cAMP/phorbol ester response element is involved in transcriptional regulation of the human replacement histone gene H3.3B

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The human histone H3.3B gene belongs to the group of replacement histone genes, which are up-regulated during differentiation of cells. Here we provide evidence that a cAMP response element/PMA response element (CRE/TRE) located in the proximal promoter contributes to the expression of the H3.3B gene. (1) Band shift and supershift analysis demonstrated the binding of AP-1 and transcription factors of the CRE-binding protein/activating-transcription-factor family to the H3.3B CRE/TRE. (2) Treatment of HeLa cells with PMA led to a 4-fold increase in H3.3B mRNA levels within 2 h, whereas transcription of the cell cycle-dependent H3 histone genes remained constant. In contrast with PMA, cAMP did not affect H3.3B transcription. (3) PMA treatment of cells transiently transfected with H3.3B promoter constructs linked to a luciferase gene caused a 4–5-fold increase in reporter gene activity, whereas mutation of the CRE/TRE element abolished the PMA response. These results demonstrate that activation of the protein kinase C pathway by PMA results in an early up-regulation of H3.3B gene expression via the CRE/TRE element. Furthermore treatment with PMA apparently leads to differential induction of H3 histone subtype genes and this in turn can result in a remodelling of chromatin structure of cells before or during differentiation processes.

INTRODUCTION

The classical function of core histone proteins has been assigned to the formation of a subunit structure of chromatin called the nucleosome [1,2], whereas linker histones are involved in the formation of higher-order chromatin structures [3]. Because the transcription machinery must gain access to specific binding sites at the DNA level, the histones have been implicated in regulating the local state of chromatin activation or repression by interacting with transcription factors, histone modifications, non-random positioning and other processes [2]. In mammals a great diversity of histone genes exists, coding for structurally varied histone proteins [4]. Replacing one particular histone subtype by another might therefore modify the function of histones in chromatin structure and in regulating gene activity.

Histone genes can be subdivided into three major groups. First, replication-dependent genes are expressed only during the S-phase of the cell cycle; secondly, replication-independent histone genes encoding replacement histones are also expressed in non-dividing quiescent or terminally differentiated cells; and thirdly, tissue-specific histone isotypes exist that are expressed exclusively in the testis of different mammalian species [4].

The replacement histone H3.3 is a minor component of the chromatin in dividing cells but can become relatively abundant in non-dividing cells [5–8], adult tissues [9,10] and differentiation-induced myelocytic leukaemia and erythroleukaemia cells [11,12]. The histone H3.3 protein is encoded by two different genes termed H3.3A and H3.3B [13,14]. Recently our group has characterized the human replacement histone gene H3.3B [15]. The H3.3B gene shows unique features within the human histone gene family: it contains three introns and three polyadenylation signals and it is located alone on chromosome 17, outside the known histone gene clusters on chromosomes 6 [16,17] and 1 [18], which contain most of the replication-dependent histone genes.

We are interested in identifying the *cis*/*trans* elements of the H3.3B promoter responsible for the regulation of the H3.3B expression to further the understanding of the differential transcriptional regulation of replication-dependent and replacement histone genes. In a previous paper we have shown that an octamer motif and a cAMP response element/PMA response element (CRE/TRE) within the proximal promoter are essential for H3.3B promoter activity *in vitro* [19]. Here we focus on the identification of nuclear proteins binding to the CRE/TRE and on the function of this site in regulating expression of the H3.3B gene in intact cells.

EXPERIMENTAL

Cell culture

Because HeLa cells are known to express protein kinase C isoenzymes responsive to PMA treatment, and because c-Jun transcription was shown to be rapidly activated after stimulation of protein kinase C [20,21], we used this cell line for studying the effects of PMA treatment on histone H3.3B expression. HeLa cells were maintained in the recommended medium supplemented with 10 % (v/v) fetal calf serum at 37 °C in an air/CO_2 (19:1) atmosphere. For cAMP/PMA treatment, cells were seeded at a density of 3.3×10^5 per 50 ml in a 150 cm^2 flask and precultured in 10 % (v/v) fetal calf serum for 18 h. Cells were then starved in 0.5 % (v/v) fetal calf serum for 48 h to decrease the interference of serum substances with the protein kinase A and C pathways. Serum starvation had a minor effect on cell proliferation (approx. 70 % decrease in cell proliferation after 48 h). Cells were subsequently treated with 100 μM cAMP (chlorophenylthio-cAMP; Boehringer Mannheim) and 100 nM PMA (Serva) in 0.5 % (v/v)

Abbreviations used: ATF, activating transcription factor; CRE, cAMP response element; CREB, CRE-binding protein; TRE, PMA response element.

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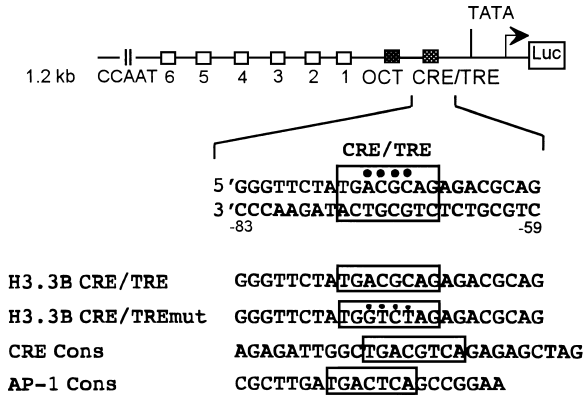

Figure 1 Sequences of the H3.3B reporter gene construct and the oligonucleotides used

The top panel shows a schematic drawing of the wild-type reporter gene construct used in transient transfection assays. The construct carries a 1.2 kb H3.3B promoter fragment linked to a luciferase gene. The six CCAAT boxes, the Oct element and the CRE/TREs are indicated. The sequence of the CRE/TRE and flanking portions is shown. Numbers below the sequence indicate nucleotide positions relative to the start site of transcription; black dots show the positions mutated by site-directed mutagenesis (drawn by analogy with [19]). The bottom panel shows the sequence of the oligonucleotides used in band-shift experiments, designated H3.3B CRE/TRE, H3.3B CRE/TREmut, CRE Cons and AP-1 Cons. Consensus sequence motifs are boxed; black dots indicate mutated positions of the H3.3B CRE/TRE.

fetal calf serum for the times indicated. Cells were washed with PBS and harvested by trypsin treatment for preparation of total RNA.

RNA isolation and Northern blot analysis

Isolation of total RNA was performed by the guanidinium isothiocyanate method [22]. For detection of H3.3B transcripts a specific probe was generated from the 500 bp *Xba*I/*Hin*dIII fragment of the 3′ flanking region of the H3.3B gene [15]; for detection of cell-cycle-dependent H3 histone mRNA species a probe was generated from the 420 bp *Sac*II/*Sac*II fragment of a consensus H3 coding region [23]; and c-*fos* mRNA was detected with a probe from the 640 bp *Acc*I/*Acc*I fragment of the c-*fos* coding region derived from a human c-*fos* gene kindly provided by Dr. R. Müller (IMT Marburg, Marburg, Germany). All probes were labelled by using the Rediprime kit (Amersham). For Northern blot analysis, total RNA was blotted on nylon membrane (Hybond-N; Amersham). Hybridization of the blots was performed in 50% (v/v) deionized formamide/5 × SSPE, 5 × Denhardt's solution/0.1% SDS at 42 °C for 16 h. Transcript size calculations were based on the electrophoretic mobilities of ribosomal RNA bands. Quantification of bands was done with the ImageQuant system (Molecular Dynamics).

Band-shift experiments and supershift analysis

Nuclear extracts were prepared exactly as described previously by Dignam et al. [24]. Figure 1 gives an overview of the oligonucleotides used in band shift experiments. The CRE and AP-1 consensus oligonucleotides were obtained from Santa Cruz Biotechnology as well as the following antibodies used in supershift experiments: CRE-binding protein/activating transcription factor (CREB/ATF) antibody (ATF-1 supershift reagent, reactive with ATF-1, CREB-1 and CREM-1), c-Fos antibody (c-Fos supershift reagent, reactive with c-Fos, Fos B, Fra-1 and Fra-2) and c-Jun antibody (c-Jun/AP-1 supershift reagent, reactive with c-Jun, Jun B and Jun D). Radioactive labelling, band-shift and supershift analyses were performed as described [19].

Transfection of cells and luminescence detection

The 1.2 kb wild-type H3.3B promoter–luciferase gene construct and the corresponding construct carrying a mutated CRE/TRE have been described elsewhere [19]. Transfections of these constructs into HeLa cells were performed as described previously [19] with the following modifications: 2×10^5 HeLa cells were precultured in 2 ml of medium, serum-starved for 48 h in 0.5% fetal calf serum and transfected for 6 h with 2 μg of DNA by using 6 μl/μg *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate (DOTAP) (Boehringer Mannheim). After removal of the transfection medium, cells were washed with PBS and then treated with 100 nM PMA/100 μM cAMP for a further 6 h. Determination of luciferase activity was done as described [19], except that the luciferase activities obtained were normalized by total cellular protein.

RESULTS

Binding of AP-1 and proteins of the CREB/ATF family to the H3.3B CRE/TRE

We have recently shown that a CRE/TRE located in the proximal H3.3B promoter is essential for promoter activity when transiently transfected in HeLa cells [19]. To identify the transcription factors binding to the CRE/TRE, we performed a series of competition band-shift and supershift experiments. Figure 2(A) shows the formation of three DNA–protein complexes when an oligonucleotide consisting of the H3.3B CRE/TRE and flanking regions was incubated with nuclear proteins from HeLa cells. The specific binding of these proteins was demonstrated by a series of competition experiments. The addition of unlabelled H3.3B CRE/TRE oligonucleotide in 40-fold and 200-fold molar excesses resulted in efficient competition, with the disappearance of all three bands at 200-fold molar excess. In contrast, an oligonucleotide containing the mutated CRE/TRE was not able to compete when added at the same molar excess, indicating that the CRE/TRE in the H3.3B oligonucleotide is indeed responsible for the binding of the nuclear proteins. Addition of an AP-1 consensus oligonucleotide as competitor led to the disappearance of only the upper band, suggesting that this band represents the binding of a protein of the AP-1 family of transcription factors. Competition with the CREB consensus oligonucleotide led to the disappearance of all three bands. Because the CREB oligonucleotide is able to bind CREB/ATF as well as AP-1, this suggests that the lower two bands represent transcription factors of the CREB/ATF family. The analogous competition experiments with labelled CRE and AP-1 consensus oligonucleotides in competition with the H3.3B CRE/TRE oligonucleotide revealed that the H3.3B CRE/TRE binds the nuclear proteins of the CREB/ATF family and AP-1 with the same affinity as the respective consensus oligonucleotides (Figures 1B and 1C) despite some base exchanges [19]. Finally, we directly identified the binding proteins as AP-1 and CREB/ATF transcription factors by using c-Fos, c-Jun and CREB/ATF antibodies in supershift experiments (Figures 3A to 3C). The addition of a CREB/ATF antibody resulted in a supershift of the lower two bands obtained after incubating an H3.3B CRE/TRE oligonucleotide with nuclear proteins (Figure 3A), demonstrating that the two lower DNA–protein complexes represent the binding of transcription factors of the CREB/ATF family. The addition of a c-Fos antibody did not give rise to a supershift, whereas the addition of

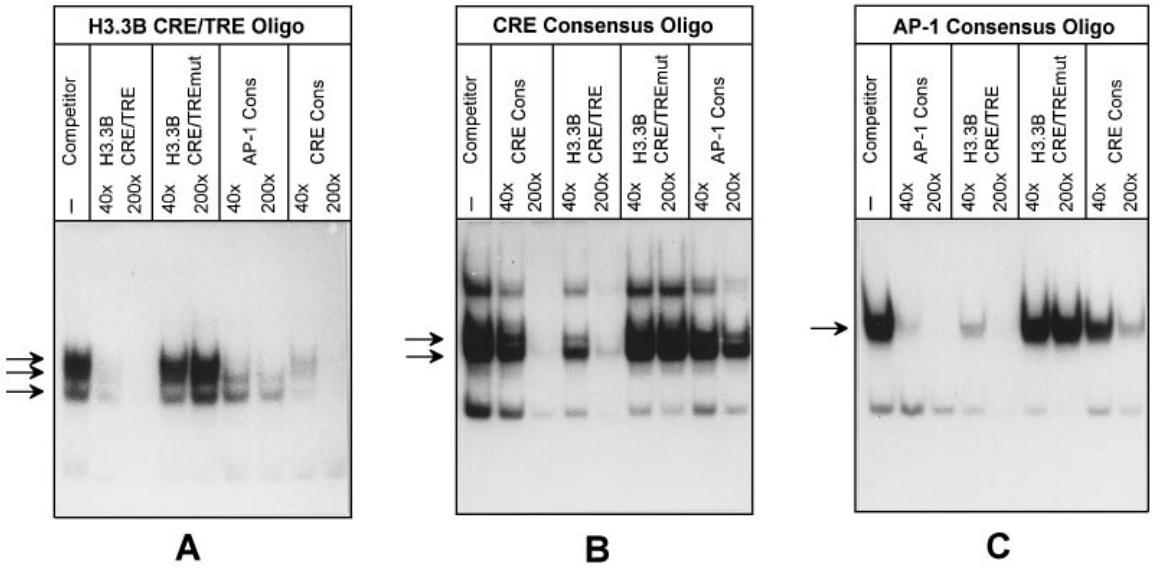

Figure 2 Band-shift analysis of nuclear proteins binding to the H3.3B CRE/TRE oligonucleotide

Nuclear proteins (10 μg) prepared from HeLa cells were incubated with 30000 c.p.m. (100 ng) of an H3.3B oligonucleotide containing the CRE/TRE (**A**), a labelled CRE consensus oligonucleotide (**B**) or a labelled AP-1 oligonucleotide (**C**) (see Table 1). Competition in DNA–protein interactions was determined by the addition of 40-fold and 200-fold molar excesses of unlabelled oligonucleotides as indicated. DNA–protein complexes were separated on a 4% (w/v) polyacrylamide gel and exposed to X-ray film. Arrows indicate the main three DNA–protein complexes obtained with the H3.3B CRE/TRE oligonucleotide (**A**), the main two complexes obtained with the CRE consensus oligonucleotide (**B**) and the band corresponding to the DNA–protein complex obtained with the AP-1 consensus oligonucleotide (**C**).

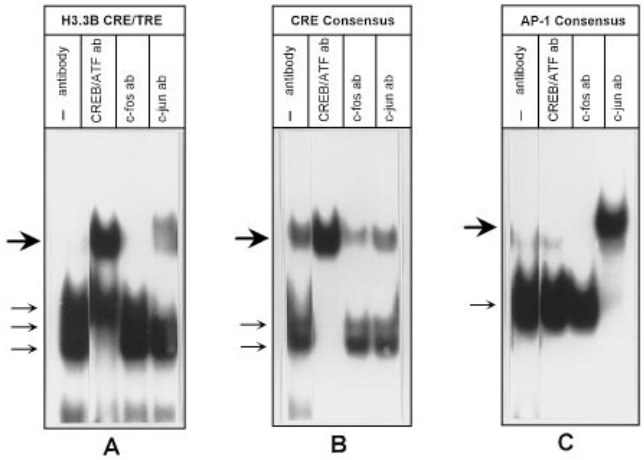

Figure 3 Supershift analysis of nuclear proteins binding to the H3.3B CRE/TRE oligonucleotide

DNA–protein complexes were obtained by incubating HeLa nuclear proteins with the H3.3B CRE/TRE oligonucleotide (**A**), a CRE consensus oligonucleotide (**B**) or an AP-1 consensus oligonucleotide (**C**) as described in the legend to Figure 2. The binding proteins were identified by further incubating the DNA–protein complexes with CREB/ATF antibody, c-Fos antibody or c-Jun antibody. The DNA–protein complexes were separated on a 4% (w/v) polyacrylamide gel. Large arrows indicate the appearance of further retarded bands due to the formation of specific DNA–protein–antibody complexes ('supershifts'). Small arrows indicate DNA–protein complexes not bound by antibodies, corresponding to the complexes obtained in Figure 2.

a c-Jun antibody resulted in a supershift of the upper band (Figure 3A), demonstrating that the nuclear binding protein is AP-1. In control experiments, analogous supershifts were performed with nuclear extracts and CRE and AP-1 consensus oligonucleotides respectively. Addition of a CREB/ATF antibody resulted in a supershift of the two bands obtained after incubating the CRE consensus oligonucleotide with nuclear extracts, whereas c-Fos and c-Jun antibodies did not (Figure 3B). The addition of a c-Jun antibody resulted in a supershift of a band obtained after incubating the AP-1 consensus oligonucleotide with nuclear extracts, whereas the addition of CREB/ATF and c-Fos antibodies did not (Figure 3C). Thus, in agreement with the competition experiments, the transcription factors binding to the H3.3B CRE/TRE were identified as AP-1 (upper band) and proteins of the CREB/ATF family of transcription factors (two lower bands). The latter two bands most probably represent ATF/ATF homodimers and CREB/ATF heterodimers respectively, as has been demonstrated for the binding of nuclear proteins from HeLa cells to a CRE consensus oligonucleotide [25].

PMA stimulates the expression of the H3.3B gene

To investigate further the function of the H3.3B CRE/TRE in intact cells, we treated HeLa cells with PMA or cAMP. Figure 4(A) shows a Northern blot analysis of total RNA extracted from cells after different durations of PMA treatment. PMA caused a detectable increase in H3.3B mRNA after only 30 min, reaching a 4-fold increase in mRNA levels after 2 h. In contrast, the expression of the H3 cell-cycle-dependent histone genes remained constant over the 6 h treatment period. However, treatment of HeLa cells with cAMP changed neither the H3.3B nor the H3 mRNA levels (Figure 4B). This indicates that in contrast with the band-shift experiments, which showed binding of both AP-1 and CREB/ATF to the H3.3B CRE/TRE, AP-1 is the dominant transcription factor binding to the H3.3B promoter under *in vivo* conditions in HeLa cells.

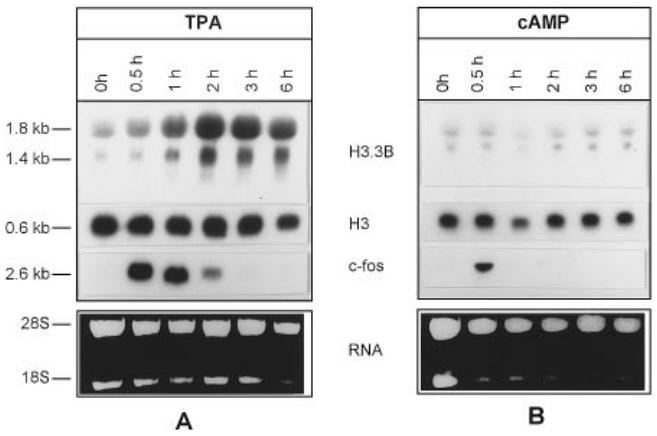

Figure 4 Induction of H3.3B mRNA by PMA and cAMP

HeLa cells were precultured for 18 h, starved in 0.5% (v/v) fetal calf serum for 48 h and then treated with 100 nM PMA (**A**) or 100 μM cAMP (chlorophenylthio-cAMP) (**B**) for the times indicated. Total RNA was prepared from cells; 20 μg of RNA was subjected to Northern blot analysis. The blots were re-probed several times with the probes indicated; the results are combined in the figure. Hybridization with the H3.3B-specific probe gives rise to 1.8 and 1.4 kb bands owing to different polyadenylation signals at the 3′ end of the H3.3B gene [15]. The H3 band, representing the mRNA species of the cell-cycle-dependent histone genes H3.1 and H3.2, migrates at 0.6 kb. Because c-*fos* expression is up-regulated in response to the treatment of cells with PMA and cAMP [30,31], we included a c-Fos probe as a positive control for the induction experiments. The bottom of the figure shows the 28 S and 18 S ribosomal RNA after staining of total cellular RNA with ethidium bromide.

Table 1 Stimulation of H3.3B promoter activity by PMA and cAMP

For induction experiments, HeLa cells were cultured as described in the legend to Figure 4. After serum starvation, cells were transfected with the H3.3B wild-type reporter gene construct (H3.3B wt) and the mutated construct (H3.3B CRE/TREmut) by lipofection for 6 h. For a detailed description of the constructs see Figure 1. Cells were then treated with 100 nM PMA or 100 μM cAMP (chlorophenylthio-cAMP) for 6 h before the cells were harvested and luciferase activity was determined. The results are means $\pm$ S.D. for three experiments. Luciferase activity is expressed relative to the unstimulated wild-type construct.

	Relative luciferase activity	
Stimulant	H3.3B wt	H3.3B CRE/TREmut
Unstimulated	1.0	0.5 $\pm$ 0.05
PMA	4.5 $\pm$ 0.5	1.3 $\pm$ 0.2
cAMP	1.5 $\pm$ 0.1	0.7 $\pm$ 0.05

The H3.3B CRE/TRE mediates the PMA-induced H3.3B transcription

Because we had observed a relatively fast induction of H3.3B mRNA, starting after only 30 min of PMA treatment, it seems likely that PMA-activated protein kinase C acts in a direct manner on the H3.3B promoter by activating AP-1, which in turn binds to the H3.3B CRE/TRE. To show that the CRE/TRE is indeed responsible for mediating the PMA effect, we transiently transfected HeLa cells with an H3.3B promoter linked to a luciferase reporter gene. Treatment of cells with PMA resulted in a 4–5-fold increase in reporter gene activity after 6 h, whereas mutation of the CRE/TRE in the 1.2 kb H3.3B promoter abolished the PMA response (Table 1). In contrast, treatment of cells with cAMP did not have an effect on reporter gene activity (Table 1), confirming the results of the induction experiments of the endogenous H3.3B mRNA, which also showed no response to treatment of cells with cAMP.

DISCUSSION

In contrast with the cell-cycle-dependent histones, the replacement histone H3.3 is synthesized independently of DNA synthesis throughout the cell cycle [7]. Furthermore H3.3B is up-regulated in terminally differentiated cells and tissues [9–12].

Previously we have described a CRE/TRE located in the proximal promoter of the human replacement histone gene H3.3B [19]. Here we show that AP-1 and transcription factors of the CREB/ATF family bind to the H3.3B CRE/TRE *in vitro*. Induction experiments revealed that PMA was capable of stimulating H3.3B transcription in intact HeLa cells, whereas cAMP was not, indicating that AP-1 is the predominant transcription factor binding to the H3.3B CRE/TRE site in HeLa cells *in vivo*. However, this does not exclude the possible binding of CREB proteins *in vivo* to the H3.3B CRE/TRE site in other cells, because the CREB/ATF family in HeLa nuclei consists mainly of ATF/ATF homodimers and CREB/ATF heterodimers, which in contrast with CREB/CREB homodimers are weak transactivators after activation of the cAMP pathway [25]. Protein–DNA interactions of ATF/AP-1 [26,27] and a Jun-related protein [28] with corresponding binding sites in the promoter of cell-cycle-dependent H3 histone genes have been described. However, we did not observe the stimulation of cell-cycle-dependent H3 transcription after treatment of cells with PMA for 6 h.

The immediate induction of H3.3B expression after treatment of cells with PMA, starting after only 30–60 min, points to a direct activation of the H3.3B gene via activated protein kinase C and the subsequent binding of AP-1 to the H3.3B promoter. In addition we showed in transient transfection assays that mutation of the CRE/TRE abolished the PMA response, demonstrating that this element is mediating the transcriptional response of the H3.3B gene after treatment with PMA. In contrast with the H3.3B gene, transcription of the cell-cycle-dependent H3 histone genes was not affected by treatment of cells with PMA, resulting in a relative increase in H3.3B mRNA level within the observed time frame of 6 h. Thus PMA has different effects on the transcription of individual histone subtype genes. These results suggest that the activation of protein kinase C by PMA results in an early accumulation of H3.3B mRNA needed for ongoing differentiation processes, or might even lead to an early remodelling of chromatin by partly replacing H3.1 and H3.2 histone proteins [29]. Previous work on histone H3 synthesis after PMA-induced differentiation of human leukaemic cells reported a decreased synthesis of histone H3.1 and H3.2 proteins and an increased synthesis of H3.3 proteins [11]. The authors concluded that this pattern of expression was due to the cessation of cell proliferation after treatment with PMA. However, in that study the earliest determination of H3 protein synthesis was made after 2 days of PMA treatment.

Besides the human H3.3B gene, an H3.3A histone gene exists [13]. As initially shown in chicken [14], both H3.3A and H3.3B genes encode the same H3.3 histone protein but differ in their nucleotide coding sequences and flanking portions. Therefore it is impossible to relate H3.3 protein levels specifically to H3.3B mRNA levels. Hybridization experiments *in situ* with mouse testis with probes derived from cDNA sequences revealed different expression patterns for both genes, indicating a basal expression of the H3.3A gene compared with a stage-specific transcription of the H3.3B gene [32]. As yet, the mouse H3.3A and H3.3B gene promoter sequences are unknown. The human H3.3A promoter [13] lacks the CRE/TRE site, in contrast with

the H3.3B promoter. Thus we can conclude that this element might be responsible for the specific transcription of the H3.3B gene in distinct cell populations during spermatogenesis, whereas the H3.3A gene might primarily contribute to basal H3.3 synthesis.

In summary we have shown that a CRE/TRE in the promoter of the human replacement histone gene H3.3B mediates PMA-induced H3.3B gene expression. Because cell-cycle-dependent H3 histone genes do not respond to PMA treatment, the activation of H3.3B gene expression by the protein kinase C pathway might result in a remodelling of chromatin structure required for ongoing differentiation processes.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft. O.W. gratefully acknowledges a fellowship from the Deutsche Forschungsgemeinschaft.

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Received 9 June 1997/19 September 1997; accepted 26 September 1997