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Transcription factor cCP2 controls gene expression in chicken embryonic stem cells

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ABSTRACT

cENS-1/cERNI **genes have been shown to be expressed very early during chicken embryonic development and as well as in pluripotent chicken embryonic stem (CES) cells. We have previously identified a promoter region, which is specifically active in CES cells compared to differentiated cells. In order to understand the molecular mechanisms which regulate the *cENS-1/cERNI* promoter, we analyzed the *cis*-acting elements of this promoter in CES and differentiated cells. We identified a short sequence, named the B region, 5′-CAAG TCCAGG CAAG-3′, that exhibits a strong enhancer activity in CES and differentiated cells. Mutation of the B region in the whole *cENS-1* promoter strongly decreases the promoter activity in CES cells, suggesting that this region is essential for activating the promoter. The B region is similar to the previously described response element for the transcription factor CP2 and we show by supershift experiments that a protein complex containing CP2 is bound to this B response element. All these results identify a nuclear factor belonging to the CP2 transcription factor family that is crucial for the activation of the *cENS-1/cERNI* promoter. The pattern of expression of *cCP2* in early chicken embryo before gastrulation is very similar to that of *cENS-1/cERNI* which strongly suggests that *cCP2* also plays an essential role in gene expression early in embryonic development.**

INTRODUCTION

Chicken embryonic stem (CES) cells are derived from the epiblast of chicken early embryos [stage X according to Eyal-Giladi and Kochav (1)]. These cells are pluripotent, can grow *in vitro* for long term and can contribute to many embryonic tissues including germ line when they are grafted into an host embryo (2). They then provide a very good model to investigate molecular mechanisms underlying the control of pluripotency and differentiation commitment. In addition to the mouse embryonic stem (ES) cell model, it could help to define more precisely the pathways conserved and involved in the maintenance of pluripotency during embryonic development.

In the mouse embryo, few regulators of ES cell pluripotency have been characterized so far. It has been shown that *Oct-3/4* is a key regulator of ES cells pluripotency and is involved in the control of other genes like *FoxD3* or *FGF4* (reviewed in 3). *Sox2*, *FoxD3*, *FGF4, FGFR2* and recently *Nanog* have also been shown to be involved in the regulation of embryonic cell pluripotency *in vivo* and *in vitro* (4–9). The *in vivo* knock-out of these genes leads to a slight decrease in the number of cells originating from the epiblast and to a rapid embryonic lethality. Moreover, it has been impossible to derive *in vitro* ES cells from these mutant embryos.

In order to identify new sets of critical genes for ES cell biology, we used a gene trap based strategy in CES cells. We have identified a gene family which is specifically expressed in CES cells and early embryo (10). The *Embryonic Normal Stem* (*ENS*) genes are strongly expressed in CES cells, but their expression rapidly decreases after the induction of CES cell differentiation by retinoic acid, or during embryoid body formation. In the embryo, *ENS* genes are highly expressed in the epiblast prior to gastrulation, and their expression is then restricted to the prospective neural plate, and later to the border of the neural plate. Up to now, three genes of this family have been characterized (10): 1) *cENS-1* which contains an open reading frame identical to *cERNI* (Early Response to Neural Induction) (11), inserted between two repeated sequences; 2) *cENS-2* which is a truncated form of *cENS-1*; 3) *cENS-3* which possesses two open reading frames corresponding to *pol* and *env*-retrovirus related gene. The coding region for *ENS-1* does not show any known homology with retroviral gene and does not seem to be conserved among birds other than galliforms. However, the occurrence of LTR-like repeated structures at both ends of *ENS* genes

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suggested that these genes might be derived from an ancestral retrovirus or transposon structure (10). In the putative U3 region of the LTR-like structures, we have previously isolated a promoter region, and shown that its activity is much stronger in CES cells than in differentiated cells (10). This result was surprising because it has been shown that retroviral LTRs are usually silenced in mouse ES cells and early mouse embryo, and correlated with a high methylation of these sequences (12,13). This silencing has not yet been observed in chicken blastula. Early chicken blastodermal cells (from which CES cells are derived) are permissive for infection by Rous Sarcoma Virus (RSV) and readily expressed RSV genes (14), suggesting that CES cells could be more permissive to retroviral gene expression than mouse ES cells.

It is also possible that *ENS* LTRs are protected from gene silencing by specific molecular regulators or by a positive position effect. The restricted expression pattern of *ENS* genes in embryonic stem cells and in the early embryo is of particular interest, and led us to study the mechanisms underlying *ENS* gene expression. Based on a systematic analysis of the regulatory elements of *cENS* genes, we report here that one *cis*-acting element, belonging to the first 310 bp of the *cENS-1/cERNI* promoter, is responsible for its activity in CES cells. These results as a whole reveal the existence of a nuclear factor, which belongs to the CP2 transcription factor family. Moreover, we observed that the pattern of expression of *CP2* in early embryo before gastrulation is very similar to that of *cENS/cERNI* which strongly suggests that *CP2* also plays an essential role in gene expression early in embryonic development.

MATERIALS AND METHODS

Cell culture and DNA transfection

The 9N2-5 CES cell line was maintained *in vitro* and transfected as previously described (10) with several modifications. Briefly, CES cells were cultivated in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen) containing 5% fetal bovine serum (FBS; PAN, Austria) supplemented with 1 ng/ml IGF1 (Sigma), 0.5 ng/ml mSCF (R&D), 1 ng/ml hIL-6 (Peprotek), 1 ng/ml hIL6-sR (Peprotek 10^{-4} M), β-mercaptoethanol, sodium pyruvate and non-essential amino acids on an irradiated feeder of STO cells at 10^4 cells/cm^2. QT6 cells (15) were cultivated in HamF12 medium (Invitrogen) containing 2% FBS complemented with 10% TPB (Tryptose Phosphate Broth, Difco). The day before transfection, the cells were plated in 12-well dishes at a density of 5×10^4 cells per well for the 9N2-5 cells or 1×10^5 per well for QT6 cells. Cells were cotransfected using Lipofectamine reagent (Invitrogen) with 300 ng of DNA construct and 30 ng of the Renilla Luciferase Reporter construct, pRL-TK, to normalize for transfection efficiency. After 5 h of incubation in diluted culture medium containing only 1% FBS, the cells were washed twice with phosphate-buffered saline (PBS) and regular medium was added to each well. On the following day, cells were washed twice with PBS and lysed with passive lysis buffer (Promega), then Firefly and Renilla luciferase luminescence assays were successively performed using Dual Luciferase Assay (Promega) as described by the manufacturer. Firefly reporter gene values were normalized to the activity of the Renilla luciferase, used as internal control.

DNA constructs

All the pGL2 luciferase reporter vectors were purchased from Promega. ENS reporter constructs were produced using PCR amplification with multiple primers that introduced a 5′-MluI restriction site at positions –738, –456, –308, –179 and –32, respectively paired with a single 3′-antisense primer that generated a 3′-MluI restriction site at position +83. Primer sequences are available in Table 1. PCR products and pGL2-basic vector were digested with MluI, purified and then ligated to produce, respectively, p738-luc, p456-luc, p308-luc, p179-luc and p32-luc. Reporter constructs and the pGL2 basic negative control vectors were used for transient transfection studies. Monomers or trimers of the –313/–180 region were constructed as follows: the –313/–180 region was amplified by PCR with BamHI –313 S and BglII –180 AS primers (Table 1). After digestion with BamHI/BglII, PCR products were ligated in the BamH1 site of pGEM-7Z. BglII restriction enzyme was then added to the ligation mix to eliminate multimers with various orientations. Multimers were then excised from pGEM-7Z by NsiI/KpnI and ligated into p179-luc or pGL2-promoter (Promega). Multimer copy numbers and identities were confirmed by DNA sequencing. Multimers (one or three copies) of the wild-type or mutated A and B response element (Table 1) were cloned in the SacI restriction site of the p179-luc construct or of pGL2-promoter. Insert identities were confirmed by DNA sequencing. Targeted mutagenesis on p456-luc for the B response elements has been performed with the Quick Change Mutagenesis kit (Stratagene) using the primers: B mutagenesis S and B mutagenesis AS.

Nuclear extracts

Nuclear extracts were prepared with the Active Motif nuclear extract kit (Active Motif) and stored at –80°C until further use. Protein concentrations were estimated using the Bio-Rad Protein Assay Kit I.

DNA binding assays

Probes used for DNA-binding assay were labeled using T4 polynucleotide kinase and [γ–^{32}P]ATP (Amersham). The labeled fragments were purified by acrylamide gel electrophoresis. Approximately 2×10^4 c.p.m. of the probe were added to a 30-µl reaction mixture in the presence or absence of 10 µg of nuclear extract in a buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol and 1 µg of poly(dI–dC)-poly(dI–dC). For competition experiments, a 100-fold molar excess of unlabeled double-stranded oligonucleotide was incubated for 10 min with nuclear extract prior to the addition of labeled double-stranded probe. After incubation on ice for 30 min, the DNA–protein complexes and unbound probes were separated by electrophoresis on a 4% glycerol and 5% polyacrylamide gel in 0.5× TBE at 160 V for 120 min. The gels were dried and autoradiographed. EMSA supershift assays were performed with a mouse monoclonal antibody directed against CP2 (anti-LSF from BD Biosciences). Then 250, 25 or 2.5 ng of this antibody was added to the reaction mix after the addition of the labeled probe.

Table 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ → 3′)
–738 S	acgcgtgtggatgtttattaggaagc
–456 S	acgcgttgaaacatttgattccac
–308 S	acgcgtagtttgctgaggaacaag
–179 S	acgcgtaggggcatcgagagagag
–32 S	acgcgttggaggataaaagaggt
+83 AS	acgcgttggcagagaacccct
BamHI –313 S	ggatccctcaaagtttgctgagga
BglII –180 AS	agatctggccttgtccatctgtgc
KpnI –456 S	ggtacctgaaacatttgattccac
SacI –238 AS	gagctccagtgtcctcaagacaaa
A RE wild-type S	cactgatggacaggagct
A RE wild-type AS	cctgtccatcagtgagct
A RE mutant S	cactaatggataggagct
A RE wild-type x3 S	cactgatggacaggactgatggacaggactgatggacaggagct
A RE wild-type x3 AS	cctgtccatcagtcctgtccatcagtcctgtccatcagtgagct
A RE mutant x3 S	cactaatggataggactaatggataggactaatggataggagct
A RE mutant x3 AS	cctatccattagtcctatccattagtcctatccattagtgagct
B RE wild-type S	caagtccaggcaagtccgagct
B RE wild-type AS	cggacttgcctggacttgagct
B RE mutant S	caattccaggcaattccgagct
B RE mutant AS	cggaattgcctggaattgagct
B RE wild-type x3 S	caagtccaggcaagtccgcaagtccaggcaagtccgcaagtccaggcaagtccgagct
B RE wild-type x3 AS	cggacttgcctggacttgcggacttgcctggacttgcggacttgcctggacttgagct
B RE mutant x3 S	caattccaggcaattccgcaattccaggcaattccgcaattccaggcaattccgagct
B RE mutant x3 AS	cggaattgcctggaattgcggaattgcctggaattgcggaattgcctggaattgagct
Probe 1 S	gatccttctttttatatcattgactcaaagtttgcta
Probe 1 AS	gatctagcaaactttgagtcaatgatataaaagaag
Probe 2 S	gatcaggtttgctgaggaacaagtccaggcaagtcctgggcaaa
Probe 2 AS	gatctttgcccaggacttgcctggacttgttcctcagcaaacg
Probe 3 S	gatccaggcaagtcctgggcaaaggcagagaaa
Probe 3 AS	gatcatttctctgcctttgcccaggacttgcct
Probe 4 S	gatccgggcaaaggcagagaaatcttttgtcta
Probe 4 AS	gatctagacaaaagatttctctgcctttgcccg
Probe 5 S	gatctcttttgtcttgaggacactgatggacagg
Probe 5 AS	gatccctgtccatcagtgtcctcaagacaaaaga
Probe 6 S	gatccttgaggacactgatggacaggtcctggca
Probe 6 AS	gatctgccaggacctctccatcagtgtcctcaag
Probe 7 S	gatctcctggctaaggattgtgaaatccttta
Probe 7 AS	gatctaaaggatttcacaatccttagccagga
B mutagenesis S	ctcaaagtttgctgaggacacagtccaggcaagtcctg
B mutagenesis AS	caggacttgcctggactgtgtcctcagcaaactttgag

Methylation interference assays

Probes were labeled as previously described (16). For sense or antisense oligoprobes, only the sense or antisense oligonucleotide was labeled. For the guanine methylation procedure, 1×10^6 c.p.m. of the probe was methylated using 1 µl of dimethyl sulfate (DMS) (Merck) for 3 min at room temperature in 200 µl of 50 mM sodium cacodylate (pH 8.0)–1 mM EDTA. The reaction was stopped by the addition of 50 µl of stop buffer [5 M sodium acetate (pH 7.0), 1 M β--mercaptoethanol]. The DNA was then precipitated with ethanol, and resuspended in 30 µl of water. The electrophoretic mobility shift assays were carried out as described above, but with 2×10^5 c.p.m. of modified ^{32}P-labeled probe and 30 µg of crude nuclear extracts. After autoradiography, the DNA was electro-eluted from the bands corresponding to the retarded probe and the free unbound probe. After precipitation with ethanol, the amounts of radioactivity of the bound and free probe were adjusted and the DNA was cleaved in 100 µl of 1 M piperidine for 30 min at 90°C. The cleaved products were separated by electrophoresis through a denaturing 18% polyacrylamide gel and were autoradiographed.

Real time PCR

Total mRNA were extracted from CES cells using RNeasy kits (Qiagen). Real-time RT–PCR was performed on a LightCycler (Roche) using the QuantiTect SYBR Green PCR Kit (Qiagen). The obtention of the single denaturation peak upon heating and of a single band after agarose gel electrophoresis (data not shown) confirmed that the real time RT–PCR reaction was specific. The PCR conditions used were: 15 min denaturation at 95°C followed by 50 cycles of 95°C for 15 s denaturation step, 58°C for 15 s and 72°C for 15 s; followed by a melting curve from 60 to 95°C.

The primers used for real time RT–PCR are: (i) QPCR GAPDH S: 5′-TGGGTGTCAACCATGAGAAA-3′; (ii) QPCR GAPDH AS: 5′-CATCCACCGTCTTCTGTGTG-3′; (iii) QPCR CP2 S: 5′-CTATCTGGAGGAGCTGACGG-3′; (iv) QPCR CP2 AS 5′-TCTGAATCATCTCGTCGCTGA-3′.

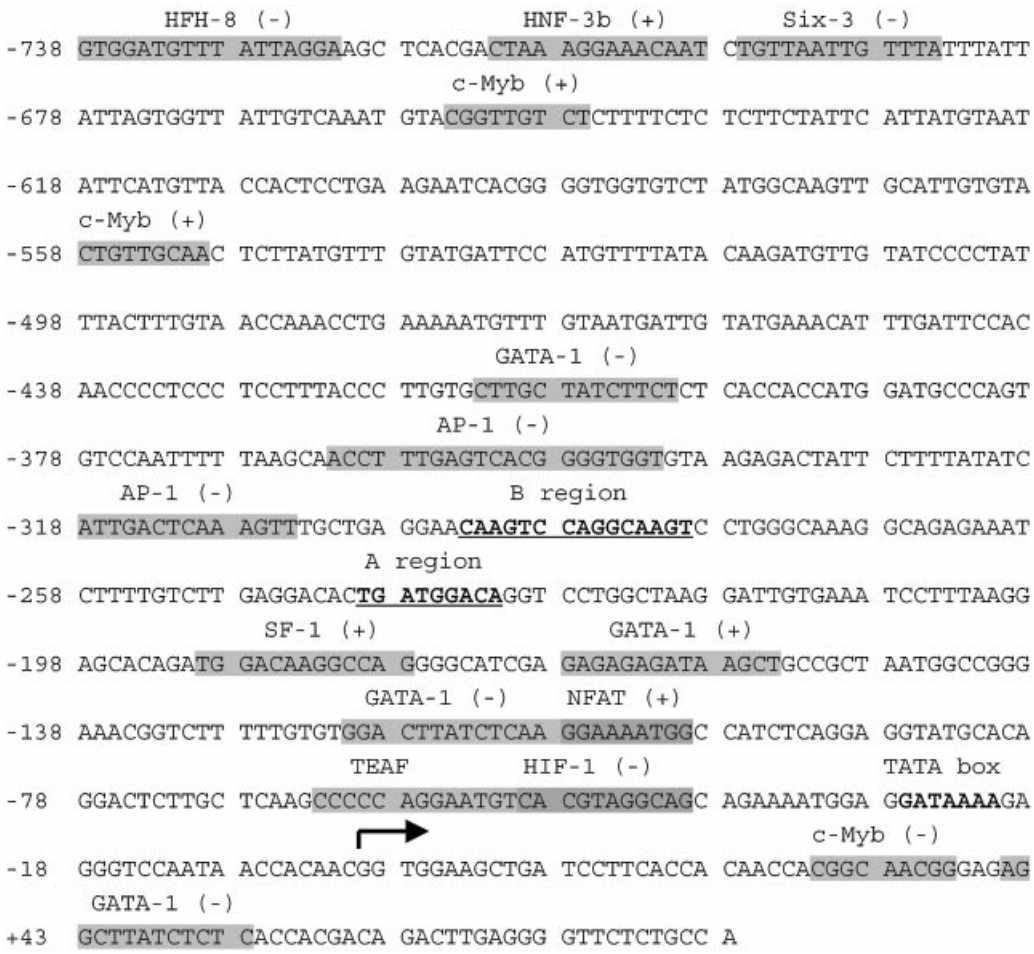

Figure 1. Structure of *cENS-1* promoter. The sequence from –738 to +83 relative to the transcription start site (TSS) is presented. The TSS is marked with right-angled arrows and the putative TATA box in bold. This sequence was analyzed by MatInspector Professional release 6.1. Transcription factors with 1.00 core similarity and more than 0.95 matrix similarities are highlighted in gray and labeled above the sequence. (+) and (–) indicate, respectively, the sense and antisense strands. The A and B response elements identified in this study are underlined.

In situ hybridization of whole-mount embryos

Whole-mount *in situ* hybridization was carried out as described by Wilkinson and Nieto (17) and revised by Streit *et al.* (18). The probe used for the experiment was amplified by PCR with the following primers: CP2 S 5′-gaatt-catggcctgggcgctgaa-3′, CP2 QPCR AS 5′-cccttggttgaggtaggt-ga-3′ and covers the first 280 bp of the CP2 coding sequence.

RESULTS

A –308/+83 cENS/cERNI promoter fragment is specifically active in CES cells

In a previous study, we identified a promoter region of *ENS* genes, which was specifically active in CES cells (10). The promoter sequence is shown in Figure 1. Analysis of the promoter sequence using MatInspector Professional release 6.1 (19) revealed several putative *cis*-acting elements for factors implicated in regulating constitutive gene expression (like AP1). It also revealed three well conserved binding elements for GATA-1 in the proximal region of the promoter. However, the analysis did not identify any candidates known to be involved in regulating ES cell specific genes. In order to localize functional *cis*-acting element(s) in the *cENS-1* promoter, luciferase reporter constructs were designed and generated for use in transient transfection assays. By using 5′ deletion mutants of the *cENS1* promoter fused to the luciferase reporter gene (cENS-luc), we carried out DNA transfection experiments in CES cells, CES cells induced to differentiate and QT6 cells, a quail fibroblast cell line. Our largest construct contained the first 738 bp of the promoter. We chose this fragment because it includes the putative U3 region of ENS genes (10). Successive deletions of cENS-luc from –738 to –456 did not reduce the expression level of the reporter gene in CES cells (Fig. 2). An additional deletion to position –308 did not decrease the promoter activity in these cells (not shown). In contrast, a further deletion to position –179 gave a 20-fold decrease, indicating that critical *cis*-acting elements are located between –308 and –179. None of these constructs showed a strong transcriptional activity either in CES cells induced to differentiate with retinoic acid for 48 h or in QT6 cells, a quail immortalized fibroblast line (15) (Fig. 2). QT6 cells were used in the following experiments as a model for differentiated cells.

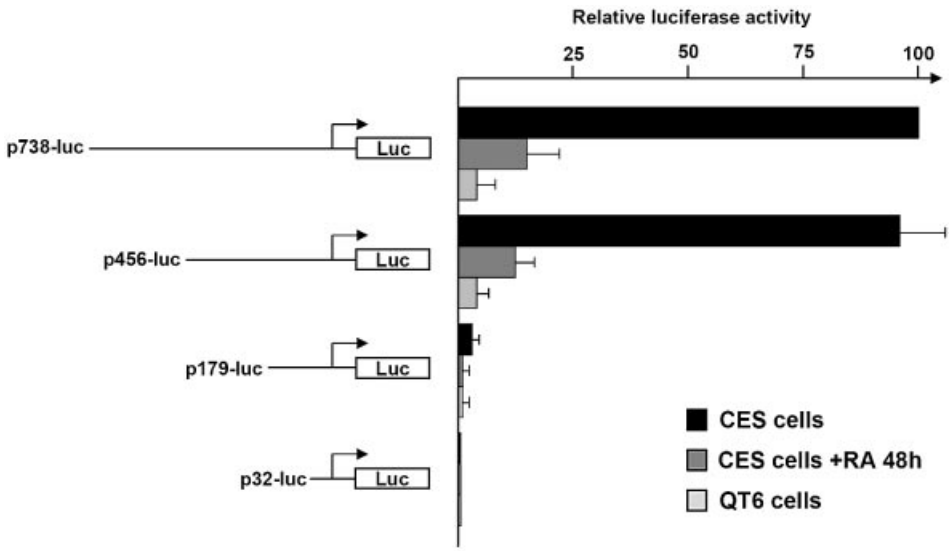

Figure 2. Functional analysis of deletion mutants of the *cENS-1* promoter. Deletions were generated as described in Materials and Methods; a schematic representation of each reporter construct is shown on the left. These constructs were transfected into CES cells, CES cells induced to differentiate with retinoic acid for 48 h and into QT6 cells. Firefly luciferase activity was normalized for transfection efficiency using the Renilla luciferase activity. Values are relative to the activity obtained with the largest promoter fragment (p738-luc) in CES cells for three independent experiments, each performed in triplicate.

A –308/–180 region is a key region for promoter activation in CES cells

To address the possible enhancer function of the DNA sequences that are located between –308 and –179, we cloned this region as one or three copies upstream of a SV40 basal promoter, which drives the expression of the luciferase reporter gene (SV40 basal promoter in pGL2-promoter). These constructs were transfected in CES cells or in QT6 cells. Three copies of the –308/–180 region increased luciferase activity 17-fold in QT6 cells and 13-fold in CES cells (Fig. 3A). One copy of the –308/–180 region also increased luciferase activity in both cell lines. This stimulatory effect was independent of the orientation of the –308/–180 region too. To test this enhancer activity on the *cENS-1* promoter, we placed one or three copies of the –308/–180 region upstream of the –179/+83 *cENS-1* promoter region (p179-luc construct). This minimal promoter contains a TATA-like box as a known *cis*-acting element, and has a weak transcriptional activity in CES cells. As with the SV40 basal promoter, one or three copies of the –308/–180 region increased luciferase activity in CES and QT6 cells (Fig. 3B). However when the –308/–180 region was placed upstream of the –179/+83 *cENS-1* promoter, in QT6 cells, the activation level decreased strongly compared to the basal SV40 promoter (from 17-fold to 4-fold with three copies of the –308/–180 region and from 10-fold to 3-fold with one copy). In contrast, the level of activation was not altered in CES cells (compare Fig. 3A and B).

As a whole, these results indicate that the –308/–180 region acts as a transcriptional enhancer for the expression of a reporter gene in undifferentiated CES cells as well as in QT6 cells. Moreover, when located upstream of the –179/+83 region, this –308/–180 region is specifically active in undifferentiated CES cells.

Two distinct protein–DNA interacting regions are located in the –308/–180 region

To investigate potential DNA–protein binding sites located in the –308/–180 region and involved in *cENS-1/ERNI* promoter activity, we performed gel retardation experiments using CES cells nuclear extracts. Seven overlapping oligonucleotides were designed to cover the –308/–180 region (Fig. 4A). The putative AP-1 site identified *in silico* and located in probe I was apparently not bound *in vitro* by nuclear proteins from CES cells (Fig. 4B, lane 2). In contrast, the oligonucleotides II, V and VI were bound by CES nuclear factors (Fig. 4B, lanes 5, 14 and 17). These three complexes were competed for by 100-fold molar excess of the respective unlabeled oligonucleotides (Fig. 4B, lanes 6, 15 and 18). The DNA–protein complexes observed with probes V and VI migrated to the same positions suggesting that they are identical and located on a DNA sequence situated between positions –251 and –230 of the ENS-1 promoter, in the overlapping region of the V and VI oligonucleotides. Complexes migrating to the same positions were also observed when probes II, V and VI were incubated with QT6 nuclear extracts (data not shown). These results are consistent with our previous data showing that the –313/–180 region was active in both cell lines when placed upstream of a SV40 basal promoter. The overlapping sequence of oligonucleotides V and VI was called the A region and the sequence located on oligonucleotide II was designated the B region.

Fine mapping of the A and B response elements

To define more accurately the nucleotides involved in DNA–protein interaction on A and B regions, we used double-stranded oligonucleotides chemically modified with dimethyl sulfate (methylation interference analysis). On both the coding

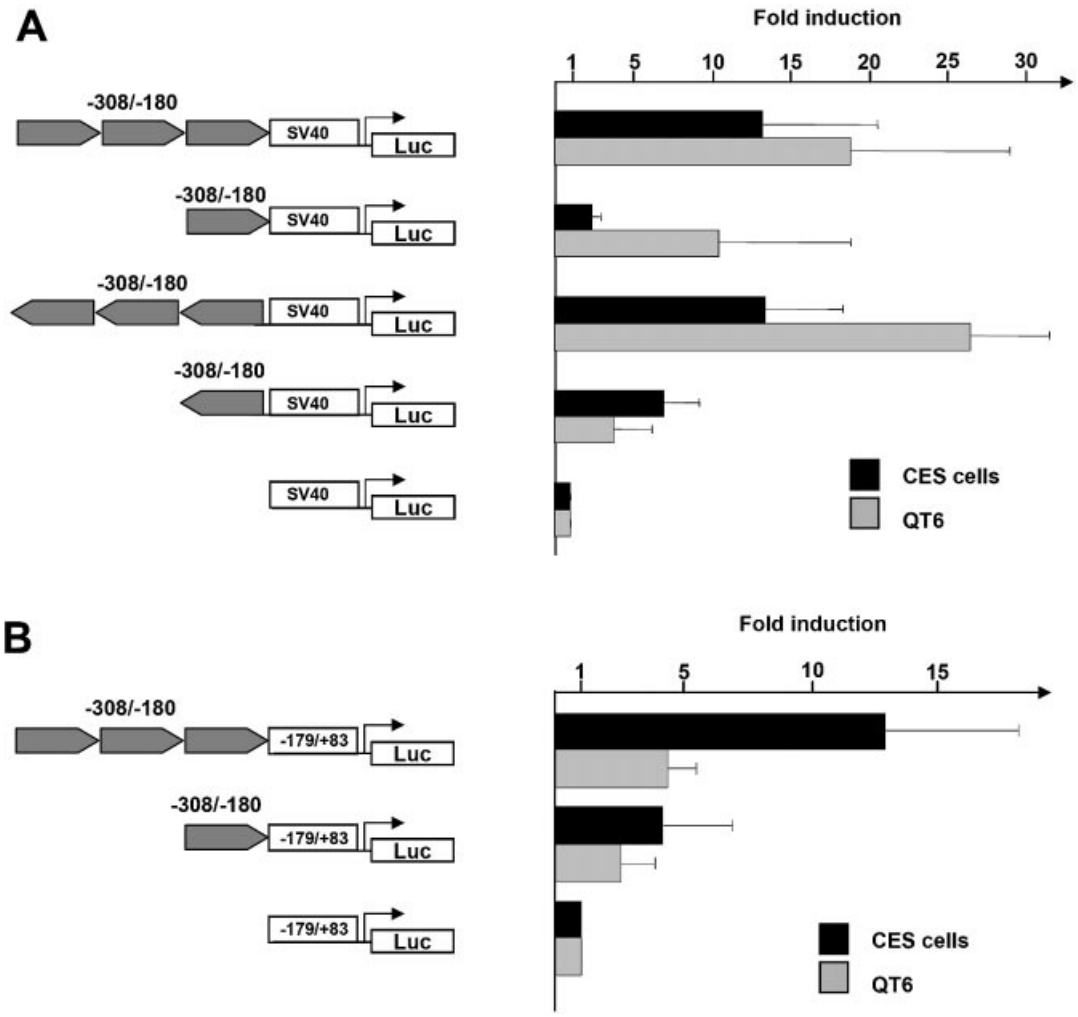

Figure 3. Functional analysis of the –308/–180 region. (**A**) One or three copies of the –308/–180 region fused to the pGL2 promoter construct were assayed by transfection experiments in CES cells and in QT6 cells. Values are relative to the activity obtained in each cell line with the empty pGL2-promoter (SV40) construct. (**B**) One or three copies of the –308/–180 region fused to the p179-luc construct were assayed by transfection into CES and QT6 cells. Values are relative to the activity obtained with p179-luc construct. Data represent ratios of firefly luciferase versus Renilla luciferase activities and values are means of at least three independent transfection experiments. The orientation of the –308/–180 is shown by the sense of the arrows.

and non-coding strands of the A region, strong interferences were detected on guanine residues which allowed us to delimit a binding region between positions –240 and –232 in the sequence 5′-Tgatggaca-3′ (Fig. 5A).

The same experiment was performed on both the coding and the non-coding strands of the B region, where strong interferences were detected on some guanine residues between positions –296 and –280 in the sequence 5′-AACAAGTCCAGGCAAGT-3′ (Fig. 5A). This response element contains a direct repeat (underlined) 5′-CAAGT CCAGG CAAGT-3′, which is important for DNA–protein interaction, as suggested by the strong interferences on the same guanine residues on each of the two CAAGT boxes (Fig. 5A). The respective places of these two response elements in the *cENS-1* promoter are shown in Figures 1 and 5B.

The A region (–240/–232) binds nuclear factors present in CES cells but is devoid of enhancer activity.

To confirm that the residues identified by methylation interference analysis were essential for DNA–protein interactions, we performed band shift experiments with a labeled probe containing the A region, in competition with wild-type or mutated oligonucleotides for the A region (not shown). These results confirmed that the residues identified by methylation interference analysis in the A region were essential for DNA–protein interaction.

To investigate the role of the A binding site in *cENS-1* promoter activity, we placed multimers of the A sequence upstream of the p179-luc construct or the SV40 basal promoter. The different constructs generated were transfected in CES or in QT6 cells and the enhancer activity was estimated with respect to the activity of empty vectors. None of these constructs revealed any enhanced activity as compared to empty vectors (not shown). These data then suggest that the A element does not have any enhancer function in the *cENS-1/cERNI* promoter region at least in the two types of cells tested.

Characterization of the B region (–294/–280)

After having identified the residues by the methylation interference assay, we confirmed that they were essential for

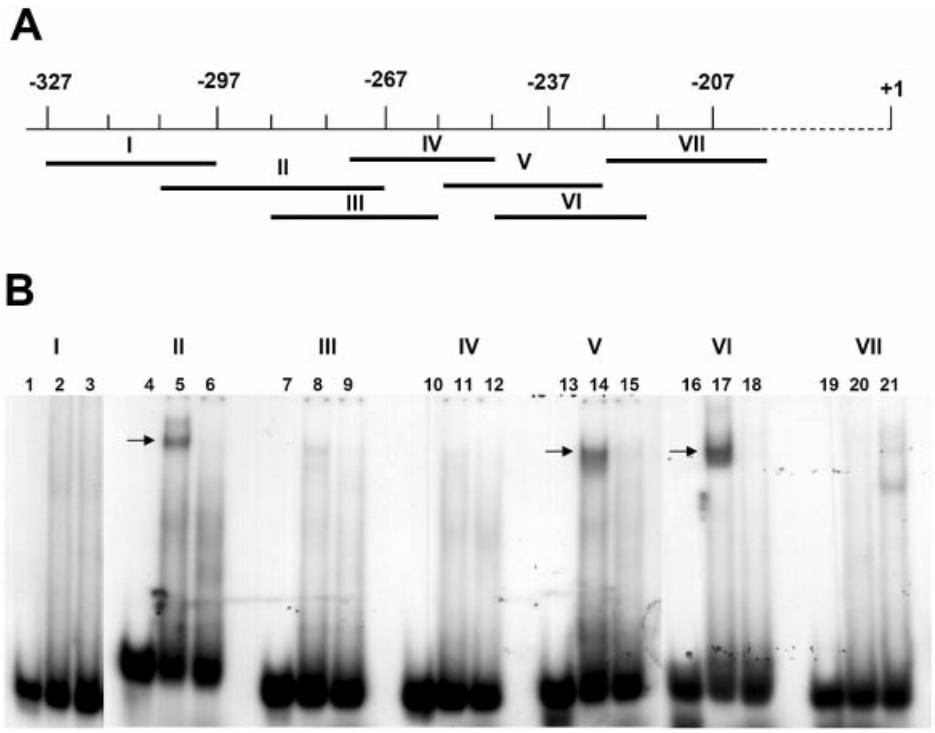

Figure 4. The –308/–180 region contains two binding sites for CES cells nuclear factors. (**A**) Map of the seven probes used to cover the –308/–180 region. Each probe is designated with a roman numeral. (**B**) EMSAs were performed with each double-stranded radiolabeled oligonucleotide probe and nuclear extracts prepared from CES cells as described in Materials and Methods. The positions of DNA–protein complexes throughout are indicated by arrows. Lanes 1, 4, 7, 10, 13, 16, 19: labeled probes alone; lanes 2, 5, 8, 11, 14, 17, 20: labeled probes with nuclear extracts from CES cells; lanes 3, 6, 9, 12, 15, 18, 21: competition experiments with 100-fold molar excess of wild-type unlabeled probes.

DNA–protein interaction through gel shift experiments with an oligonucleotide-labeled probe covering the B region (B RE) (Fig. 6A); the results are shown in Figure 6B. A unique DNA–protein complex was detected with the labeled probe incubated with CES nuclear extracts (Fig. 6B, lane 2). A competition with 10- or 100-fold molar excess of wild-type B oligonucleotide strongly decreased the signal corresponding to the DNA–protein complex, confirming the specificity of this interaction (Fig. 6B, lanes 3 and 4). A competition with a 100-fold molar excess of oligonucleotides mutated, respectively, on the C_1, G_4, C_{11} and G_{14} residues (Fig. 6A), which are important for DNA–protein interaction as proven by methylation interference analysis, did not decrease the signal (Fig. 6B, lanes 5 to 8). These results confirmed that each of these four nucleotides are essential for the interaction with nuclear binding proteins. It also demonstrates that each part of the direct repeat is necessary for DNA–protein complex formation. To determine whether each of the nucleotides of the B sequence participates in this interaction, mutated labeled probes covering the first part of the repeat were used in gel shift retardation assays (Fig. 6A and C). Results showed that the interaction with nuclear proteins was abrogated only when the C_1 or the G_4 residues were mutated (Fig. 6C, lanes 1 and 4). A probe, in which the T_5 residue is mutated, exhibited a weaker signal compared to the wild-type probe (Fig. 6C, lane 5), suggesting that this nucleotide improves the stabilization of the DNA–protein complex. The same results were obtained with probes mutated in the second part of the direct repeat (not shown). The two parts of this direct repeat are separated by a linker of five nucleotides (CCAGG). In order to check the function of this spacing sequence, we tested probes containing spacers of different lengths. Band shift assays performed with these oligonucleotides are shown in Figure 6D. Deletion or addition of three nucleotides to the linker decreased the interaction between DNA and nuclear factors (Fig. 6D, lanes 6 and 10), while the addition of six bases totally abolished the interaction (Fig. 6D, lane 14). The optimal B response element is then summarized by the sequence **C**AA**G**TNNNNN**C**AA**G**T where bold characters indicate bases essential for the DNA–protein complex formation.

The B region is essential for promoter activation in CES cells

To test the enhancer activity of the B response element, multimers of this region were placed upstream of the SV40 basal promoter. The different constructs were transfected in parallel into CES cells and QT6 cells. One copy of the B region in sense or antisense orientation increased the activity of the basal SV40 promoter from 5–7 fold in CES cells to 10-fold in QT6 cells. Three copies of the B region in sense or antisense orientation also strongly increased the activity of the basal SV40 promoter from 10-fold in CES cells to 20-fold in QT6 cells (Fig. 7A). In comparison, the mutated B response element was inactive in the two cell lines. These data prove that the B region exhibits an enhancer activity, like the –308/–180 region, and strongly suggest that it could be a key activator of the *cENS-1* promoter. This hypothesis is also confirmed by targeted mutagenesis of the B response element

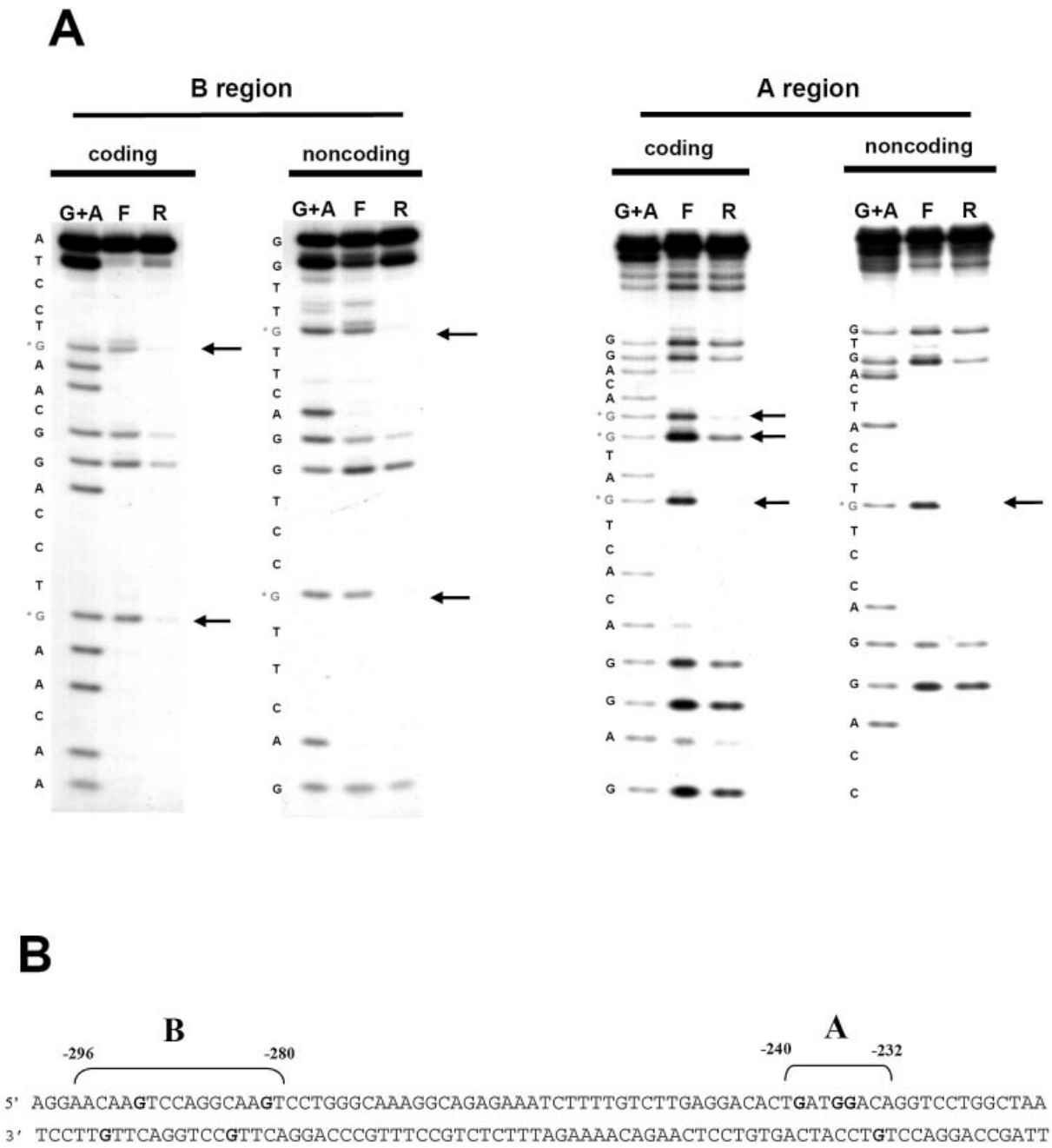

Figure 5. Fine mapping of A and B binding sites by methylation interference. (**A**) Methylation interference assays were performed with methylated –251/–223 (A region) or –297/–277 (B region) probes radiolabeled at the 5′-end of the sense or antisense strands. The probes were incubated with CES cell nuclear extracts. The free (F) and retarded (R) probes were then sequenced and compared with the G+A sequence obtained with the non-methylated probes. The G residues implicated were identified by a disappearance or a decrease in the signal. (**B**) Summary of the interactions. The guanine nucleotides identified by methylation interference assays are presented in bold on a double-stranded DNA fragment.

in the p456-luc construct. In this construct, the cytosine residue of the first CNRG box in the B response element was substituted by an adenine residue (see Materials and Methods), leading *in vitro* to the loss of the protein–DNA interaction (Fig. 6C). Compared to the wild-type –456/+83 *cENS-1* promoter, the promoter with a mutated B element exhibited a strong decrease in its activity in CES cells (Fig. 7B) showing that the B response element plays an essential role within its natural sequence environment. All these data support the fact that the B element is essential for the *cENS-1* promoter activation in CES cells.

The B response element is bound by the transcription factor CP2

Analysis of the B response element revealed that it is similar to the CP2 consensus binding sequence CNRG-N_6-CNRG (20).

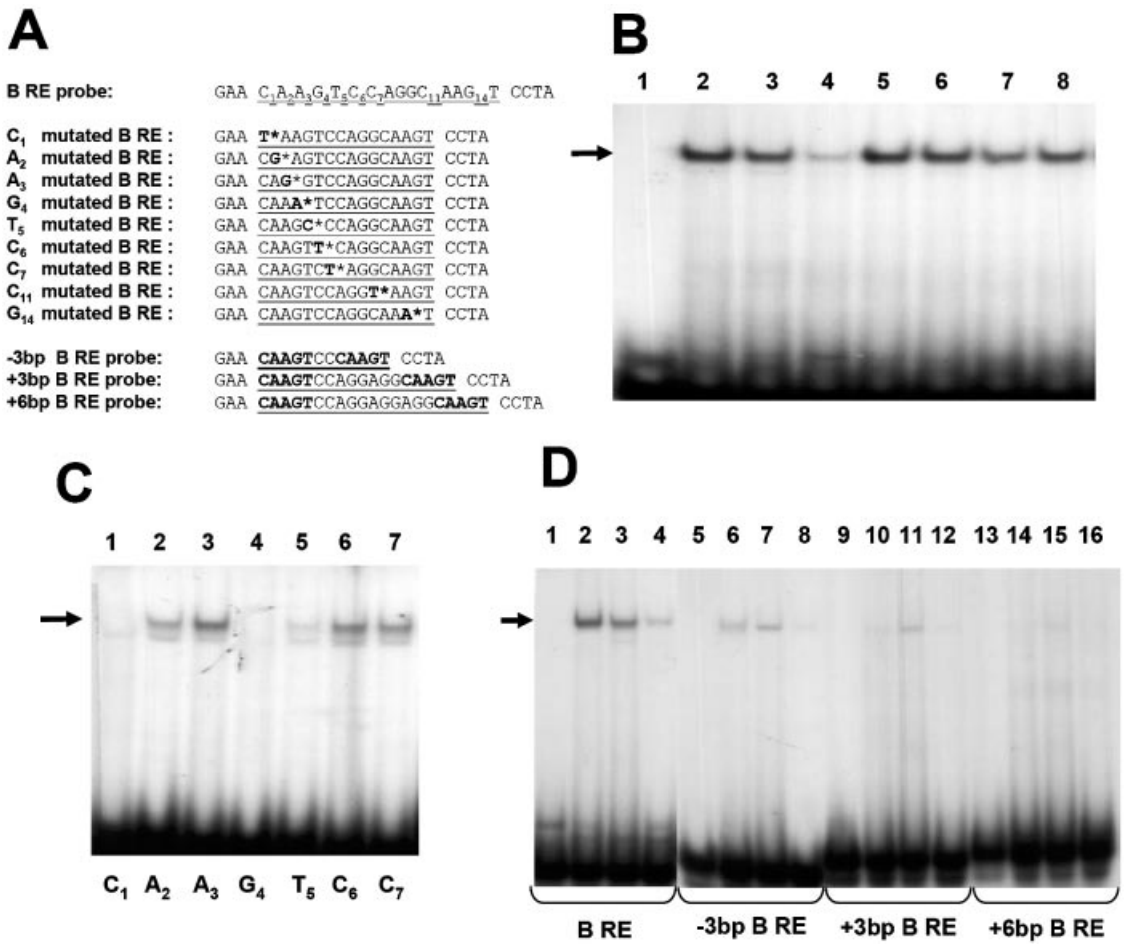

Figure 6. Binding of chicken nuclear proteins to the B region. (**A**) Sequences of the oligonucleotides used. DNA binding sequence is underlined and mutations are marked with an asterisk and bold characters. Mutated nucleotides on the B region are numbered. (**B**) EMSAs were performed with the B wild-type labeled probe alone (lane 1) or with nuclear extracts prepared from CES cells (lane 2). The position of the DNA–protein complex is indicated by an arrow. Competition experiments were performed with a 10-fold molar excess (lane 3) or 100-fold molar excess (lane 4) of the unlabeled wild-type B oligonucleotide or with a 100-fold molar excess of the B mutated C_1, G_4, C_{11} and G_{14} unlabeled oligonucleotide (lanes 5, 6, 7 and 8, respectively). (**C**) B mutated C_1, A_2, A_3, G_4, T_5, C_6, C_7 oligonucleotides were labeled and used for EMSAs with CES cell nuclear extracts (lanes 1, 2, 3, 4, 5, 6 and 7, respectively). (**D**) EMSAs were performed with each double-stranded radiolabeled probe alone (lanes 1, 5, 9, 13) or with CES cell nuclear extracts (lanes 2, 6, 10, 14). The position of the DNA–protein complex is indicated by an arrow. Competition experiments were performed with a 100-fold molar excess of either the mutated B unlabeled oligonucleotide (lanes 3, 7, 11, 15) or the B wild-type unlabeled oligonucleotide (lanes 4, 8, 12, 16).

The CP2 response element, like the B response element, is composed of a direct repeat essential for protein binding. In order to test whether the B response element is bound *in vitro* by the transcription factor CP2, we performed supershift experiments using a mouse monoclonal antibody, which recognizes human CP2. Addition of anti-CP2 to the reaction after complex formation generated antibody-dependent supershifts (Fig. 7C, lanes 4 to 6, top arrow) which were not seen with use of an IgG control antibody (Fig. 7C, lane 7). Increasing dilutions of the anti-CP2 progressively restored the unshifted DNA–protein complex (Fig. 7C, lanes 5 to 6, bottom arrow). The anti-CP2 incubated with nuclear extracts of CES cells did not interfere with the DNA–protein complex formed with the A response element (not shown). To confirm the specificity of the anti-CP2, we performed immunoblots using protein extracts from CES and HeLa cells (Fig. 7D). A band of 64 kDa was detected in undifferentiated and differentiated CES cells (lane 1 and 2) and HeLa cells (lane 3) at the described size of CP2 (21). These results strongly suggest that the B response element binds the chicken CP2 transcription factor.

Expression pattern of chicken *CP2* in CES cells and early embryo

Chicken *CP2* has been previously shown to regulate the αA-crystallin promoter (21). Based on the *cCP2* cDNA sequence published in that reported work, we amplified the chicken *CP2* by PCR from RNAs extracted from CES cells indicating that *cCP2* is expressed in CES cells. *In silico* transduction of this *cCP2* coding sequence (GenBank accession no. AY298725) revealed an amino acid sequence, which is very similar to those of human (22) and mouse (23) and is also identical to the published sequence of chicken CP2 (21), but with a small deletion of 10 amino acids (from position 323 to position 332). This small deletion is localized in the oligomerization domain (from position 266 to 403) (24) but preserved the DNA

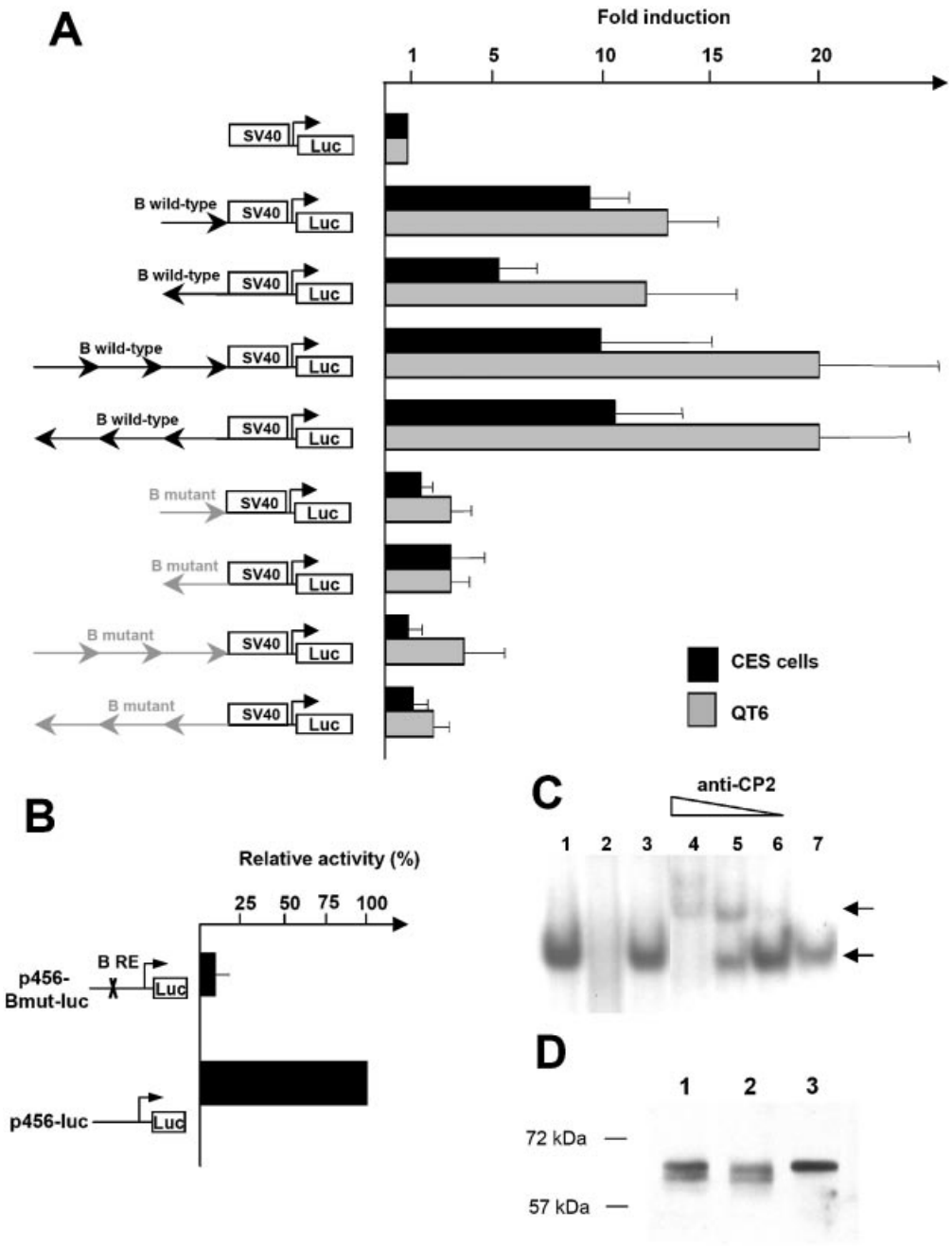

Figure 7. Functional analysis of the B region. (**A**) Multimerized wild-type or mutated B region fused to the pGL2-promoter construct were assayed by transfection into QT6 and CES cells. For each cell line, values are relative to the activity obtained with the pGL2-promoter construct. Data represent ratios of firefly luciferase versus Renilla luciferase activities, and values are the means of three independent experiments. (**B**) The B region was disrupted by targeted mutagenesis on the p456-luc construct to give p456 Bmut-luc. The cytosine residue of the first CNRG box in the B response element was substituted by an adenine residue (see Materials and Methods). These two constructs were transfected into CES cells and promoter activity assayed. Values are relative to the activity obtained with the p456-luc. (**C**) Labeled wild-type B oligonucleotide was used as a probe with CES cell nuclear extracts (lane 1). Complex is indicated by a black arrow. Competition with 100-fold molar excess of unlabeled wild-type or mutated B oligonucleotides is shown, respectively, on lanes 2 and 3; 250, 25 and 2.5 ng of monoclonal mouse anti-CP2 or 250 ng of IgG control antibody were incubated with CES nuclear extracts after the addition of B labeled probe (lanes 4 to 7, respectively). Antibody-dependent supershift is indicated by the top black arrow. (**D**) Immunoblots realized with nuclear extracts from undifferentiated and differentiated CES cells (lanes 1 and 2, respectively) and HeLa cells (lane 3) incubated with a monoclonal mouse anti-CP2.

binding domain (from position 189 to 239) (21) and the serine 291, whose phosphorylation is important for CP2 activation (25). CP2 expression was detected in undifferentiated and differentiated CES cells by immunoblot using a mouse monoclonal anti-CP2 antibody (Fig. 7D). These results are consistent with the fact that the B region is active in undifferentiated and differentiated cells when placed upstream of the SV40 basal promoter. The spatial pattern of the *cCP2* gene expression was examined during embryogenesis by whole-mount *in situ* hybridization. By using a probe located in

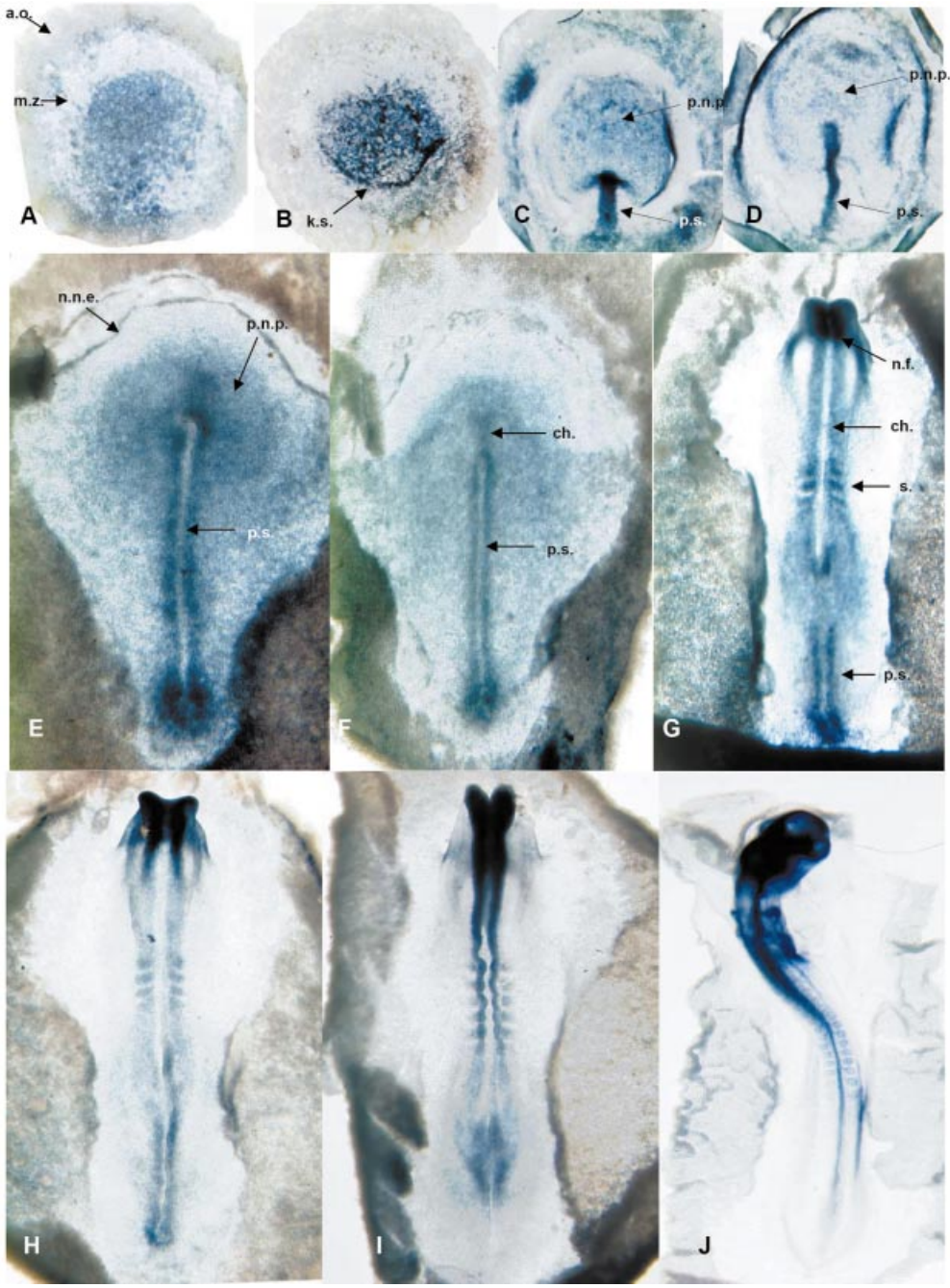

Figure 8. Expression of *CP2* in chicken embryo. Whole-mount *in situ* hybridization of *CP2* mRNA. Embryos were hybridized to an RNA antisense probe located in the coding sequence of *CP2*. (**A**) Stage X embryo (EG) dorsal side; a.o., area opaca; m.z., marginal zone. (**B**) Stage XIII embryo (EG) dorsal side; k.s., koller's sickle. (**C**) Stage 3 embryo dorsal side; p.s., primitive streak; p.n.p., prospective neural plate. (**D**) Stage 3+ embryo dorsal side. (**E**) Stage 4+ embryo; n.n.e., non-neural ectoderm; n.p., neural plate. (**F**) Stage 5 embryo; ch., chord. (**G**) Stage 7+ embryo; n.f., neural fold; s., somite. (**H**) Stage 8 embryo. (**I**) Stage 9 embryo. (**J**) Stage 12 embryo.

the coding sequence of *cCP2*, we observed a particular expression pattern during early chick embryogenesis. *cCP2* is expressed in the epiblast of embryos at the pre-primitive streak stage (Fig. 8A and B). In embryos at the primitive streak stage, it is also expressed in the extending primitive streak and in the prospective neural plate (11) (Fig. 8C–F). At stages 7 and 8, the *cCP2* expression was mostly localized in the neural folds and the somites of the embryo (Fig. 8G–I) but also in the regressing primitive streak. Later at stage12, *cCP2* was ubiquitously expressed in the whole embryo (Fig. 8J). The pattern of expression before stage 12 is quite similar to that previously described for cENS-1/cERNI with few differences (10,11).

DISCUSSION

The *cENS-1/cERNI* gene is expressed specifically in CES cells, in the epiblast of chicken blastula as well as in the

prospective neural plate of chick gastrula (10,11). Even though the functions of *cENS-1/cERNI* remain elusive either for the biology of CES cells or for the neural induction of embryonic ectoderm, its specific expression pattern is of particular interest to understand the mechanisms which lead to both the formation of the neural plate and the maintenance of cell pluripotency. These genes are so far the only genes identified with a specific expression in undifferentiated CES cells (10). They can then be considered as endogenous reporter genes to identify transcription mechanisms and signaling pathways that might account for determining the pluripotency state of embryonic stem cells. Few mechanisms which regulate ES-specific gene expression have been identified so far. *Oct-3/4* is one of the genes whose specific expression in pluripotent cells (26–28) and implication for transcriptional regulation of ES-specific genes (3) are the best understood in the mouse. Analyzing the transcriptional regulation of *cENS-1/cERNI* will then provide new insights on ES-specific transcription regulatory mechanisms but also on neural induction. Therefore, the primary aim of our studies was to identify first specific regulatory elements located on the ENS promoter and then *trans*-activating factors that might be involved in the cell-specific activity of this promoter. Functional dissection of the *cENS-1* promoter through testing in pluripotent ES cells versus differentiated cells, either differentiated cells derived from ES cells or fibroblast cells, lead us to identify two main ways of regulating the *cENS-1* promoter. One way, described in this study, is mediated by the CP2 transcription factor, which works as a transcriptional activator. Another still not fully identified pathway, induces a selective repression of the *cENS-1* promoter in differentiated cells.

Identification of CP2 transcription factor as essential for *cENS-1* promoter activation

Molecular and functional assays clearly led to identifying a consensus CP2 responsive element (5′-CAAGTCC-AGGCAAGT-3′) in the *cENS-1* promoter. Specific mutation of this response element strongly abrogates the function of the promoter in transient transfection assay in CES cells. Moreover, bandshift assay clearly demonstrated the presence of the nuclear factor CP2 in CES cells. Cloning of the chicken *CP2* product in CES cells confirmed the identity and expression of *cCP2* in these cells. *CP2* (also known as *LSF* or *LBP-1c*) belongs to a transcription factor family whose founding member is the *Drosophila grainyhead* (also known as *NTF-1* or *Elf-1*) gene, an important factor in developmental patterning in the fly (29–31). The phylogenetic tree of this transcription factor family is composed of two distinct branches (32). The first contains *grainyhead* and its homologue mammalian *grainyhead* (*MGR*), *brother of MGR* (*BOM*) and *sister of MGR* (*SOM*). The second contains *dCP2* and the mammalian *CP2*, *LBP1-a* (*NF2d9* for the mouse homologue) and *LBP-9* (*CRTR-1* in the mouse) genes. *CP2* controls the expression of a wide range of genes in human, mouse or chicken cells (21–23,33–38). RT–PCR amplification of *cCP2* and immunoblots using two distinct anti-CP2 antibodies revealed that it is expressed in QT6 and CES cells (not shown), in addition to published data indicating that *cCP2* is expressed ubiquitously in chick embryonic tissues from stage 13 (21). Our present data show that *cCP2* is differentially expressed in cell layers and tissues of the early chicken embryo. First, *cCP2* is expressed in the epiblast of chick gastrula in the tissue from which CES cells are derived. However, during gastrulation, *cCP2* expression is stronger in the primitive streak and the Hensen node and is absent in the non-neural ectoderm of stage 3 and 4 embryos. This is consistent with a previous study which demonstrated that a graft of the Hensen node induced *cENS-1/cERNI* expression in the area opaca of chicken embryo (11). This also suggests that *cCP2* could be essential for *cENS-1/cERNI* expression *in vivo* because its absence in the non-neural ectoderm is correlated with the absence of *cENS-1/cERNI* in this tissue. As expression of the *cENS-1* promoter in transient transfection assays strongly depends on the integrity of the CP2-response element, we might consider that CP2 is the major transcription activator of *cENS-1* gene. Nevertheless, we cannot exclude a contribution from other transcription factors, whose binding sites were identified *in silico* in the promoter sequence. However, the observation that major expression of *cCP2* in the early embryo (before and around gastrulation) strongly correlates with that of *cENS-1* (10), is highly supportive of a major role attributable to *cCP2* in the regulation of the expression of *cENS-1* during early chicken embryogenesis. In the mouse, the expression pattern of *CP2* is not described at gastrulation stages. Preliminary experiments indicate that CP2 is expressed in mouse ES cells and that the B region is also active in mouse ES cells (unpublished results). Together, these data support the idea that CP2 could be an important regulator of gene expression in embryonic stem cells.

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