Transcriptional regulation of the human replacement histone gene H3.3B

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Abstract In contrast to the cell-cycle-dependent histone genes, replacement histone genes are transcribed independently of DNA replication and their expression is upregulated during differentiation. We have investigated the transcriptional regulation of the recently characterized human replacement histone gene H3.3B. Using reporter gene assays of promoter-luciferase gene-constructs, we show that promoter activity largely depends on an intact Oct and CRE/TRE element within the proximal 145 bp of the promoter. DNase I footprinting revealed binding of proteins to a 40-bp region covering these two elements. Band shift experiments identified binding proteins as Oct-1 and factors of the CREB/ATF and AP-1 family, respectively. The unexpected transcriptional regulation of this replacement histone gene is discussed.

Key words: Histone gene regulation; Replacement histone gene; Histone H3.3B

1. Introduction

Histones are the primary constituents of eukaryotic chromatin. This group of basic proteins consists of five classes termed H1, H2A, H2B, H3 and H4. Except H4, these classes of nuclear proteins consist of several subtypes [1,2]. Histone genes may be subdivided into three major groups. First, replication-dependent genes are expressed only during S-phase of the cell cycle; second, replication-independent histone genes encoding replacement histones are also expressed in nondividing quiescent or terminally differentiated cells and third, tissue-specific histone isotypes that are exclusively expressed in the testis of different mammalian species [3,4].

H3.3B is a minor component of the chromatin in dividing cells but can become relatively abundant in nondividing cells [5–8] and adult tissues [9,10], and is expressed in erythroleukemia cells during the precommitment period of differentiation [11]. The expression of this gene is independent from DNA replication [7]. Recently, we have characterized the human replacement histone gene H3.3B [12]. Among the histone gene family, the H3.3B gene shows unique features: it contains three introns and three polyadenylation signals and it is solitarily located on chromosome 17, outside the known histone gene clusters on chromosome 6 [13,14] and 1 [15] which contain the majority of the replication-dependent histone genes.

We have investigated 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. Using transient transfection assays of deletion constructs of the H3.3B promoter linked to a luciferase reporter gene, DNase I footprint analysis and band shift experiments, we demonstrate that an Oct element and a CRE/TRE motif largely contribute to the expression of this gene. This unexpected finding points to a fine tuned transcriptional regulation rather than just basal expression of this replacement histone gene variant.

2. Material and methods

2.1. Probes and promoter constructs

For transcription of probes used in RNase protection analysis, the *Nsi*I/*Xba*I fragment of the 3′-flanking region of the H3.3B gene (see Fig. 1) was subcloned into Bluescript KS. For generation of the luciferase reporter gene constructs, the following H3.3B promoter fragments were cloned into the *Sma*I/*Xho*I site of the luciferase vector pGL3 basic (Promega): the 4.2-kb fragment corresponding to the *Hin*dIII/*Sal*I fragment of the H3.3B promoter, the 1.2-kb *Pst*I/*Sal*I fragment, the 586-bp *Eco*RI/*Sal*I fragment, the 349-bp *Bam*HI/*Sal*I fragment and the 292-bp *Hin*fI/*Sal*I fragment (see Fig. 1). The latter 292-bp H3.3B promoter fragment was used to generate all minor constructs by limited exonuclease III digestion using the Double Stranded Nested Deletion Kit (Pharmacia) according to the manufacturer's instructions. For mutation of the Oct, CRE/TRE, and CCAAT-boxes, the 1.2-kb H3.3B promoter construct was subjected to site directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Stratagene) as described in the manufacturer's manual. The correct sequence of all constructs was verified by automated DNA sequencing.

2.2. RNA isolation and RNase protection analysis

Isolation of total RNA and RNase protection analysis were performed as described previously [4]. Human RNA was kindly provided by Dr. E.J. Bellefroid, Université de Liège [16].

2.3. Transfection of cells and luminescence detection

For transfection experiments, HeLa cells (ATCC CCL2) were cultivated in MEM, 10% fetal calf serum at 37°C and 5% CO_2. Transfections were performed as recommended by the DOTAP manual (Boehringer Mannheim). 2×10^5 cells were cultured in 2 ml medium and transfected with 2 μg of plasmid DNA and 50 ng pCMVβGAL (Clontech) using 12 μl DOTAP in 20 mM HEPES pH 7.4 containing 150 mM NaCl for 17–19 h. After transfection, cells were washed with PBS, incubated with 125 μl lysis buffer provided with the Galacto-Light Plus kit (Tropix) and harvested with a rubber policeman. 20 μl of cell lysate was subjected to measurement of luciferase activity in a luminometer using the luciferase assay substrate provided with the Luciferase Assay System kit (Promega). For determination of the co-transfected β-galactosidase activity, 10 μl of cell lysate was incubated with 66 μl reaction buffer provided with the Galacto-Light Plus kit for 1 h at room temperature and luminescence was measured in a luminometer (AutoLumat LB 953, Berthold) using the accelerator provided with the kit. All measurements were performed in duplicates. To take into account different transfection efficiencies as well as differences in the amount of cell lysates subjected to luminescence measurement, the luciferase activity was normalized by the corresponding co-transfected β-galactosidase activity corrected by the endogenous β-galactosidase activity. The obtained luciferase activities were then expressed relatively to the activity of the 1.2-kb wild-type construct.

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Abbreviations: CRE, cAMP responsive element; TRE, TPA responsive element; TPA, tetraphorbolacetate

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2.4. DNase I footprint analysis

Nuclear extracts were prepared exactly as previously described by Dignam et al. [17]. A 230-bp promoter fragment containing the TATA, Oct, CRE/TRE and the first two CCAAT-boxes was end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (MBI Fermentas). 50 000 cpm of the DNA probe was incubated with increasing amounts (20–60 μg) of nuclear proteins in 30 μl binding buffer containing 20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 20% (v/v) glycerol and 60 μg/ml poly(dI-dC) for 60 min on ice. At the end of the incubation, 200 ng DNase I (Boehringer Mannheim) in 10 μl 20 mM $MgCl_2$, 60 μg/ml poly(dI-dC) was added to the samples with nuclear extracts. Control samples without nuclear proteins were treated in the same way except that the amount of added DNase I was 50 ng. The mixture was incubated for 90 seconds at room temperature and DNase I digestion was terminated by the addition of 100 μl of 20 mM Tris/HCl pH 7.5, 20 mM EDTA, 0.4% SDS, 800 μg/ml tRNA and 80 μg/ml proteinase K. After phenol/chloroform extraction and ethanol precipitation, the samples were analyzed on a denaturing 8 *M* urea/5% (v/v) polyacrylamide gel.

2.5. Band shift experiments

The following oligonucleotides from the H3.3B promoter were used in band shift experiments. H3.3B Oct: 5′-CGTTGGTGTATGCAAATAAGGGTT-3′ and H3.3B CRE/TRE: 5′-GGGTTCTATGACGCAGAGACGCAG-3′. The CRE and AP-1 consensus oligonucleotides were obtained from Santa Cruz Biotechnology. About 0.01 pmol (30 000 cpm) of the [γ-^{32}P]-ATP-labeled double-stranded oligonucleotide was incubated with 10 μg of nuclear proteins, in the presence or absence of unlabeled competitor, in a total volume of 16 μl, containing 10 mM HEPES pH 7.9, 5% (v/v) glycerol, 125 μg/ml poly(dI-dC), 50 mM KCl, 1 mM EDTA, 1 mM DTT and 0.5 mM PMSF for 30 min at room temperature. At the end of incubation, 2 μl of loading buffer with 5% glycerol was added and samples were analyzed on a 4% non-denaturing polyacrylamide gel. In the supershift analysis, the mixture of [γ-^{32}P]-ATP-labeled double-stranded oligonucleotide and nuclear proteins was further incubated with serial dilutions of an Oct-1-specific antibody (Oct-1 supershift reagent, Santa Cruz Biotechnology) for 45 min at room temperature and then analyzed by gel electrophoresis as described above.

3. Results

3.1. Expression of the Human H3.3B Gene

We have examined the expression of the recently characterized human histone gene H3.3B gene using RNase protection assay. The probe used in this assay was transcribed from the *NsiI*/*XbaI* fragment of the 3′-flanking region (see Fig. 1). Fig. 2 shows that the H3.3B gene is transcribed in several human cells and tissues investigated, albeit at different levels. Three polyadenylation sites corresponding to three H3.3B mRNA species differing in length have been described [12]. The predominant mRNA of the H3.3B gene appears to be the longest 1.8-kb transcript and to a lesser extent the smaller 1.4-kb transcript. In contrast, we were not able to detect a protected fragment corresponding to the shortest transcript of 1.1 kb in length (not shown). Since HeLa cells express the H3.3B gene at a relatively high rate, we used this cell line to further study the mechanism of transcriptional regulation of this gene.

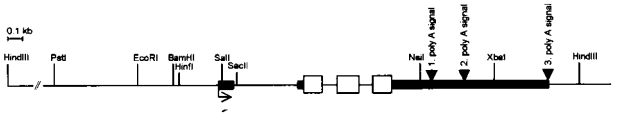

Fig. 1. Restriction map of the human replacement histone gene H3.3B (EMBL accession No. Z48950). Arrow indicates start site of transcription, black boxes flanking portions and open boxes intervening sequences.

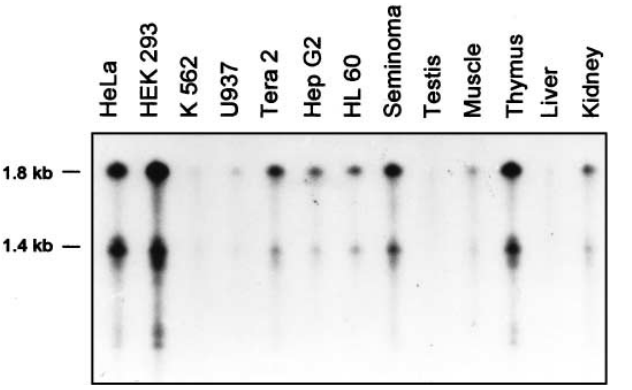

Fig. 2. Expression of the human replacement histone gene H3.3B. Total RNA from different cell lines and human tissues was subjected to RNase protection assay using a probe from the H3.3B 3′ flanking region.

3.2. Identification of promoter elements

To identify *cis* acting elements in the H3.3B promoter, 4.2 kb of the promoter region were cloned proximal to the luciferase reporter gene and a series of 5′ → 3′ deletion constructs were transiently expressed in HeLa cells (Fig. 3). Successive deletion of the promoter, taking into account the 6 CCAAT-boxes, down to the proximal 145-bp fragment did not result in a significant decrease in relative luciferase activity. However, further deletion of 45 bp led to a drop of the promotor activity to basal expression levels (Fig. 3). This 45-bp fragment contains a fully conserved Octamer element (ATGCAAAT) and a CRE/TRE element with the core motif CGTCA on the complementary strand [18–21] located at −105 and −128 relative to the *SalI* cloning site (see Fig. 1), corresponding to −75 and −93 relative to the start of transcription (sequences included in Fig. 4). We then investigated the functional relevance of these two elements within the large 1.2-kb promoter context by mutating 4 bases of each element, respectively (Fig. 4). Compared with the 1.2-kb wild-type construct, mutation of the Oct and CRE/TRE element caused a 25% and 45% reduction of luciferase activity (Fig. 4). Mutation of both elements led to a 70% decrease of reporter gene activity, indicating that the Oct and CRE/TRE elements and corresponding transcription factors are responsible for nearly the entire rate of transcriptional activity of the H3.3B promoter. Since CCAAT boxes have been demonstrated to be important for the transcription of replication-dependent histone genes [22,23], we also investigated the function of the first two CCAAT boxes of the H3.3B promoter. Mutation of these elements did not result in a significant change of promoter activity, confirming the results of the deletion studies, which had shown no effect on promoter activity upon successive deletion of the 6 CCAAT boxes (Figs. 3 and 4). This suggests different functions of the CCAAT boxes in the promoters of replication-dependent and replacement histone genes.

3.3. Binding of nuclear proteins to the proximal H3.3B promoter region

Because the deletion and mutation analysis indicated that the major part of the H3.3B promoter activity resides within the proximal 145 bp, we performed a DNase I footprint experiment to delineate the binding of *trans*-acting factors to this region. A DNA fragment of 230 bp, including the prox-

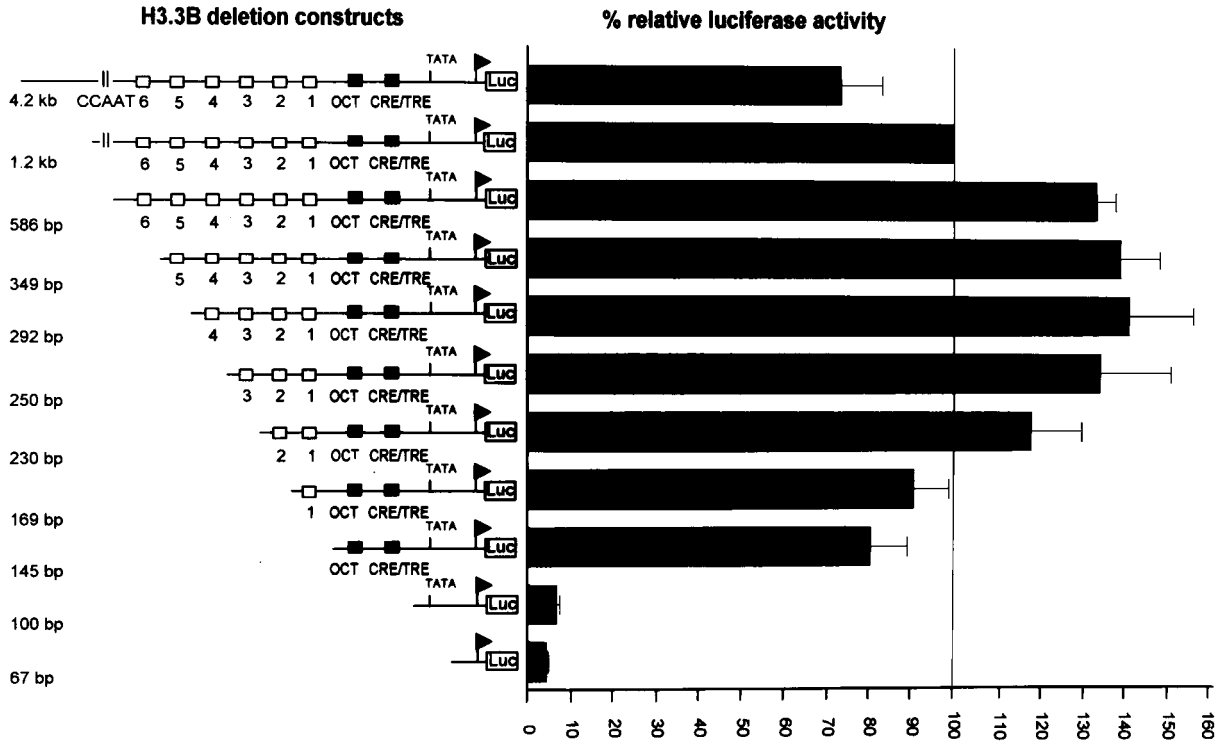

Fig. 3. Transient expression of H3.3B promoter deletion constructs. The deletion constructs are schematically shown in the left panel. The right panel shows the corresponding luciferase activity relative to the 1.2-kb construct. The data are mean and SD values of four experiments.

imal 145 bp of the H3.3B promoter, was incubated with nuclear extracts from HeLa cells and was partially digested with DNase I. This resulted in a 40-bp protected region containing the Oct and CRE/TRE elements (Fig. 5), whereas the region of the first two CCAAT-boxes did not reveal binding of nuclear proteins (not shown). This confirmed the results of the reporter gene analysis by demonstrating the binding of nuclear proteins to the region of the Oct and CRE/TRE elements of the H3.3B promoter.

3.4. Identification of binding proteins

To further specify the nuclear proteins binding to the 40-bp region of the proximal H3.3B promoter, we performed band shift experiments using two overlapping oligonucleotides spanning this region. These oligonucleotides, containing the Oct and CRE/TRE element, respectively, were incubated with nuclear proteins from HeLa cells. Fig. 6A shows specific binding of nuclear proteins to the Oct element-containing oligonucleotide, giving rise to a single protein-DNA-complex. Since

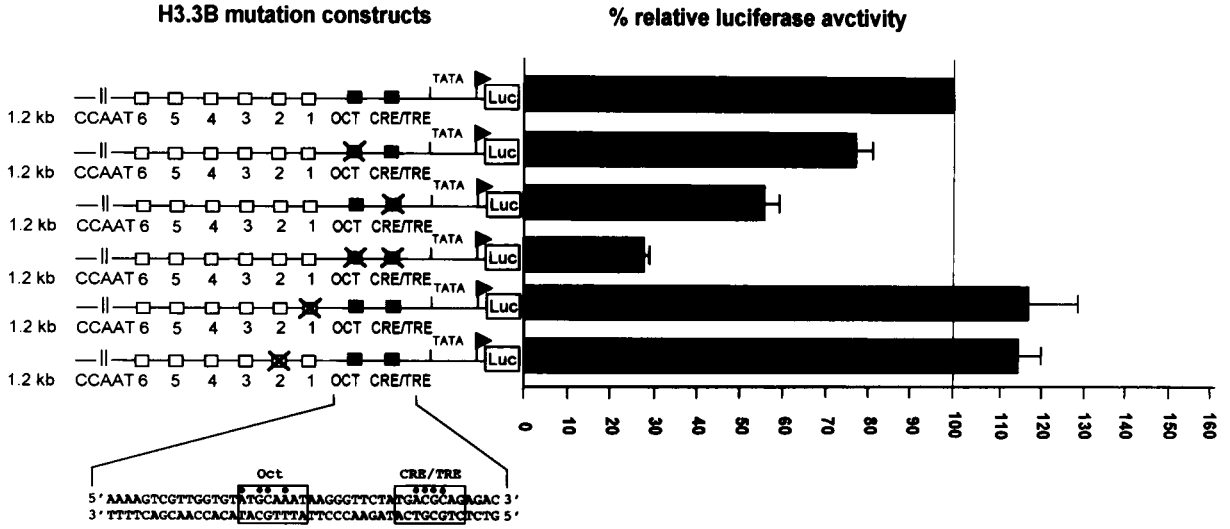

Fig. 4. Transient expression of H3.3B mutation constructs. For details see Fig. 3. The inserted sequence shows the Oct and CRE/TRE element and flanking nucleotides in the proximal H3.3B promoter region. Dots indicate positions mutated by site directed mutagenesis.

the different species of octamer binding proteins form protein-DNA-complexes with different electrophoretic mobility [24], we conclude that only one type of octamer binding protein interacts with the H3.3B Oct element in HeLa cells. Supershift experiments using a specific antibody identified the binding proteins as Oct-1 (Fig. 6B). The analogous experiment using the H3.3B CRE/TRE oligonucleotide resulted in the appearance of three distinct protein-DNA-complexes (Fig. 7A). These complexes apparently consist of proteins of the CREB/ATF family of transcription factors (lower two bands) and of AP-1 (upper band) as shown by co-migration of protein-DNA-complexes obtained with a CRE and AP-1 consensus oligonucleotide of identical size, respectively (Fig. 7B).

4. Discussion

In this paper we show that promoter activity of the human replacement histone gene H3.3B essentially depends on two elements: a fully conserved octamer element and a CRE/TRE element both located within the proximal 145 bp of the promoter. We further demonstrate that the transcription factor Oct-1 and proteins of the CREB/ATF and AP-1 family of transcription factors are capable of binding to these elements, respectively.

The finding of the octamer box and Oct-1 involved in transcriptional regulation of a replacement histone gene is somewhat surprising, since the Oct element and Oct-1 has been implicated in the cell-cycle-dependent transcription of the histone gene H2B [25–27]. It therefore appears likely that additional factors or flanking sequences must exist which convert the same element and corresponding transcription factor into

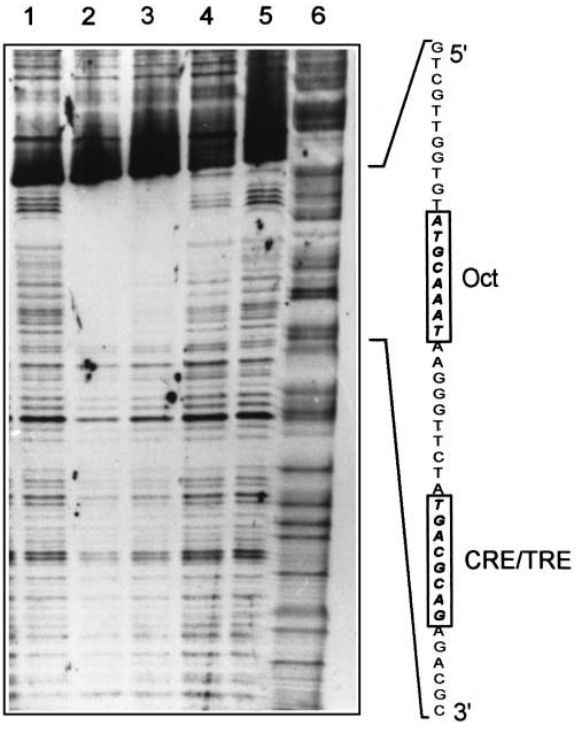

Fig. 5. DNase I footprint analysis of the proximal H3.3B promoter region. Lanes: 1 and 5, control samples without nuclear extract; 2, 3 and 4, samples incubated with 60, 40 and 20 μg nuclear proteins, respectively; 6, G/A sequencing ladder. The sequence of the protected promoter region is given on the right.

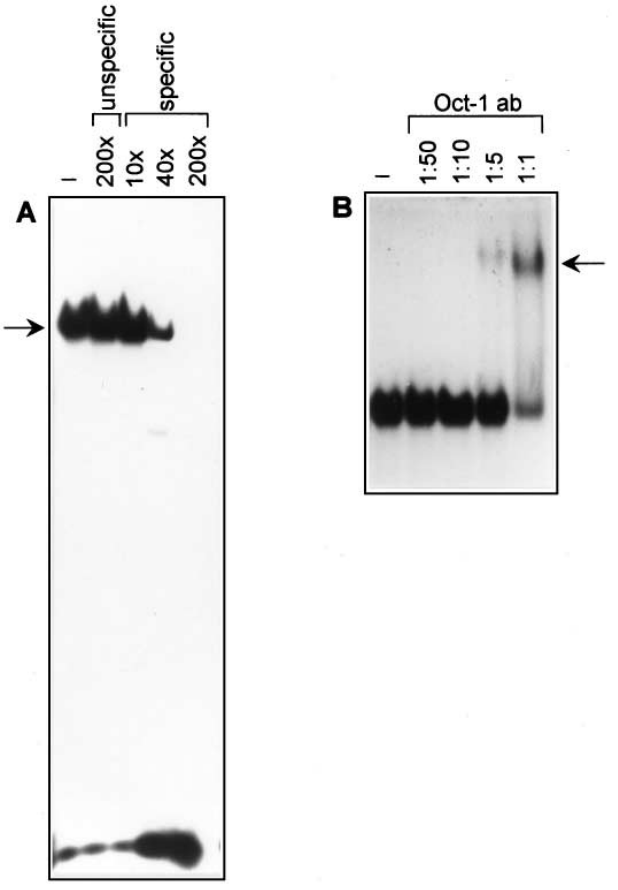

Fig. 6. Band shift analysis of nuclear proteins binding to the H3.3B octamer element. (A) An oligonucleotide consisting of the H3.3B Oct element and flanking sequences was incubated with nuclear extracts without (−) and in the presence of 10, 40 and 200-fold molar excess of specific and unspecific competitor, respectively. (B) Supershift analysis of the DNA/protein complexes shown in A using an Oct-1 specific antibody in serial dilutions. Arrows indicate DNA/protein (A) and DNA/protein/antibody complexes (B), respectively.

a cell-cycle-dependent element in the case of H2B or into a replication independent element as part of the H3.3B promoter. An example for such a selective transactivation of Oct-factor-dependent transcription is the B cell coactivator Bob1 which differentially coactivates octamer sites bound by Oct-1-depending on the promoter context [28–30].

The second element involved in transcriptional regulation of the human H3.3B gene has the sequence TGACGCAG. We designated this element as CRE/TRE element because (a) this element and flanking sequences is able to bind transcription factors of the CREB/ATF and AP-1 family, (b) it contains the essential CRE core motif CGTCA on the complementary strand [20], and (c) the same TGACGCAG motif has been identified as the combined cAMP- and TPA-responsive element in the promoter of the human cholecystokinin gene [19]. Interestingly, it has been shown that nuclear proteins bind to a CRE element of a replication-dependent H3 histone gene throughout the cell cycle, suggesting that this element facilitates basal transcription of this gene outside the S phase [31]. Another function of the CRE/TRE element in the H3.3 B promoter could be the coupling of H3.3B expression to ongoing differentiation processes of cells mediated by the protein kinase C pathway. Preliminary data show that H3.3B expres-

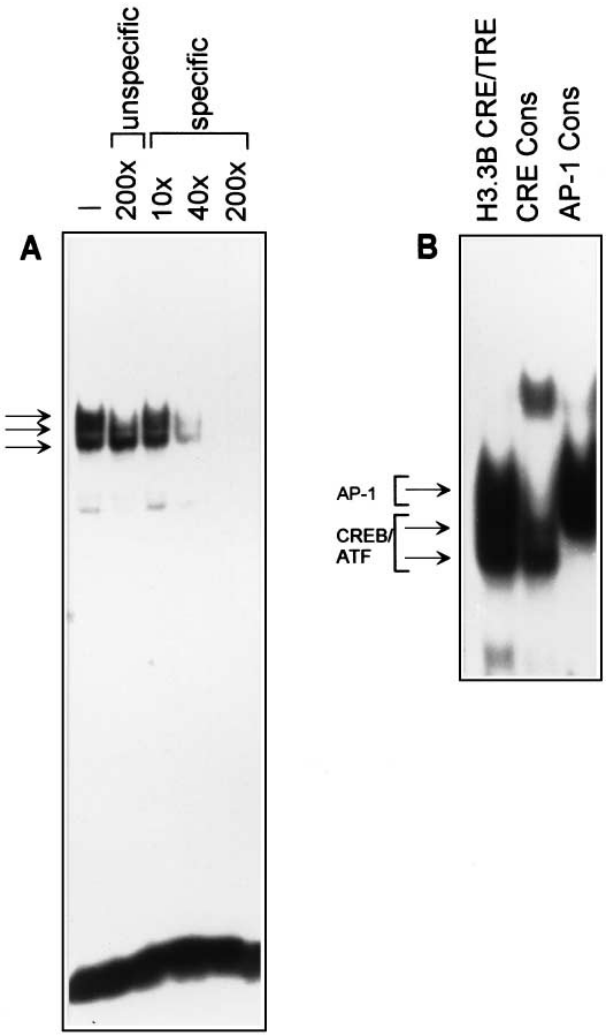

Fig. 7. Band shift analysis of nuclear proteins binding to the H3.3B CRE/TRE element. (A) An oligonucleotide consisting of the H3.3B CRE/TRE element and flanking sequences was incubated with nuclear extracts without (–) and in the presence of 10, 40 and 200-fold molar excess of specific and unspecific competitor, respectively. Arrows indicate the resulting three DNA/protein complexes. (B) Co-migration of the same three DNA/protein complexes as in A (first lane) with DNA/protein complexes formed using nuclear extracts and CRE and AP-1 consensus oligonucleotides, respectively.

sion is indeed upregulated following treatment of cells with TPA (Witt et al., unpublished data).

Comparison of the structure of the H3.3B promoter with the promoter of the histone gene H1°, which also belongs to the group of replacement histone genes, reveals no homologies except the existence of a TATA box and an octamer-like element (ATTTGCT) [32]. However, reporter gene assays in HeLa cells indicate a function of this Oct-like element only in cooperation with other more upstream located elements [32]. On the other hand, a retinoic-acid-responsive element appears to be of major relevance for the H° promoter activity [32,33], which is not contained in the H3.3B promoter. This indicates that the transcriptional regulation of these two replacement genes involves different *cis*/*trans*-acting elements.

In addition to the H3.3B gene, a H3.3A histone gene exists [34]. Both genes encode the same H3.3 histone protein but differ in their nucleotide coding sequence and flanking portions. In situ hybridization experiments with mouse testis using probes derived from cDNA sequences revealed different expression patterns of both genes, indicating a basal expression of the H3.3A gene versus a stage-specific transcription of the H3.3B gene. Thus, the H3.3B CRE/TRE element might be involved in this specific regulation, since the H3.3A promoter is lacking the CRE/TRE site. As yet, the mouse H3.3A and H3.3B promoter sequences are unknown.

In addition to promoter sequences, the 3′ end of histone mRNAs has been shown to be important in regulating histone mRNA levels of replication-dependent histone genes [35]. A conserved stem-loop structure contained in all replication-dependent histone mRNAs is important for efficient mRNA processing and degradation thereby linking mRNA levels of these histone genes to the S-phase of the cell-cycle [36–38]. Since the H3.3B mRNA is lacking this conserved stem-loop structure and contains a poly-A tail instead, it may be possible that basal transcription of the H3.3B gene throughout the cell-cycle is just due to the lack of the 3′ stem-loop. Experiments using chimeric constructs of the H3.3B gene and a replication-dependent gene are in progress to investigate this possibility.

In summary, we have identified an Oct and CRE/TRE element as major determinants of the H3.3B promoter activity, suggesting that these elements are involved in the DNA replication independent transcription featured by the human H3.3B gene, and these elements may also contribute to the increased expression of this gene during differentiation processes. Experiments are in progress to further study the in-vivo function of the CRE/TRE element in regulating the transcription of the human replacement histone gene H3.3B.

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