Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta xx (2007) xxx–xxx
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbaexp

Promoter paper

Functional characterization of the promoter of human kinetochore protein HEC1: Novel link between regulation of the cell cycle protein and CREB family transcription factors

Liansheng Cheng *, Liangwei Li, Xinxian Qiao, Jing Liu, Xuebiao Yao

Anhui Province Key Laboratory of Molecular Medicine, School of Life Science, University of Science and Technology of China, Hefei, Anhui, 230027, China

Received 25 February 2007; received in revised form 10 July 2007; accepted 23 July 2007

Abstract

HEC1 (highly expressed in cancer), which localizes to kinetochore in cell mitosis, plays an essential role in chromosome segregation for M phase progression. To clarify the mechanism of its transcriptional regulation, we searched out and isolated its 5′-flanking region. Mapping of this region identified that it is a TATA-less promoter and contains several putative binding sites for different transcription factors. The results from HeLa cells transfected with pGL3 luciferase reporter vectors containing progressive deletion of the *HEC1* 5′-flanking region demonstrated that two elements containing binding sites for cAMP responsive element binding (CREB) protein and activating transcription factor 4 (ATF4 or CREB2) are critical for transcriptional activity. Mutation of the two elements, not downstream E2F box, resulted in a significant reduction of the promoter activity. Gel shift and supershift assays also demonstrated specific binding of transcription factors to their putative binding sites. Furthermore, overexpression of either CREB or ATF4 enhanced the activation of the *HEC1* promoter and overexpression of both of them had an additive effect on the activation of the *HEC1* transcription. Conversely, overexpression of dominant negative mutants of either CREB or ATF4 resulted in downregulation of *HEC1* mRNA significantly. Our study provided a new insight into a potential mechanism of how transcription factors of CREB family are involved in the regulation of kinetochore protein HEC1 in cancer-related cells.

Keywords: HEC1; Kinetochore; Promoter; CREB; ATF4

1. Introduction

Cell mitosis is a highly ordered and precisely regulated process. In order to distribute the two replicated sister chromatids into two daughter cells equally, cell cycle progression is supervised strictly by spindle checkpoint during metaphase to anaphase. Sister chromatid will not separate until all chromosomes have undergone bipolar spindle attachment [1]. Chromosome movement on the spindle during mitosis and meiosis is powered and regulated by the centromere, a discrete locus on each chromosome. In mitosis, a complex structure, known as kinetochore, assembles on centromeric chromatin to generate a microtubule-binding interface that links chromosomes to the mitotic spindle [2]. Numerous proteins have been involved in kinetochore assembly and microtubule attachment. Among them, $Ndc80^{HEC1}$ complex which is conserved from fungi to humans plays a direct role in mediating interactions between kinetochores and microtubules [3]. Human *HEC1* (highly expressed in cancer) cDNA was isolated first by an improved version of the yeast two-hybrid system using the retinoblastoma protein (p110RB) as a bait [4]. The protein encoded by this gene contains 642 amino acids with a predicted molecular mass of 72 kDa. It localizes to kinetochore and mediates the formation of kinetochore–microtubule structure [5]. A striking feature of HEC1 is its long series of typical leucine heptad repeats at its C-terminal region [6]. Subsequently, one of the components isolated from budding yeast spindle pole preparation shows homology to human HEC1 and was named Ndc80 [7]. Both in human and in yeast, $Ndc80^{HEC1}$ interacts with other three kinetochore proteins (Nuf2, Spc25, and Spc24) to form the Ndc80 complex through its coiled-coil domain for helping to constitute

* Corresponding author. Tel.: +86 551 3606294; fax: +86 551 3601443.
E-mail address: charlson@ustc.edu.cn (L. Cheng).

0167-4781/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbaexp.2007.07.005

the outer plate of kinetochore. Ndc80^{HEC1} complex is required for recruitment of spindle checkpoint components, including Mps1 and Mad1/Mad2 complexes, to centromeres [8,9] and plays a crucial role in establishing the stable kinetochore–microtubule interaction [10]. Recent study suggested that the Ndc80^{HEC1} complex does not act as a targeting scaffold for kinetochore proteins, but rather plays a crucial role in stabilizing microtubule attachments by maintaining the structural integrity of binding sites for microtubule plus ends at the outer kinetochore. The stabilization of microtubule attachments allows the formation of mature kinetochore fibers capable of aligning and segregating chromosomes properly [11].

In HeLa cells the results from RNAi study showed that HEC1 is required for other two checkpoint proteins Mad1 and Mad2 binding to kinetochores and HEC1/Nuf2 complex function to prevent microtubule-dependent stripping of Mad1 and Mad2 from kinetochores if it has not formed stable kinetochore–microtubule attachments [12]. Furthermore, although cell injected with anti-HEC1 antibodies were able to undergo cytokinesis, disordered sister chromatid alignment and separation as well as multiple micronuclei were observed and cells were ultimately nonviable [13]. The evidences that inactivation of HEC1 either by genetic deletion or by antibody neutralization will result in severe and lethal disruption of mitotic progression combined with the fact that HEC1 is always expressed abundantly in the S and M phases of rapidly dividing cells but not in terminal differentiated cells suggested strongly that HEC1 is required for cell proliferation and has strong association with the occurrence and development of cancer. The activation of transcription of the *HEC1* gene may be one of a large number of initial physiological processions which transform normal cells to cancer cells. However, the mechanism of how the *HEC1* gene expression is regulated is still poorly understood.

In the present study, we first cloned a 2039-bp fragment from the 5′-flanking region of human *HEC1* gene and identified several putative binding sites for transcription factors. By luciferase reporter vector and electrophoretic mobility shift assay (EMSA) we identified the core sequence of 5′-flanking region and the critical elements for transcriptional activity. We showed that the −177/+73 region, which contains two boxes for CREB and ATF4, is indispensable as a core promoter for the *HEC1* gene transcription. Finally, by fluorescent quantitative PCR (FQ-PCR) assay, we further identified the effect of level of CREB and ATF4 on the *HEC1 gene* expression.

2. Materials and methods

2.1. Cell culture

Human cervical carcinoma HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dullbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% calf serum at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Mapping and Isolation of the HEC1 promoter

The GenBank Database, the Genomatix Suite of sequence analysis tools MatInspector (professional version 7.4) [14] and TFSEARCH (version 1.3) (Yutaka Akiyama: "TFSEARCH: Searching Transcription Factor Binding Sites", http://www.rwcp.or.jp/papia/) were used for analyzing the *HEC1* promoter. Genomic DNA isolated from HeLa cells by Wizard Genomic DNA Purification Kit (Promega, Madison, WI) was used as the template for PCR to obtain 5′-flanking region of the *HEC1* gene using the specific primer HecP-2KF (5′-cccacctcttc-cactcccacccttggcc-3′) and HecP-2KR (5′-ccagtttcctgacacgacccagggaccg-3′). The PCR product was cloned using pGEM-T vector (Promega, USA) and sequenced for confirming the sequence correctness. It was then used for construction of luciferase reporter plasmids.

2.3. Construction of luciferase reporter

Progressive deletion reporter in the *HEC1* promoter region was constructed by PCR using the *Mlu*I and *Xho*I sites of the luciferase reporter vector pGL3-basic (Promega, USA). These PCR fragments were generated using a common reverse primer Hec1P-R and ten different forward primers designed by Primier Primer 5.0 software (Table 1(1)). The acquired luciferase reporter plasmids were then sequenced to make sure their correction. Mutageneses of three transcription factor binding sites in the promoter region were carried out using the plasmids pHec1P-3 and pHec1P-9 as the template by the QuikChange II XL Sitedirected mutagenesis system (Stratagene, USA). The appropriate pairs of complementary primers were designed according to the manufacturer's instruction and the constructed plasmids were named pHec1P-mCREB, pHec1P-mATF4, pHec1P-mE2F, and pHec1P-mCREB+mATF4 for mutation of pHec1P-3 and pHec1P-9-mCREB, pHec1P-9-mATF4, pHec1P-9-mE2F, and pHec1P-9-mCREB+mATF4 for mutation of pHec1P-9 (Table 1(2)).

2.4. DNA transfection and luciferase assays

1×10^6 cells were transiently transfected with total 4 μg plasmid DNA containing pCMV-SPORT-β-gal as a control and 10 μl LipofectAMINE reagent in 500 μl serum-free OPTI-MEM (Invitrogen, Carlsbad, CA). Cells were incubated with transfection solution for 6 h and recovered for 48 h in complete

Table 1
Primers used for construction of luciferase reporter

(1) Primers for stepwise deletion plasmid constructs[a]	
Hec1P-1(−51/+73)	5′-gaaACGCGTgaattctttcaaattcgaacggct-3′
Hec1P-2(−123/+73)	5′-gaaACGCGTgaattcttccagcgacgaggcggtc-3′
Hec1P-3(−177/+73)	5′-gaaACGCGTgaattccccacagaccccagttcct-3′
Hec1P-4(−244/+73)	5′-gaaACGCGTgctgcccacaggtcttccg-3′
Hec1P-5(−501/+73)	5′-gaaACGCGTcctgggagcccattccacc-3′
Hec1P-6(−718/+73)	5′-gaaACGCGTgcagcgaggaaatcctatt-3′
Hec1P-7(−928/+73)	5′-gaaACGCGTactcagagcagaaaggcaagc-3′
Hec1P-8(−1165/+73)	5′-gaaACGCGTccacctttggaagcagcaact-3′
Hec1P-9(−1406/+73)	5′-gaaACGCGTcggaaccaaaagaaccttgaa-3′
Hec1P-10(−1966/+73)	5′-gaaACGCGTcccacctcttccactcccac-3′
Hec1P-R/A-750(−705/+73)	5′-ctcACGCGTgcagcgaggaaatcctatt-3′
Hec1P-R/A-850(−769/+73)	5′-ctcACGCGTctttcatcaccatcactc-3′
Hec1P-R/A-900(−834/+73)	5′-ctcACGCGTacaacgctagttaatggcaca-3′
Hec1P-R/A-950(−879/+73)	5′-ctcACGCGTattatttctgaactaaaggtg-3′
Hec1P-R/A-1050(−980/+73)	5′-ctcACGCGTttaaaataatgcttggttcctcc-3′
Hec1P-R/A-1100(−1018/+73)	5′-ctcACGCGTcagacggttgcctaattttca-3′
Hec1P-R/A-1150(−1060/+73)	5′-ctcACGCGTaagttcaacctaacagtattcc-3′
Hec1P-R/A-1200(−1125/+73)	5′-ctcACGCGTaatgttgtttccatcaatctt-3′
Hec1P-R/A-1300 (−1200/+73)	5′-ctcACGCGTattatttctgaactaaaggtg-3′
Hec1P-R	5′-gaaCTCAGAccagtttcctgacacgaccc-3′
(2) Primers for mutation of E2F, ATF4 and CREB sites[b]	
mE2F F	5′-aaattcgaacggc***tttgaaaggcc***gaggaaggacct-3′
mE2F R	5′-***ggcctttcaaa***gccgttcgaattt-3′
mCREB F	5′-cgcgcgtcgtgcgtaa***tcaattca***gcgccggcggag-3′
mCREB R	5′-***tgaattga***ttacgcacgacgcgcgc-3′
mATF4 F	5′-cagctgggcgcgaccag-3′
mATF4 R	5′-ctggtcgcgcccagctgaccccaggaactg-3′

[a] The *Mlu*I and *Xho*I site underlined in the primers for cloning.
[b] The bold italics indicated the mutation sites.

Please cite this article as: L. Cheng et al., Functional characterization of the promoter of human kinetochore protein HEC1: Novel link between regulation of the cell cycle protein and CREB family transcription factors, Biochim. Biophys. Acta (2007), doi:10.1016/j.bbaexp.2007.07.005

medium. Then cell extracts were prepared by lysing the cells with 1 ml of freshly diluted 1× Reporter Lysis Buffer (Promega, USA). The lysate was centrifuged at 13,000 rpm for 2 min to pellet the cell debris and the supernatants were collected to a new tube. A 10-μl aliquot of the extract was added to 25 μl of the luciferase assay substrate and the luminescence of the samples was read immediately on a TD-20/20 Luminometer. At the same time, a 10-μl of the cell extract was mixed with 290 μl of o-nitrophenyl-β-D-galactopyranoside (ONPG) solution (880 μg/ml ONPG, 67 mM Na_3PO_4, 1 mM $MgCl_2$, 45 mM β-mercaptoethanol, pH 7.5). The absorbance of the mixture was determined at 420 nm after 30 min of incubation at 37 °C. Each assay was performed in triplicate and corrected with transfection efficiency by β-galactosidase activity.

2.5. Electrophoretic mobility shift assay

The nuclear extracts were prepared as described previously with little modification [15]. Briefly, cells were scrapped and resuspended in 100 μl of cold buffer A (10 mM HEPES–KOH with pH 7.9, 0.05% NP-40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF), and then were allowed to swell on ice for 10 min and centrifuged briefly to discard the supernatant fraction. The pellet was resuspended in 100 μl of cold buffer C (20 mM HEPES–KOH with pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 20 min for high salt extraction. After centrifugating for 2 min at 4 °C, the supernatant fraction containing DNA binding proteins was stored. The nuclear extract (5 μg of protein) was then incubated with 1 μg of poly(dI–dC) (Pharmacia Biotech Inc.) on ice for 20 min, and a ^{32}P-labeled double-stranded oligonucleotide containing CRE (cAMP response element) element (5′-gtcgtgcgtaatgacgtcagcgccggcgga-3′) or ATF4-binding site (5′-tcctggggtgacgcagctgggcgcgac-3′) was added at room temperature for 20 min. The resulting DNA–protein complexes were resolved in 4% nondenaturing polyacrylamide gel. In the supershift experiments, 1 μg of rabbit polyclonal anti-CREB or anti-ATF4/CREB2 antibody (Santa Cruz Biotechnology) was added to the reaction mixture and incubated on ice for 1 h before the addition of the probe.

2.6. Real-time fluorescent quantitative PCR

The genes of CREB, ATF4 and their dominant negative mutants which were generated by mutation of phosphorylated site for CREB and deletion of their bZIP (basic leucine zipper) regions for ATF4 were obtained from Dr. Eric J. Nestler and Dr. Thomas Green (UT Southwestern Medical Center, Dallas, Texas). These genes were then subcloned into pcDNA3.0 expression vector (Invitrogen, Carlsbad, CA) and the constructed plasmids were named pCREB, pmCREB, pATF4, and pmATF4 respectively. HeLa cells were transfected with 2 μg of constructed plasmids for 48 h as described above and total RNAs were then extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed with poly $(dT)_{15}$ primer as described in manufacturer's instruction (Promega, USA). To identify gene expression changes, FQ-PCR analysis was performed with an ABI Prism 7900HT Sequence Detection System using SYBR® universal PCR master mix according to the manufacturer's specifications (Applied Biosystems Incorporated (ABI), foster City, CA). SYBR Gene Expression Assays are available for the *HEC1* gene with primers HEC1-F (5′-ttagcagagtatcacaaattggcta-3′) and HEC1-R

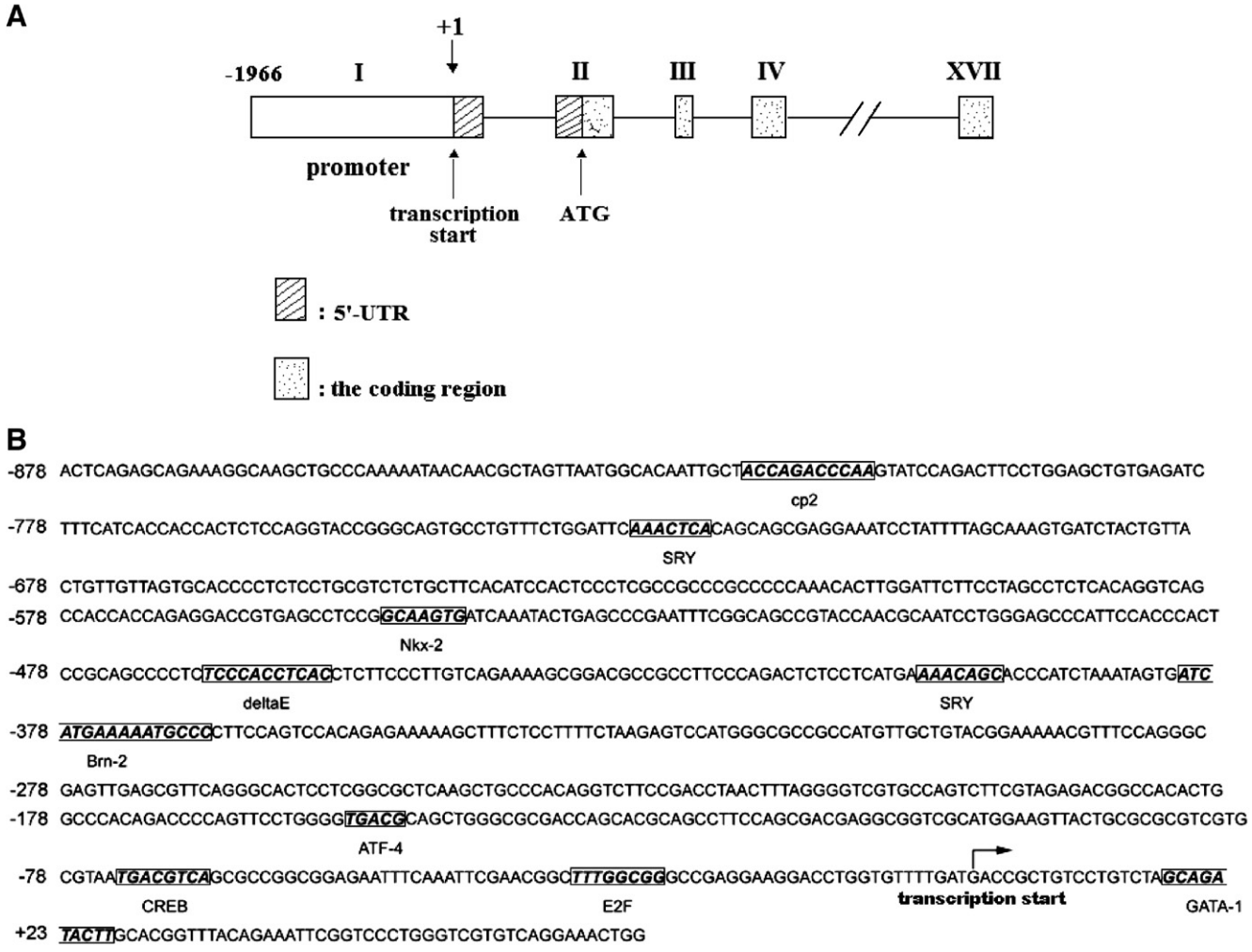

Fig. 1. Structural analysis of the *HEC1* 5′-flanking region. (A) Schematic representation of the genomic structures of the *HEC1* gene. Exons are numbered by upper-roman. (B) Putative binding sites for transcription factors are indicated with boxes. The transcription start site is indicated by arrow.

Please cite this article as: L. Cheng et al., Functional characterization of the promoter of human kinetochore protein HEC1: Novel link between regulation of the cell cycle protein and CREB family transcription factors, Biochim. Biophys. Acta (2007), doi:10.1016/j.bbaexp.2007.07.005

(5′-aagaggtacataaacttgagccctg-3′). Human *β-actin* gene was amplified as endogenous control with primers B-actin-F (5′-ttgccgacaggatgcagaa-3′) and B-actin-R (5′-gccgatccacacggagtactt-3′). The thermal cycler conditions were as follows: hold for 10 min at 95 °C, followed by three-step PCR for 50 cycles of 95 °C for 30 s followed by 50 °C for 30 s and 72 °C for 30 s. All samples were performed in triplicate. To normalize the relative expression of gene of interest to the β-actin control, standard curves were prepared for HEC1 and β-actin in each experiment. Amplification data were analyzed with an ABI Prism Sequence Detection Software version 2.1.

3. Results

3.1. Mapping of the putative cis-acting elements in the HEC1 promoter

With an accession number ***NM006101***, *HEC1* mRNA was reported first by Chen et al. in 1997 [6]. After searching for Entrez Gene by BLAST tool, we located the *HEC1* gene at chromosome 18p11.32. It is composed of seventeen exons and spans more than 45 kb of genomic DNA. The exon I of the *HEC1* gene contains the 5′-untranslated region (5′-UTR) and the exon II contains a 10-bp untranslated region and the translation start site (Fig. 1A). According to the *HEC1* genomic sequence searched in GenBank, we designed the primer HecP-2KF and HecP-2KR for cloning its 5′-flanking region. Subsequently, we extracted the genomic DNA from HeLa cells and it was then used to isolate a 2039-bp fragment by PCR. The PCR products were sequenced and it covers the region from −1966 to +73 relative to the transcription start site.

To explore potential transcription factor binding sites existed in the *HEC1* promoter region, we employed two computer softwares, MatInspector (professional version 7.4) and TFSEARCH (version 1.3), while the former was used to inspect possible transcription factor binding sites and the latter for potential promoter regions.

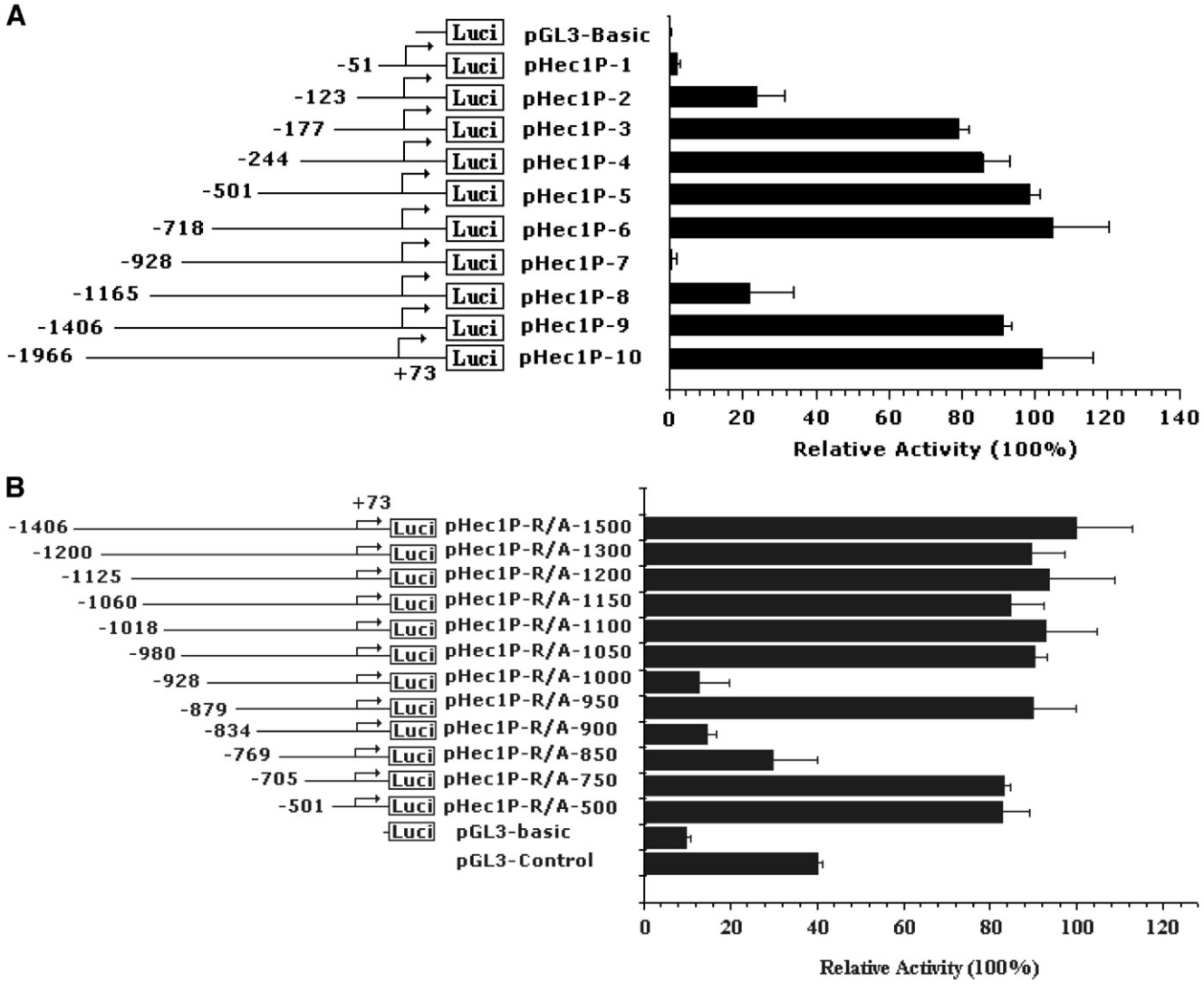

Fig. 2. Deletion analysis of *HEC1* promoter activity in HeLa cells. (A) Schematic representation of a series of deletion constructions containing different lengths of *HEC1* promoters fused to luciferase reporter gene. The bent arrow indicates the transcription initiation site. 4 μg of each reporter plasmid was transfected into HeLa cells. Cells were harvested for luciferase assays at 48 h after transfection. Results are normalized relative to the luciferase activity of pHec1P-10, which was set as 100%. (B) Schematic representation of DNA constructions containing various lengths of the repressor/activator region of the *HEC1* promoter fused to luciferase reporter gene. Each construction was transfected into HeLa cells and its promoter activity was measured as described above. Results are normalized relative to the luciferase activity of pHec1P-9, which was set as 100%.

Please cite this article as: L. Cheng et al., Functional characterization of the promoter of human kinetochore protein HEC1: Novel link between regulation of the cell cycle protein and CREB family transcription factors, Biochim. Biophys. Acta (2007), doi:10.1016/j.bbaexp.2007.07.005

The search was performed using 0.95 and 0.90 as cutoffs for core and matrix similarity, respectively, and revealed that the *HEC1* promoter is a TATA-less promoter and contains several putative binding sites of transcription factors for cp2, SRY, Nkx-2, deltaE, Bm-2, ATF4, CREB and E2F (Fig. 1B). This result was used as the reference for the design of luciferase reporter vectors.

3.2. Transcriptional activity of HEC1 promoter deletions and mutants

In order to localize the *cis*-acting elements that are important for transcriptional activity of the *HEC1* promoter, a series of 5′-deletion constructions were generated by PCR and cloned into the promoterless pGL3-basic luciferase reporter vector. The resulting constructions were named pHec1P-1 to -10 which contain different sections of 5′-flanking region and transiently transfected into HeLa cells. As shown in Fig. 2A, transfection of cells with pHec1P-5 (from nucleotides −501 to +73 relative to the transcription start site) which contains most of elements of promoter showed that it has the same transcriptional activity as pHec1P-10 (−1966 to +73) and yielded an 88-fold increase in promoter activity relative to pGL3-basic. Deletion from nucleotides −501 to −177 (pHec1P-3) reduced the *HEC1* promoter activity only by 15%. Further deletion from nucleotides −177 to −123 (pHec1P-2) reduced the activity by 70% but it was still 10 times higher than the pGL3-basic vector. However, deletion to nucleotide −51 (pHec1P-1) cancelled the promoter activity completely. These results suggest that the sequence between nucleotide −177 and −51 contains elements that contribute the main transcriptional activity to the *HEC1* promoter. The 5′-flanking region including up to nucleotide −177 could be referred as the proximal promoter, and the 5′-flanking region including up to −123 relative to the translational start site could be referred as the minimal promoter. Interestingly, the segments from −928 to −718 bp repressed the observed reporter gene expression completely and this repression could be restored partly or completely in the presence of the segments from −1165 to −928 bp or the segments from −1406 to −928 bp respectively (Fig. 2A). These findings indicate a strong repressor domain between −928 and −718 bp and a strong activator domain between −928 and −718 bp in the *HEC1* gene promoter.

To further characterize the region regulating the transcription activity of the *HEC1* promoter, fragments containing varying lengths of sequences of repressor/activator region were generated by PCR. The PCR fragments were subcloned into pGL3-basic vector and sequenced as described above. These constructions were designated as pHec1P-R/A-750, -850, -900, -950, -1050, -1100, -1150, -1200, and -1300 (Fig. 2B). The plasmids pHec1P-R/A-500, -1000, and -1500 are same as the plasmids pHec1P-5, -7, and -9. The promoter activities of these deletion constructions were also tested in HeLa cells as described above. As shown in Fig. 2B, deletions from −980 to −928 and from −879 to −834 resulted in almost complete abolition in transcription activity compared to pGL3 basic. These results suggest that potential positive regulatory elements exist within the −980 to −928 and −879 to −834 region, while potential negative regulatory elements exist within the −928 to −879 and −834 to −718 region. Our results further indicated that the transcription activity of the *HEC1* promoter may be regulated precisely in different cell cycle by cooperation of multiple manipulators.

3.3. Effects of ATF4-binding site and CRE element on the HEC1 promoter activity

From the results of computational analysis and transient transfection of HeLa cells, two elements, TGACG and TGACGTCA harboring in sequence −177 to −51 and corresponding to consensus binding sites for transcription factors ATF4 and CREB respectively, were identified as potential and the most important transcription factor binding sites which is responsible for the promoter activity. In order to examine the role of the two elements in the regulation of the *HEC1* promoter, we modified ATF4-binding site and CRE element of luciferase reporter vectors pHec1P-3 and pHec1P-9 and constructed a number of new plasmids, pHec1P-mE2F, pHec1P-mATF4, pHec1P-mCREB, and pHec1P-mCREB/mATF4 for mutation of pHec1P-3 and pHec1P-9-mCREB, pHec1P-9-mATF4, pHec1P-9-mE2F, and pHec1P-9-mCREB+mATF4 for mutation of pHec1P-9, which contain the mutated E2F-binding site, ATF4-binding site, CRE element and both of latter two elements respectively (Fig. 3A). As shown in Fig. 3B and C, transfection of HeLa cells with pHec1P-mATF4 and pHec1P-mCREB decreased promoter activity up to 2-fold and 7-fold compared with the unmodified pHec1P-3, and transfection with pHec1P-9-mATF4 and pHec1P-9-mCREB decreased promoter activity up to 2-fold and 5-fold compared with the unmodified pHec1P-9 respectively. However, transfection of cells with pHec1P-mCREB+mATF4 or pHec1P-9-mCREB+mATF4 almost abolished the promoter activity completely compared with cells treated with pHec1P-3 or pHec1P-9 respectively. Although the result showed that the −123/+73 promoter region containing the consensus E2F binding site has no transcriptional activity (Fig. 2A), as an important transcription factor binding site [16] and an additional control, we also constructed the plasmids pHec1P-mE2F and pHec1P-9-mE2F which contain the mutated E2F element and transfected them into HeLa cells. The result showed that even if this site was mutated, transcriptional activities of pHec1P-mE2F and pHec1P-9-mE2F were the same as pHec1P-3 and pHec1P-9 respectively as expected (Fig. 3B and C). These results indicated that the region between −177 and −51 which includes an ATF4-binding site and a CRE element is critical for transcriptional activity of the *HEC1* promoter.

3.4. Binding analysis of transcription factors to the promoter

To prove the sequence from −177 to −51 really contains the binding sites for transcription factors ATF4 and CREB as supposed by computational analysis, we next performed electrophoretic mobility shift assays to identify the actual DNA–protein interaction. A probe, which spans from nucleotides −84 to −55 relative to transcription start site and contains the CREB binding site, was labeled with ^{32}P-ATP and incubated with nuclear extracts from HeLa cells. One distinct binding complex was generated in electrophoretic gel, and it could efficiently be removed

Please cite this article as: L. Cheng et al., Functional characterization of the promoter of human kinetochore protein HEC1: Novel link between regulation of the cell cycle protein and CREB family transcription factors, Biochim. Biophys. Acta (2007), doi:10.1016/j.bbaexp.2007.07.005

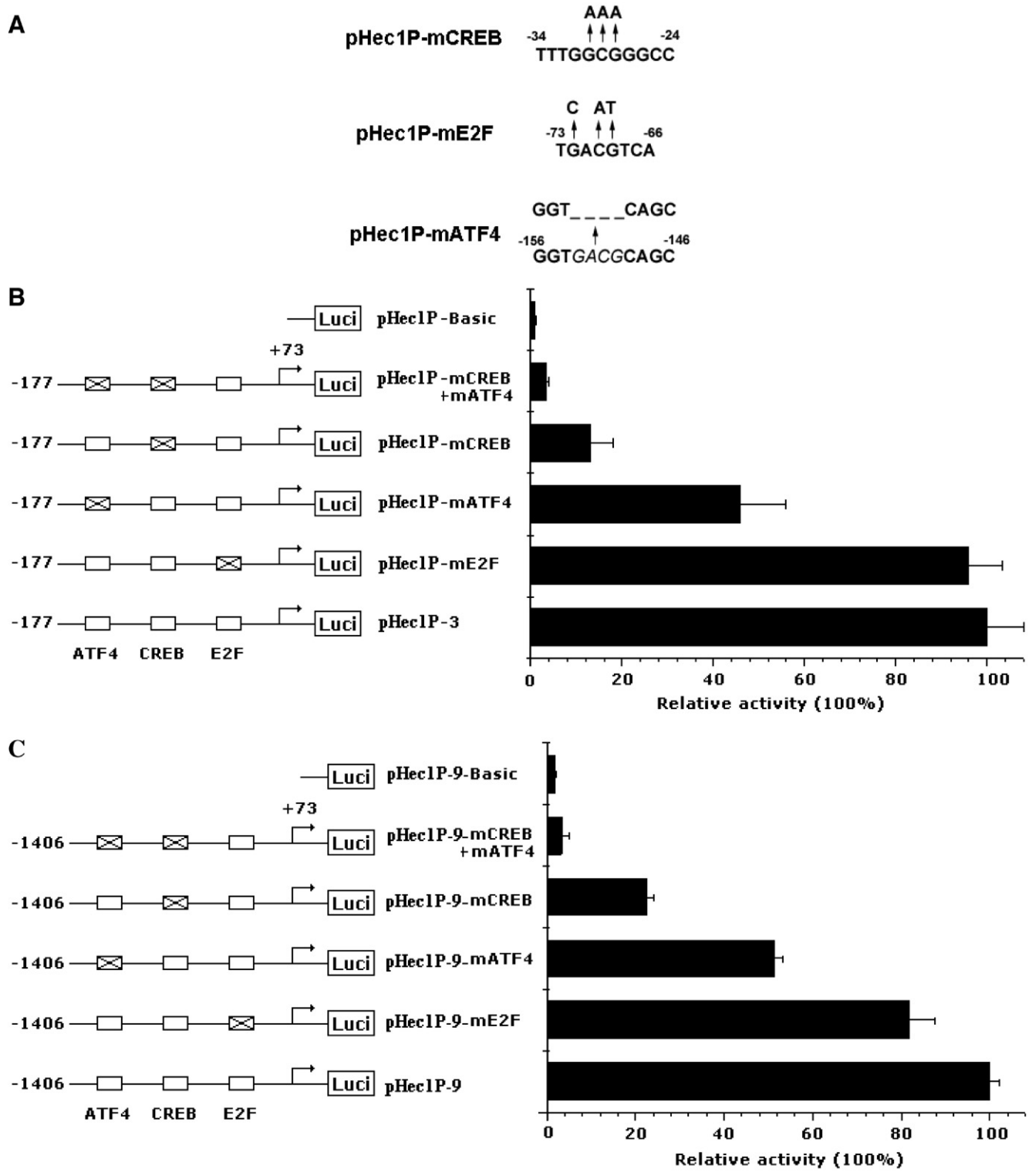

Fig. 3. CREB and ATF4 are required for maximal basal activity of the *HEC1* proximal promoter. Mutations were introduced into the binding sites for E2F, CREB and ATF4 of the *HEC1* promoter by PCR as described in Materials and methods. (A) The schematic representation of the mutated binding sites for three transcription factors. (B, C) HeLa cells were transfected with 4 μg pGL3-basic control or vectors containing the mutated binding sites for three transcription factors. Luciferase activities of different vectors were assayed as described in Materials and methods. Results are normalized relative to the luciferase activity of pHec1P-3 and pHec1P-9 respectively, which was set as 100%.

by an excess of unlabeled homologous CREB probe but not by the heterologous NF-κB probe (Fig. 4A). Furthermore, the binding pattern obtained in the gel shift assays was characterized using antibodies against either CREB or ATF4 and the DNA–protein complex could be supershifted only by formation of complex of DNA, CREB and antibody (Fig. 4A). Similarly, with

Please cite this article as: L. Cheng et al., Functional characterization of the promoter of human kinetochore protein HEC1: Novel link between regulation of the cell cycle protein and CREB family transcription factors, Biochim. Biophys. Acta (2007), doi:10.1016/j.bbaexp.2007.07.005

another labeled probe which spans from nucleotides −162 to −134 and contains a specific ATF4-binding site, a binding complex was also observed in electrophoretic gel (Fig. 4B). The competitive reaction also showed that ATF4 specifically binds to this probe because the shift could just be cancelled by 100-fold unlabeled ATF4 probe but not by NF-κB probe (Fig. 4B). Furthermore, this band of DNA–protein complex could be removed only by anti-ATF4 antibody but not by anti-CREB antibody. Therefore, at least two nuclear protein factors from HeLa cell extracts, one of which should be CREB and the other of which should be ATF4, can specifically interact with their corresponding elements located in the −177/−51 promoter region.

3.5. Regulation of HEC1 expression by overexpression of transcription factor

To further elucidate the actions by which CREB and ATF4 contributed to the regulation of the *HEC1* gene expression, we evaluated the impacts of contents of wild-type and mutated CREB and ATF4 on gene transcription by FQ-PCR. First, HeLa cells were treated with plasmids containing the wild-type *CREB* or *ATF4* gene, and total RNAs were isolated and reverse-transcripted with poly(dT) primer and M-MLV reverse transcriptase. The cDNAs were then subjected to amplify and products were quantified using the green fluorescent dye. As shown in Fig. 5, comparing with the control cells treated with pcDNA3.0 vector, transfection of 1×10^6 cells with 2 μg plasmid of wild-type CREB (pCREB) or ATF4 (pATF4) led to a 2.2- or 1.6-fold increase of *HEC1* mRNA respectively, and transfection of cells with 2 μg plasmid of mutated CREB (pmCREB) or ATF4 (pmATF4) led to a 0.6- or 0.7-fold decrease of *HEC1* mRNA respectively. Moreover, cotransfection with 2 μg both pCREB and pATF4 increased the *HEC1* mRNA level up to 4.1-fold. Conversely, cotransfection with 2 μg both pmCREB and pmATF4 decreased the *HEC1* mRNA level up to 0.5-fold. These results demonstrated that the *HEC1* promoter was regulated by the nature of transcription factors CREB and ATF4 corporately.

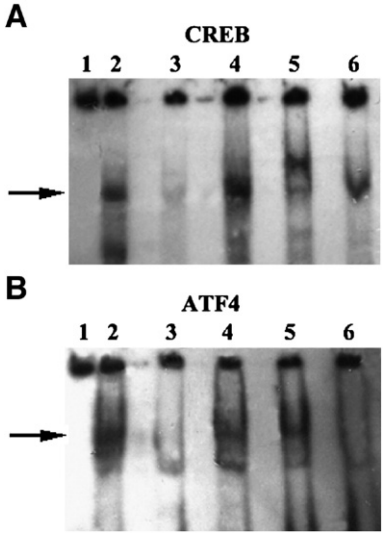

Fig. 4. Electrophoresis mobility shift assay of the activating region. (A) ^{32}P-labeled CREB probe was run on gel without incubation with HeLa nuclear extract as negative control (lane 1). CREB was detected by incubation of 5 μg of nuclear extract with ^{32}P-labeled CREB probe (lane 2), and 100-fold unlabeled CREB or NF-κB probe was added to this system as specific or nonspecific competitor respectively (lanes 3 and 4). Supershift was assayed by incubation of anti-CREB or -ATF4 antibody with nuclear extract and ^{32}P-labeled CREB probe respectively (lanes 5 and 6). The shift band is indicated by arrow. (B) EMSA assay of binding of ATF4 to putative site was done as the case of that of CREB with the replacement of CREB probe with ATF4 probe.

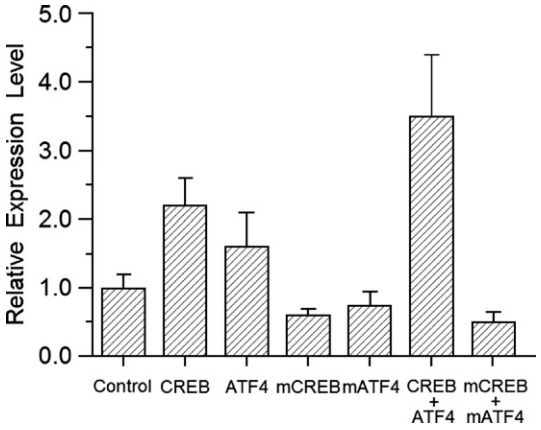

Fig. 5. The effect of ectopic expression of key transcription factors on the expression of HEC1 in HeLa cells. HeLa cells were transfected with parent pcDNA3.0 vector or vectors containing wild-type CREB (pCREB), wild-type ATF4 (pATF4), both wild-type CREB and ATF4 (pCREB+pATF4), dominant negative mutant of CREB (pmCREB), dominant negative mutant of ATF4 (pmATF4), or both dominant negative mutants of CREB and ATF4 (pmCREB+pmATF4) respectively. The expression of HEC1 on mRNA level was analyzed by FQ-PCR as described in Materials and methods.

4. Discussion

HEC1, a kinetochore protein named by its high expression in cancer cell, expresses in most cancer cells and is crucial for faithful chromosome segregation [6]. HEC1 is expressed most abundantly in actively dividing cells but not in terminally differentiated cells. To explore the potential mechanism of the regulation of HEC1 expression, we isolated the 5′-flanking region of the human *HEC1* gene and identified *cis*-acting elements involved in the regulation of human *HEC1* gene. We then constructed a series of deletions from the *HEC1* promoter and assayed their transcriptional activities by luciferase reporter gene. The results revealed that the −177/−51 region relative to the transcription start site contains the basic transcriptional activity of the *HEC1* promoter and the presence of two key elements at positions −154 to −150 bp and −73 to −66 bp as ATF4- and CREB- binding site respectively. To elucidate the roles of two elements on promoter activity, we mutated their core sequences for binding of transcription factors respectively. The results showed that mutation of anyone of them would result in significant decrease of promoter activity and maximal transactivation of HEC1 requires the combined use of both the sequences. Furthermore, we also identified the presence of a strong upstream inhibitory domain at positions −928 to −718 bp. It is postulated that there is the negative element which could be bound by a dominant repressor protein, which once bound to this element, will antagonize the activity of other activator or enhancer

Please cite this article as: L. Cheng et al., Functional characterization of the promoter of human kinetochore protein HEC1: Novel link between regulation of the cell cycle protein and CREB family transcription factors, Biochim. Biophys. Acta (2007), doi:10.1016/j.bbaexp.2007.07.005

elements in this domain. Transcriptional repression in eukaryotes is achieved through what called silencer, which is now generally considered to be a short, specific sequence of nucleotides which are located in the 5′ upstream promoter region of a given gene [17]. These sequences are capable of recruiting repressor proteins to the promoter, which in turn carry out specific functions. At the present time a large number of silencers have been identified, but for relatively few has a specific and defined binding sequence been determined [18]. Totally, they can be divided into two types, silencer elements which are position-independent elements that direct an active repression mechanism and negative regulatory elements (NREs) which are position-dependent elements that direct a passive repression mechanism. As shown in Fig. 2A and B, transcriptional repression by repressors harboring in domain at positions −928 to −718 bp could be restored after the addition of the segments from the −928 to −879 bp and from −834 to −718 bp. So it can be postulated that there are two strong activators which counteract the function of repressors at positions from −928 to −879 bp and from −834 to −718 bp. An example consistent with this finding is the promoter of the human Pi class glutathione S-transferase (GSTP1) gene [19–21]. The NRE of the GSTP1 promoter was shown to suppress GSTP1 expression in MCF7 cells, but introduction of a half helical turn (5 bp in B-DNA) between the NRE and an essential sequence that bound a Jun-Fos heterodimer in a multidrug-resistant derivative of MCF7 cells abrogated repressor activity. Therefore, we proposed that the repressors of the *HEC1* promoter may be the passive silencers and their function should be regulated by the context of the repressor binding sites. The presence of negative regulatory elements in specific genes is becoming increasingly evident. Although the 'switching on and off' of gene expression through repressor and activator elements is to be considered as a important mechanism of regulation of the expression of some important proteins such as HEC1, the knowledge of the negative regulatory domain of the *HEC1* promoter remains to be less understood and needs to be further investigated.

CREB was named in 1987 to refer to proteins that bind to the cAMP responsive element on the somatostatin promoter [22]. Over the years, more than 20 identical or homologous cDNA clones encoding proteins that can bind to the CRE element site have been isolated. All these cDNAs encode proteins which bind to the consensus sequence TGACGTCA and has been found to mediate transcriptional responses to a variety of growth factor and stress signals [23]. Growth factors induce Ser/Thr kinases phosphorylate CREB at Ser-133, promoting its association with the coactivator CREB-binding protein (CBP) and inducing transcriptional activation [24]. CREB activity is also regulated by additional CREB-regulatory partners for recruitment of the transcriptional apparatus to the promoter [25] and by TORC family of latent cytoplasmic coactivators which translocate to the nucleus and bind to CREB in response to extracellular stimuli [26]. CREB is now considered as a key regulator to control the cellular gene expression because genome-wide studies identified ~5000 putative CREB target genes in human genome [25,27]. Furthermore, the aberrant expression of several cell cycle and growth factor genes regulated by CREB is often associated with certain cancers [28–30]. Therefore, the abilities of enhancing proliferation and/or survival of myeloid progenitor cells by CREB suggested its potential role as a proto-oncogene in regulation of cell proliferation. In our study, to examine whether CREB could bind the *HEC1* promoter, EMSA assays were performed using an oligonucleotide of the region from −84 to −55 as the probe. A DNA–protein complex band was observed and it was eliminated when the unlabeled probe was used as competitor but not when the nonspecific probe was used. The specific interaction between CREB and its palindromic sequence was also identified by supershift analysis with the anti-CREB antibody. Subsequently, to study transcriptional regulation of the *HEC1* gene by CREB, HeLa cells were transfected with the plasmid containing the wild-type *CREB* gene and the increased expression of *HEC1* mRNA was observed. Correspondingly, expression of *HEC1* mRNA in cells treated with the dominant negative CREB which can competitively bind the CBP with the leucine zipper dimerization domain but cannot bind the CRE element for deletion of bZIP proteins was inhibited as expected. Therefore, our results demonstrated that the nature of CREB imposes a significant effect on the expression of HEC1.

Another nuclear protein factor was detected using the probe from −162 to −134. By a homology search, a classical ATF4-binding site, TGACG, is identified in this region and EMSA assays also verified the specific binding of ATF4 to this site as the case of CREB. ATF4 is another member of the ATF/CREB bZIP transcription factor family and plays a crucial role in response to multiple intracellular stress pathways and in regulation of cell proliferation and differentiation [31–33]. Hence ATF4 is a master transcription factor for which temporal expression and activity are under tight cellular control [34,35]. Here, our studies provided first direct evidence of correlation between the expression of kinetochore protein and ATF4. It has been reported that ATF4 maybe either a transcriptional activator as noted above or a repressor through competitive binding to CRE element with CREB or sequestration of the components of the general transcription machinery such as TATA binding protein (TBP), TFIIB, and the RAP30 subunit of TFIIF [36,37]. However, in our study the results demonstrated that ATF4 is a transcriptional activator of the *HEC1* promoter. Both mutation and ectopic expression studies showed that ATF4 plays an important role in upregulation of promoter activity. Considering the preference that ATF4 has been shown to form heterodimers with a variety of bZIP proteins rather than the ATF/CREB family of proteins [36], we proposed that it may interact with another bZIP protein to form a complex which binds to ATF4-binding site and thereby enhances the *HEC1* transcription.

In this study, we demonstrated that −177 to +73 region of the *HEC1* gene functions as a core promoter in HeLa cells. However, this region contains the ATF4-binding site and CRE element but lacks any basal promoter elements such as a TATA box or initiator elements. These elements determine start site placement, as does TFIID with TATA-containing promoters [38,39]. CREB has been found to stimulate transcription in part by recruiting TFIID via a direct interaction with $TAF_{II}130$ and enhance the recruitment of a complex containing TFIID, TFIIB, and RNA polymerase II to a linked promoter [40]. However, the

Please cite this article as: L. Cheng et al., Functional characterization of the promoter of human kinetochore protein HEC1: Novel link between regulation of the cell cycle protein and CREB family transcription factors, Biochim. Biophys. Acta (2007), doi:10.1016/j.bbaexp.2007.07.005

fact that CREB target gene activation by cAMP stimulus through recruitment of TFIID complexes might be impaired on TATA-less promoters indicated that core promoter configuration imposes important constraints on transcriptional activation [41]. Notably, many of the TATA-less genes appears to be involved in cell cycle progression, DNA repair, and apoptosis-cellular functions that are not typically regulated by cAMP. Therefore, CREB is likely to activate these genes in a selective fashion which depends on cell context and costimulatory signals and the mechanism of regulation of their transcriptional activation by CREB remains to be clarified.

In summary, our data show that CREB family transcription factors can bind to the *HEC1* promoter, and thereby activate its expression at mRNA levels. Using HeLa cells, we demonstrated that treatment of cells with plasmids containing either CREB or ATF4 results in an increased expression of *HEC1* mRNA alone or in combination. Conversely, overexpression of their dominant negatives attenuates the activity of the *HEC1* promoter. Our present data added evidence to a novel link between cell cycle protein and CREB family transcription factors. Considering the relationship between HEC1 expression and cancer cell growth, our finding promoted us to gain a further understanding of new molecular mechanisms by which cell growth stimuli regulate the progression of cell proliferation and cancerization through a pathway of regulation of transcriptional activity.

Acknowledgements

This work was supported by Chinese 973 project (No. 2002CB713700), National Natural Science Foundation of China (No. 30400078), and Anhui Provincial Natural Science Foundation (No. 050430201).

References

[1] Z. Storchova, D. Pellman, From polyploidy to aneuploidy, genome instability and cancer, Nat. Rev., Mol. Cell Biol. 5 (2004) 45–54.
[2] D.W. Cleveland, Y. Mao, K.F. Sullivan, Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling, Cell 112 (2003) 407–421.
[3] T. Hori, T. Haraguchi, Y. Hiraoka, H. Kimura, T. Fukagawa, Dynamic behavior of Nuf2–Hec1 complex that localizes to the centrosome and centromere and is essential for mitotic progression in vertebrate cells, J. Cell Sci. 116 (2003) 3347–3362.
[4] T. Durfee, K. Becherer, P.L. Chen, S.H. Yeh, Y. Yang, A.E. Kilburn, W.H. Lee, S.J. Elledge, The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit, Genes Dev. 7 (1993) 555–569.
[5] J.G. DeLuca, B. Moree, J.M. Hickey, J.V. Kilmartin, E.D. Salmon, hNuf2 inhibition blocks stable kinetochore–microtubule attachment and induces mitotic cell death in HeLa cells, J. Cell Biol. 159 (2002) 549–555.
[6] Y. Chen, D.J. Riley, P.L. Chen, W.H. Lee, HEC, a novel nuclear protein rich in leucine heptad repeats specifically involved in mitosis, Mol. Cell. Biol. 17 (1997) 6049–6056.
[7] P.A. Wigge, O.N. Jensen, S. Holmes, S. Soues, M. Mann, J.V. Kilmartin, Analysis of the Saccharomyces spindle pole by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, J. Cell Biol. 141 (1998) 967–977.
[8] A. Nabetani, T. Koujin, C. Tsutsumi, T. Haraguchi, Y. Hiraoka, A conserved protein, Nuf2, is implicated in connecting the centromere to the spindle during chromosome segregation: a link between the kinetochore function and the spindle checkpoint, Chromosoma 110 (2001) 322–334.
[9] S. Martin-Lluesma, V.M. Stucke, E.A. Nigg, Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2, Science 297 (2002) 2267–2270.
[10] C. Ciferri, J. De Luca, S. Monzani, K.J. Ferrari, D. Ristic, C. Wyman, H. Stark, J. Kilmartin, E.D. Salmon, A. Musacchio, Architecture of the human ndc80–hec1 complex, a critical constituent of the outer kinetochore, J. Biol. Chem. 280 (2005) 29088–29095.
[11] J.G. DeLuca, Y. Dong, P. Hergert, J. Strauss, J.M. Hickey, E.D. Salmon, B.F. McEwen, Hec1 and nuf2 are core components of the kinetochore outer plate essential for organizing microtubule attachment sites, Mol. Biol. Cell 16 (2005) 519–531.
[12] J.G. DeLuca, B.J. Howell, J.C. Canman, J.M. Hickey, G. Fang, E.D. Salmon, Nuf2 and Hec1 are required for retention of the checkpoint proteins Mad1 and Mad2 to kinetochores, Curr. Biol. 13 (2003) 2103–2109.
[13] Y. Chen, D.J. Riley, L. Zheng, P.L. Chen, W.H. Lee, Phosphorylation of the mitotic regulator protein Hec1 by Nek2 kinase is essential for faithful chromosome segregation, J. Biol. Chem. 277 (2002) 49408–49416.
[14] K. Higo, Y. Ugawa, M. Iwamoto, T. Korenaga, Plant cis-acting regulatory DNA elements (PLACE) database: 1999, Nucleic Acids Res. 27 (1999) 297–300.
[15] M. Nicolas, V. Noe, K.B. Jensen, C.J. Ciudad, Cloning and characterization of the 5′-flanking region of the human transcription factor Sp1 gene, J. Biol. Chem. 276 (2001) 22126–22132.
[16] I. Kovesdi, R. Reichel, J.R. Nevins, Identification of a cellular transcription factor involved in E1A trans-activation, Cell 45 (1986) 219–228.
[17] A.H. Brand, L. Breeden, J. Abraham, R. Sternglanz, K. Nasmyth, Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer, Cell 41 (1985) 41–48.
[18] S. Ogbourne, T.M. Antalis, Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes, Biochem. J. 331 (1998) 1–14.
[19] G.J. Moffat, A.W. McLaren, C.R. Wolf, Functional characterization of the transcription silencer element located within the human Pi class glutathione S-transferase promoter, J. Biol. Chem. 271 (1996) 20740–20747.
[20] G.J. Moffat, A.W. McLaren, C.R. Wolf, Transcriptional and post-transcriptional mechanisms can regulate cell-specific expression of the human Pi-class glutathione S-transferase gene, Biochem. J. 324 (1997) 91–95.
[21] C.J. Henderson, A.W. McLaren, G.J. Moffat, E.J. Bacon, C.R. Wolf, Pi-class glutathione S-transferase: regulation and function, Chem. Biol. Interact. 111–112 (1998) 69–82.
[22] M.R. Montminy, L.M. Bilezikjian, Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene, Nature 328 (1987) 175–178.
[23] T. Hai, C.D. Wolfgang, D.K. Marsee, A.E. Allen, U. Sivaprasad, ATF3 and stress responses, Gene Expr. 7 (1999) 321–335.
[24] J.C. Chrivia, R.P. Kwok, N. Lamb, M. Hagiwara, M.R. Montminy, R.H. Goodman, Phosphorylated CREB binds specifically to the nuclear protein CBP, Nature 365 (1993) 855–859.
[25] X. Zhang, D.T. Odom, S.H. Koo, M.D. Conkright, G. Canettieri, J. Best, H. Chen, R. Jenner, E. Herbolsheimer, E. Jacobsen, S. Kadam, J.R. Ecker, B. Emerson, J.B. Hogenesch, T. Unterman, R.A. Young, M. Montminy, Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues, Proc. Natl. Acad. Sci. 102 (2005) 4459–4464.
[26] R.A. Screaton, M.D. Conkright, Y. Katoh, J.L. Best, G. Canettieri, S. Jeffries, E. Guzman, S. Niessen, J.R. Yates, H. Takemori, M. Okamoto, M. Montminy, The CREB coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector, Cell 119 (2004) 61–74.
[27] S. Impey, S.R. McCorkle, H. Cha-Molstad, J.M. Dwyer, G.S. Yochum, J.M. Boss, S. McWeeney, J.J. Dunn, G. Mandel, R.H. Goodman, Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions, Cell 119 (2004) 1041–1054.
[28] D.B. Shankar, K.M. Sakamoto, The role of cyclic-AMP binding protein (CREB) in leukemia cell proliferation and acute leukemias, Leuk. Lymphoma 45 (2004) 265–270.
[29] D.B. Shankar, J.C. Cheng, K.M. Sakamoto, Role of cyclic AMP response element binding protein in human leukemias, Cancer 104 (2005) 1819–1824.

Please cite this article as: L. Cheng et al., Functional characterization of the promoter of human kinetochore protein HEC1: Novel link between regulation of the cell cycle protein and CREB family transcription factors, Biochim. Biophys. Acta (2007), doi:10.1016/j.bbaexp.2007.07.005

[30] D.B. Shankar, J.C. Cheng, K.M. Sakamoto, The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia, Cancer Cell 7 (2005) 351–362.
[31] D.T. Rutkowski, R.J. Kaufman, All roads lead to ATF4, Dev. Cell 4 (2003) 442–444.
[32] F. Elefteriou, J.D. Ahn, S. Takeda, M. Starbuck, X. Yang, X. Liu, H. Kondo, W.G. Richards, T.W. Bannon, M. Noda, K. Clement, C. Vaisse, G. Karsenty, Leptin regulation of bone resorption by the sympathetic nervous system and CART, Nature 434 (2005) 514–520.
[33] X. Yang, K. Matsuda, P. Bialek, S. Jacquot, H.C. Masuoka, T. Schinke, L. Li, S. Brancorsini, P. Sassone-Corsi, T.M. Townes, A. Hanauer, G. Karsenty, ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin–Lowry Syndrome, Cell 117 (2004) 387–398.
[34] I. Lassot, E. Estrabaud, S. Emiliani, M. Benkirane, R. Benarous, F. Margottin-Goguet, p300 modulates ATF4 stability and transcriptional activity independently of its acetyltransferase domain, J. Biol. Chem. 280 (2005) 41537–41545.
[35] J.A. Sayer, E.A. Otto, J.F. OToole, G. Nurnberg, M.A. Kennedy, C. Becker, H.C. Hennies, J. Helou, M. Attanasio, B.V. Fausett, B. Utsch, H. Khanna, Y. Liu, I. Drummond, I. Kawakami, T. Kusakabe, M. Tsuda, L. Ma, H. Lee, R.G. Larson, S.J. Allen, C.J. Wilkinson, E.A. Nigg, C. Shou, C. Lillo, D.S. Williams, B. Hoppe, M.J. Kemper, T. Neuhaus, M.A. Parisi, I.A. Glass, M. Petry, A. Kispert, J. Gloy, A. Ganner, G. Walz, X. Zhu, D. Goldman, P. Nurnberg, A. Swaroop, M.R. Leroux, F. Hidebrandt, The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome and activates transcription factor ATF4, Nat. Genet. 38 (2006) 674–681.
[36] T. Hai, M.G. Hartman, The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis, Gene 273 (2001) 1–11.
[37] G. Liang, T. Hai, Characterization of human activating transcription factor 4, a transcriptional activator that interacts with multiple domains of cAMP-responsive element-binding protein (CREB)-binding protein, J. Biol. Chem. 272 (1997) 24088–24095.
[38] A.L. Roy, S. Malik, M. Meisterernst, R.G. Roeder, An alternative pathway for transcription initiation involving TFII-I, Nature 365 (1993) 355–359.
[39] L. Weis, D. Reinberg, Transcription by RNA polymerase II: initiator-directed formation of transcription-competent complexes, FASEB J. 6 (1992) 3300–3309.
[40] E.A. Felinski, J. Kim, J. Lu, P.G. Quinn, Recruitment of an RNA polymerase II complex is mediated by the constitutive activation domain in CREB, independently of CREB phosphorylation, Mol. Cell. Biol. 21 (2001) 1001–1010.
[41] M.D. Conkright, E. Guzman, L. Flechner, A.I. Su, J.B. Hogenesch, M. Montminy, Genome-wide analysis of CREB target genes reveals a core promoter requirement for cAMP responsiveness, Mol. Cell 11 (2003) 1101–1108.

Please cite this article as: L. Cheng et al., Functional characterization of the promoter of human kinetochore protein HEC1: Novel link between regulation of the cell cycle protein and CREB family transcription factors, Biochim. Biophys. Acta (2007), doi:10.1016/j.bbaexp.2007.07.005