*B*) −215CATpmM. (*C*) −215CATpmC.

The effects of these mutations on the overall level of expression were determined. While mutation of the CAAT box element (−215CATpmC) had a small but reproducible effect on expression, the M box mutation (−215CATpmM) drastically decreased expression (Fig. 2*A*, Table 1), even in the presence of site I. This result suggests that interactions at site I cannot stimulate transcription if the M box is disrupted. It appears that integrity of the M box is essential for transcription from this promoter, and we therefore refer to this element as the master switch.

We then examined the transcription of the site II point mutants during the HeLa cell cycle by nuclear run-on analysis of samples harvested at various times during the cell cycle. Mutation of the M box (−215CATpmM) abrogates cell cycle-regulated transcription, while the CAAT box mutant (−215CATpmC) retains cell cycle regulation (Fig. 2 *B* and *C*). Transcription of the M box mutant remains constant as cells enter and progress through S phase and continue into mitosis. On the other hand, the CAAT box mutant is transcribed in a manner indistinguishable from that of the wild-type promoter. These results indicate that the M box is the human histone H4 gene CCE.

The differential effect of two clusters of point mutations within distal site II indicates that this site can be divided into discrete functional domains. One domain, the CCE/M box, is required for cell cycle-regulated transcription, as well as for overall expression of the gene. The other domain, the CAAT box, is not critical for either cell cycle regulation or overall expression but participates in DNA–protein interactions. This is consistent with the detection *in vivo* of a large footprint over this region, within which multiple DNA–protein interactions occur *in vitro*.

The CCE Is the M Box. We used gel retardation assays to examine the effects of these distal site II point mutations on activity of three distinct site II binding proteins, factors

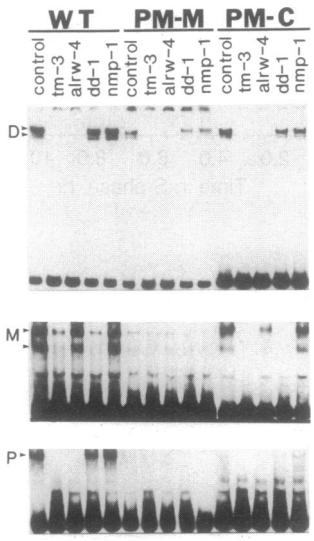

FIG. 3. Analysis of *in vitro* protein–DNA interactions at the proximal FO108 promoter. Gel retardation analysis with HeLa S3 nuclear proteins was performed using radiolabeled fragments prepared from wild-type [−215CAT (WT)] and mutant [−215CATpmM (PM-M) and −215CATpmC (PM-C)] promoter plasmids. Protein–DNA complexes were formed in the presence of the indicated competitor oligonucleotide: control, no competitor; tm-3, wild-type oligonucleotide that includes all site II sequences; alrw-4, oligonucleotide with a truncated HiNF-M binding site, the HiNF-P binding site, and the GGTCC element; dd-1, oligonucleotide with two copies of the HiNF-M binding site; nmp-1, nonspecific competitor; D, M, and P, complexes HiNF-D, -M, and -P, respectively. Double arrowheads for HiNF-M and HiNF-P indicate two electrophoretic forms of each factor (22).

Table 2. Summary of the effect of distal site II point mutations

Promoter	Cell cycle*	*In vivo* comp†	Level exp‡	Factor§ D	M	P
Wild-type (−215)	+	+	+	+	+	+
M mutant (−215pmM)	−	−	−	±	−	−
C mutant (−215pmC)	+	+	+	+	+	−

*Cell cycle-regulated transcription.
†Efficiency in *in vivo* competition.
‡Level of expression in exponentially growing cells *in vivo*.
§*In vitro* binding to histone nuclear factors (HiNF-D, -M, and -P).

HiNF-D, HiNF-M, and HiNF-P (22) (Fig. 3). The wild-type DNA fragment binds each factor, as expected, under the appropriate conditions. The M box mutant binds HiNF-D at a reduced level and fails to bind HiNF-M or HiNF-P, while the CAAT box mutation, which does not significantly affect cell cycle-dependent transcription, abolishes HiNF-P binding but has no appreciable effect on HiNF-M or HiNF-D binding. HiNF-P is similar to H4TF-2, the histone transcription factor characterized by Heintz and colleagues (26, 30) that binds the corresponding region of the Hu4A histone H4 promoter. Our data suggest that HiNF-P/H4TF-2 binding is not rate-limiting for cell cycle-regulated transcription. Rather, the loss of transcription and cell cycle control correlates primarily with the loss of HiNF-M binding *in vitro* (Table 2). In support of our conclusion that the CCE is the master switch, we find that among several protein–DNA interactions within the proximal H4 promoter, it is the binding of HiNF-M to the CCE that correlates with cell cycle regulation and expression of this gene, suggesting that this interaction is the key regulatory event.

The CCE/M Box Mediates *in Vivo* Competition. *In vivo* competition experiments were used to investigate further the role of protein–DNA interactions in the proximal promoter of the FO108 H4 gene. Plasmids carrying only promoter sequences were cotransfected with the wild-type H4CAT test construct (−215CAT) into HeLa cells at a 3:1 competitor:test ratio (Fig. 4). Expression of −215CAT was monitored 48 hr later. While the nonspecific competitor pUC19 had no effect on −215CAT expression, the wild-type competitor (−215) drastically reduced its expression. The M box mutant (−215pmM) was unable to compete, presumably because of its inability to bind a critical limiting factor. However, the CAAT box mutation (−215pmC) retained the capacity to function as a competitor *in vivo*. Another competitor (SM), in which an M box homology (5′-GGTTTTC-3′) corresponding to the most conserved set of nucleotides among H4 genes is juxtaposed to site I sequences, competed with the test plasmid as efficiently as the wild-type competitor.

The fact that *in vivo* competition via the M box occurred in the presence of site I sequences lends further credibility to

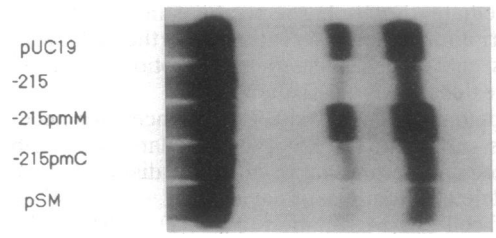

FIG. 4. *In vivo* competition analysis of distal site II point mutants and site I. HeLa cells were cotransfected at a 3:1 competitor:test molar ratio with the test plasmid −215CAT and site II competitor plasmids that contained only promoter sequences. Each plate received 20 μg of total DNA (test and competitor plasmids, and salmon sperm DNA). Cells were harvested 48 hr later and the level of test gene expression was determined by CAT assays as described in the legend to Fig. 1.

our interpretation that binding to the CCE is the rate-limiting step in H4 gene transcription. Interestingly, even at a 6:1 competitor:test ratio, a site I only plasmid had no effect on expression of the test construct (data not shown). Furthermore, deletion of site I in the competitor plasmid did not affect its ability to inhibit test gene expression. The efficient competition between the M box competitor plasmids and the test plasmid at a 3:1 ratio suggests that the trans-acting factor(s) involved, HiNF-M, is titratable and of relatively low abundance.

DISCUSSION

In the present study we report the delineation of the human H4 histone CCE *in vivo*. We show that mutations within the CCE result in abrogation of cell cycle-dependent transcription, loss of *in vitro* binding to its cognate DNA-binding protein, HiNF-M, and an inability of the entire proximal promoter to act as a competitor *in vivo*. The results suggest that the H4 CCE is the master switch for transcription of this gene *in vivo* and that its interaction with HiNF-M is a rate-limiting step for overall expression as well as for cell cycle-regulated transcription.

Maximal activation of the FO108 H4 promoter occurs during S phase (3). At this time, all protein–DNA and protein–protein interactions are intact to produce fully functional complexes on sites I and II capable of interacting with the general transcription apparatus. In normal cells, HiNF-D is down-regulated outside of S phase, while transformed cells demonstrate constitutive HiNF-D activity (31). Transformed cells therefore remain in a poised state that supports a basal level of transcription outside of S phase with modulation of only HiNF-M DNA-binding and/or transcriptional activity during the cell cycle. On the other hand, normal cells regulate the level of HiNF-D binding. Changes in M binding *in vivo* may be readily masked by interactions of HiNF-D, which contacts H4 site II on both sides of HiNF-M (22). Furthermore, modulation of HiNF-M transcriptional activity may be only partially dependent on its binding to the CCE, so that persistent binding would not preclude periodic cell cycle-regulated activity. Cell cycle-dependent changes in post-translational modifications of the H4-site II DNA-binding factors or the nature of their associations with the DNA and/or each other could stabilize their interactions and contribute to the formation of the optimal transcription complex. Phosphorylation has been shown to be involved in the regulation of binding of the factors that interact at H4 site II (22).

When cells have terminally differentiated (32–34) or reached density-dependent quiescence (29, 33) and down-regulated histone gene transcription, there is a loss of HiNF-D- and HiNF-P-binding activity *in vitro*. In terminally differentiated HL60 cells we also detected by genomic footprinting the loss of *in vivo* protein–DNA interactions at site II but not at site I (32). We propose that the irreversible shutdown of this gene under these conditions is the result of disassembly of factors from site II.

In this study the promoter element responsible for cell cycle-dependent transcription of a human histone H4 gene has been identified. The H4 CCE is part of the multipartite proximal promoter region site II and functions as the master switch for transcriptional activation of this gene *in vivo*. Our results suggest that optimal expression of this H4 gene requires binding of the HiNF-M DNA-binding factor to the CCE and most likely the subsequent formation of a fully functional complex, perhaps facilitated by the CCE/HiNF-M interaction. Further investigation of the human histone H4 promoter and regulation of its associated DNA-binding proteins may help to elucidate the mechanisms that govern cell proliferation and differentiation.

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