A CDE/CHR-like element mediates repression of transcription of the mouse *RB2 (p130)* gene

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Abstract The bipartite repressor elements, termed cell cycle-dependent element (CDE)/cell cycle regulatory element (CCRE)-cell cycle homology region (CHR) control the growth-dependent transcription of the *cyclin A*, *cdc25C*, *cdc2* genes. Here, we have identified a functional element displaying the signature of the CDE–CHR in the promoter of the mouse *RB2 (p130)* gene, encoding the retinoblastoma protein family (pRB)-related protein p130. This element locates close to the major transcription start site where it makes major groove contacts with proteins that can be detected in a cellular context using in vivo genomic footprinting techniques. Inactivation of either the CDE or CHR sequence strongly up-regulates the *p130* promoter activity in exponentially growing cells, a situation where endogenous *p130* gene expression is almost undetectable. Electrophoretic mobility shift assays suggest that two different protein complexes bind independently to the *p130* CDE and CHR elements, and that the protein(s) bound to the CDE might be related to those bound on *cyclin A* and *cdc2* promoters.

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Key words: Retinoblastoma protein family; p130; Cell cycle-dependent element; Cell cycle homology region; Promoter; Transcriptional repression

1. Introduction

The orderly progression through the mammalian cell cycle is controlled, at least in part, by the level of expression of genes encoding rate-limiting regulators of cell proliferation, including members of the cell cycle 'machinery' such as cyclins, cdks, cdk's inhibitors or various regulators of transcription such as E2F, retinoblastoma protein family (pRB), B-myb, c-myc, etc. Although multiple positive transcriptional events are undoubtedly involved in the coordinated activation of these genes, there is now increasing evidence that the promoters of several of them contains a phase specific and dominant repressor element located in the transcription start site region and whose function is to ensure the correct timing and/or level of expression of those genes [1–4]. One of these cell cycle-regulated negative elements, termed cell cycle-dependent element (CDE)/cell cycle regulatory element (CCRE)-cell cycle homology region (CHR), has been shown to be important for the cell cycle-dependent expression of both the *cyclin A* and *cdc25C* genes and possibly the *cdc2, b-myb, survivin* and *Plk* (polo-like kinase) genes [2,5–12]. In the promoter of these genes, the CHR (5′-A/TG/TGAA-3′) is cooperating with a contiguous GC-rich motif (5′-G/CGCGG-3′), termed CDE or CCRE, to form a bipartite cell cycle-regulated repressor element. Inactivation of either region of this two-components module results in high constitutive transcriptional activity of these promoters throughout the cell cycle [2,5,7,9,11].

Searching for novel examples of CDE/CHR-like sequences in the transcription start site region of genes encoding cell cycle regulators, we now report the cloning of the promoter of the mouse *p130* gene encoding the pRB-related protein p130, within which we have identified and characterized a functional CDE/CHR-like regulatory element.

Members of pRB (p107 and p130) are key signal transducers connecting the cell cycle machinery to the transcriptional control of genes whose products regulate cell growth and differentiation processes [1,3,13]. Their cellular function depends on their ability to bind to and modulate the activity of various transcription factors [1,3]. Most studies on p130 have focused on its interaction with the E2F/DP family of transcription factors [1,3,13]. The hypophosphorylated form of p130 interacts with E2F/DP bound to DNA, acting either as a sequestrating factor by blocking the transactivating domain of E2Fs or as a co-repressor by recruiting histone deacetylases factors [1,3,13,14]. E2F complexes containing p130 predominate in the G_0 state of many differentiated cells and tissues where they are thought to play a major role in the maintenance of the quiescent state of the cells [1,3,13,15,16]. Indeed, E2F–p130 complexes are believed to function by repressing the expression of genes that are required for G_0/G_1-S-phase progression and DNA biosynthesis, a process which is critical for cell cycle exit and terminal differentiation [1,3,13]. Consistent with this, p130 proteins and E2F complexes containing p130 are almost undetectable in exponentially growing fibroblasts [1,3,13–16].

Here we show that the promoter of the mouse *p130* gene contains a CDE/CHR-like sequence located nearby its major transcription initiation site. This sequence is likely to represent a CDE/CHR-like functional repressor element active in growing cells since, (i) it is occupied in vivo by proteins, (ii) mutation of either the CDE or the CHR sequence leads to a strong up-regulation of the *p130* promoter activity in proliferating cells, a situation where endogenous *p130* gene expression is almost undetectable, (iii) it binds protein complexes that might be related to the unidentified protein(s) bound on the CDEs of *p130*, *cyclin A* and *cdc2* genes.

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2. Materials and methods

2.1. Cell culture, synchronization

Swiss 3T3 and NIH 3T3 fibroblasts were grown at 37°C in a 5% CO_2-containing atmosphere, in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS) (Biomedia). Relative luciferase activities of the the wild-type and CDE–CHR mutant p130 promoter-luciferase constructs in cells synchronized in G_0 or at the G_1/S or G_2-M boundaries. For synchronization, cells were treated 18 h with 0.5 mM hydroxyurea to block at the G_1/S transition or with 0.1 μg/ml Nocodazole to block in G_2-M (prometaphase) or serum-starved at confluence during 18 h to block in G_0. Cells were then harvested and assayed for luciferase and β-galactosidase activities. Diagram is showing the average of two independent experiments.

2.2. RNA isolation and primer extension

Total cellular RNA was prepared by guanidinium thyocyanate–phenol/chloroform extraction according to standard protocols. For primer extension, the oligonucleotide mp130-2 with sequence 5′-GCTGGAGGAGGCGGTGGCGACTGGTTG-3′ was ^{32}P-labelled with T4-polynucleotide kinase (Amersham) to a specific activity of 10^7 dpm/50 ng and purified by gel electrophoresis. Primer extension analysis was performed according to standard protocols using 10 μg of total RNA, 10^5 dpm of radiolabelled oligonucleotide and a mixture of 1.25 U AMV reverse transcriptase (Gibco, Life Technologies) and 100 U MMLV reverse transcriptase (Gibco, Life Technologies). To identify the position of the transcription start site, the products of the primer extension reaction were run alongside a G-specific ladder of the transcription start site region of the mouse *p130* promoter which had been generated by ligation mediated PCR amplification (LMPCR) on in vitro DMS-methylated genomic DNA (see genomic footprint protocols below) [4].

2.3. Isolation of the murine p130 promoter and analysis of promoter activity

The murine *p130* proximal promoter was cloned using the Mouse Genome Walker kit from Clontech, according to the supplier's instructions using two nested oligos whose sequences were derived from the published mouse p130 c-DNA sequence [17]: p130-1: 5′-CTCTTCCTCCGAGCTGGCTGCAGCAGC-3′ and the nested p130-2: 5′-GCTGGAGGAGGCGGTGGCGACTGGTTG-3′. A 350 bp PCR fragment was generated, end blunted, and cloned into the luciferase-based reporter vector pGL3 (Promega). The resulting reporter construct, pGL3p130-300 was sequenced and used for functional analyses after transfection. SOE-PCR performed with the LF23 oligonucleotide (5′-GGGCGGGCGCTTCTAGATAGATCTGGCTGCGG-3′) was used to create mutations in both the CDE and CHR sequences of the mp130 promoter using pGL3p130-300 as a template. The same method was used to create specific mutations in either the CDE (LF24 5′-GGGCGGGCGCTTCTAGATTTGAATGGCTGCGG-3′) or the CHR (LF25 5′-GGGCGGGCGCTTCGCGGTAGATCTGGCTGCGG-3′). The resulting vectors pGL3- p130-300, pGL3p130-CDE/CHRmut, pGL3p130-CDEmut, or pGL3p130-CHRmut were co-transfected into NIH3T3 cells together with the β-galactosidase expression vector pCH110. Luciferase activity values were normalized to the β-galactosidase activity to account for variations in transfection efficiencies. Transfections, β-galactosidase and luciferase assays were performed as described previously [4,18].

2.4. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts from NIH 3T3 cells were prepared as previously described [4,6]. All probes were ^{32}P-end-labelled with T4 polynucleotide kinase (N.E. Biolabs) and purified on polyacrylamide gels. EMSAs were performed as followed: 3 μg of nuclear extracts were incubated for 15 min at room temperature in a total volume of 20 μl of EMSA buffer (10 mM Tris–HCl (pH 7.9), 40 mM KCl, 10% glycerol, 0,05% Nonidet P-40 and 1 mM DTT) with 1 μg poly(dI:dC) (Pharmacia) and 1 ng of end-labelled double-stranded probes before samples were run on 4.5% acrylamide non-denaturing gels in O.25×TBE at 22°C and 10 V/cm. The (+) strand sequence of double-stranded oligonucleotides used as probes were: p130 CDE/CHR (5′-GGGCGGGCGCTTCGCGGTTTGAATGGCTGCGG-3′), p130CDEmut (5′-GGGCGGGCGCTTCTAGATTTGAATGGCTGCGG-3′), p130CHRmut (5′-GGGCGGGCGCTTCGCGGTAGATCTGGCTGCGG-3′). *Cyc A*, *cdc2*, *cdc25C*, *b-myb* and *E2* oligonucleotides used for competition were as previously described [4,6,10,19].

2.5. In vivo DMS genomic footprinting

In vivo DMS genomic footprinting with the LMPCR procedure was performed essentially as described elsewhere [4,20]. 2×10^6 Swiss 3T3 cells per 14 cm diameter dish were treated with the guanosine methylating agent DMS at 0.2% for 5 min at room temperature in their cell culture medium (DMEM/FCS) buffered with HEPES (20 mM final), pH 7.4. After DMS treatment, cells were washed three times with cold PBS/2% β-mercaptoethanol and then collected in 1 ml of lysis buffer (50 mM Tris pH 8, 20 mM EDTA, 1% SDS, 2% β-mercaptoethanol). Genomic DNA was isolated as described [4]. As a reference, genomic DNA (1 mg/ml in water) from the same cell type was methylated in vitro with 0.5% DMS for 4 min at room temperature. Piperidine cleavage at methylated bases was performed in piperidine 1 N at 95°C for 30 min. 2 μg of cleaved genomic DNA was used for LMPCR that were carried out as previously described [4,20]. The following primers were designed to analyze the upper strand of the transcription start site region of the mouse p130 promoter: ASp130-1 5′-AGGCGGTGGCGACTGGTTGC-3′, ASp130-2 5′-TTGCCTCCAGATGCCATGGC-3′ and ASp130-3 5′-TGCCTCCAGATGCCATGGCGCACC-3′. Samples were loaded onto a 5% sequencing gel and run at 50 W. Dried gels were analyzed with a phosphorimager from Molecular Dynamics.

3. Results and discussion

3.1. The molecular cloning of the mouse 5′ flanking region of the mouse RB2 (p130) gene reveals the presence of a putative CDE/CHR-like element in the transcription start site region

Searching for novel examples of CDE/CHR-like sequences in the transcription start site region of genes encoding cell cycle regulators, we identified in the recently cloned 5′ flanking region of the human *RB2 (p130)* gene [21], a sequence (nt +39–+49) resembling the CDE/CHR element of the *cyclin A*, *cdc2*, *cdc 25C*, and *b-myb* promoters (Fig. 1C). To confirm the presence of such an element and test its function we cloned and sequenced the corresponding proximal promoter region of the mouse *p130* gene using a PCR approach with oligonucleotides derived from the known mouse *p130* cDNA sequence [17,23,24] (Fig. 1A) (GenBank Submission, AF228064). The position of two major transcription initiation sites was determined by primer extension (Fig. 1B). The mouse and human *p130* genes show 67% of sequence identity in their 5′ flanking regions (Fig. 1A). Both of them lack a typical TATA box but contain several GC boxes and potential binding sites for numerous transcription factors [21]. Interestingly, the putative CDE/CHR-like element located nearby one of the major transcription start sites was highly conserved between human and mouse (Fig. 1A,B). As proposed for the other previously described CDE–CHR [2], the location of this CDE/CHR-like sequence immediately upstream of the initiation site raises the interesting but yet unproven possibility that its occupancy somehow interferes with the process of transcriptional initiation of the *p130* gene.

3.2. The CDE–CHR-like sequence is occupied by proteins in vivo

A major feature of the CDE/CHR elements that have so far been described is that they make major groove contacts with proteins (G-residues contacted) which are detectable in a cellular context using in vivo genomic footprinting techniques [5–9]. Therefore, we performed an LMPCR-based genomic footprint analysis [4,20] of the CDE/CHR region of the mouse

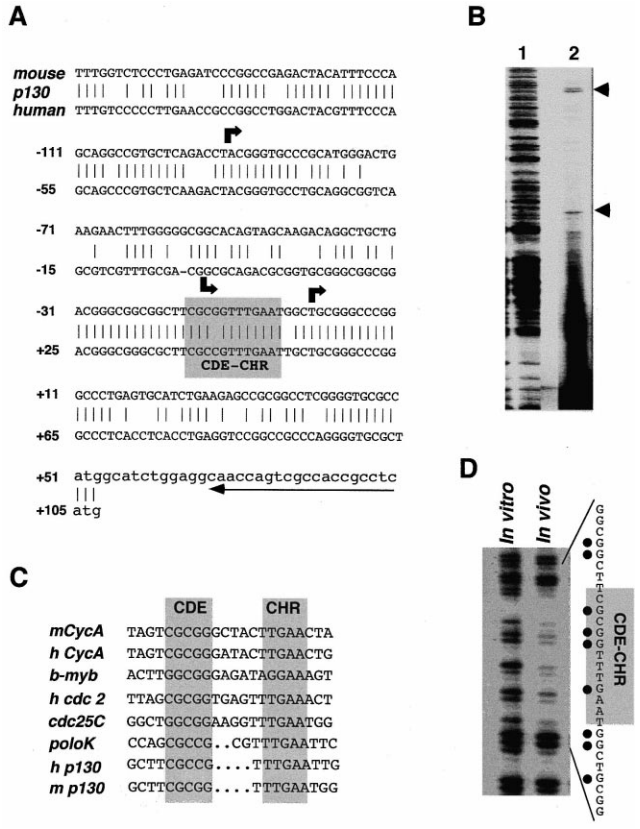

Fig. 1. Promoter sequence and transcription initiation site of the mouse *p130* promoter. Identification of a putative CDE–CHR sequence. A: Sequence alignment between human and mouse *p130* promoters. The putative CDE–CHR element is shaded. Transcription initiation sites are indicated by a thick arrow. The thin arrow underlining the sequence indicates the oligonucleotide used for the primer extension assay. The numbering is relative to the first transcription initiation site. B: Determination of the transcription initiation site of the mouse *p130* gene. Primer extension was performed with the oligonucleotide indicated in A, using total RNAs isolated from NIH 3T3 cells, as described in Section 2. The major extension products are indicated by an arrow. Size standards, on the left, consist of a G-ladder generated in a DMS-LMPCR in vitro footprint reaction. C: Sequence alignments of the *p130*, *cdc25C*, *b-myb*, *cyclin A*, *cdc2*, *polo kinase* promoter sequences in the region of the CDE and CHR elements. Core sequences are shaded. D: In vivo footprint analysis of the CDE–CHR element of the mouse *p130* promoter. LMPCRs were performed on in vivo DMS-methylated genomic DNA templates obtained from exponentially growing Swiss 3T3 cells treated with DMS. The lane indicated as 'in vitro' refers to similar LMPCR carried out with naked DNA methylated in solution with DMS. Amplified DNA ladders that are visible correspond to guanosines of the upper strand of the mouse *p130* promoter around the start site. The position of the CDE–CHR element is shaded in the sequence. Protected residues are indicated by a closed circle in the sequence.

p130 promoter in growing fibroblasts using either in vivo or in vitro DMS-methylated genomic DNA as templates (Fig. 1D). This analysis revealed that at least four G-residues (major groove) inside the *p130* CDE/CHR sequence are protected by proteins in vivo (Fig. 1D). This result strongly suggests that the CDE/CHR-like sequence of the *p130* gene is indeed a functional element in vivo that makes major groove contacts with proteins in exponentially growing mouse fibroblasts.

3.3. Transcription of the p130 gene is strongly up-regulated upon mutation of the CDE–CHR element

Another important feature of the previously identified CDE/CHR elements is that inactivation of either the CDE or the CHR leads to a significant up-regulation of promoter activity [2]. To test whether this was also true for the *p130* gene, we performed a functional analysis of the mouse *p130* CDE–CHR by transiently transfecting NIH 3T3 cells with either the wild-type or the CDE/CHR mutated versions of *p130* promoter-luciferase reporter constructs. As shown in Fig. 2A, mutation of either the CDE or the CHR element in the context of the *p130* promoter resulted in a 15 and 20-fold increase of the basal promoter activity, respectively, as measured by luciferase activity in growing cells. Interestingly, this increase was even stronger, (up to 30-fold) when cells where transfected with a *p130* reporter construct containing mutations in both the CDE and the CHR. Altogether, the dramatic up-regulation of promoter activity upon mutation in either the CDE-like or the CHR-like sequence, the location of those sequences in the transcription start site region and

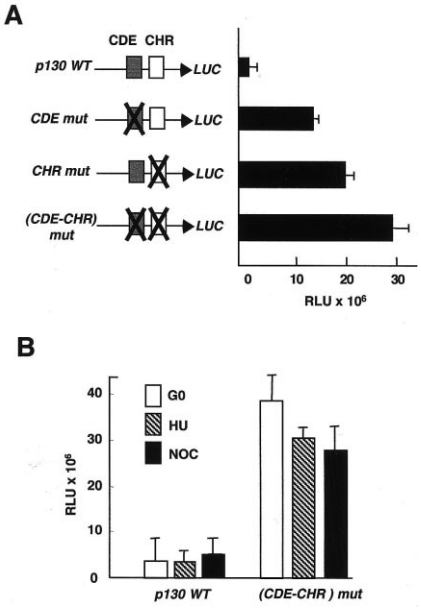

Fig. 2. Effect of mutations within the CDE and CHR elements on *p130* promoter activity. A: Relative luciferase activities of reporter constructs driven by either the wild-type or the CDE–CHR-mutated versions of the *p130* promoter. NIH 3T3 cells were co-transfected either with the wild-type *p130* promoter-driven luciferase reporter vector (p130wt) or with the CDE–CHR mutated versions of the promoter (CDEmut, CHRmut, (CDE–CHR)mut) together with A CMV-driven β-galactosidase construct. 24 h after transfection, luciferase activity was measured on exponentially growing cells and results were normalized to β-galactosidase activity. Diagram is showing the average of three independent experiments. B: Relative luciferase activities of the the wild-type and CDE–CHR mutant p130 promoter-luciferase constructs in cells synchronized in G_0 or at the G_1/S or G_2-M boundaries. NIH 3T3 cells were co-transfected either with the wild-type or with the CDE–CHR mutated *p130* promoter luciferase construct together with A CMV-driven β-galactosidase construct. 24 h after transfection, cells were treated 18 h with hydroxyurea to block at the G_1/S transition or with nocodazole to block in G_2-M (prometaphase) or serum-starved to block in G_0. Cells were then harvested and assayed for luciferase and β-galactosidase activities. Diagram is showing the average of two independent experiments.

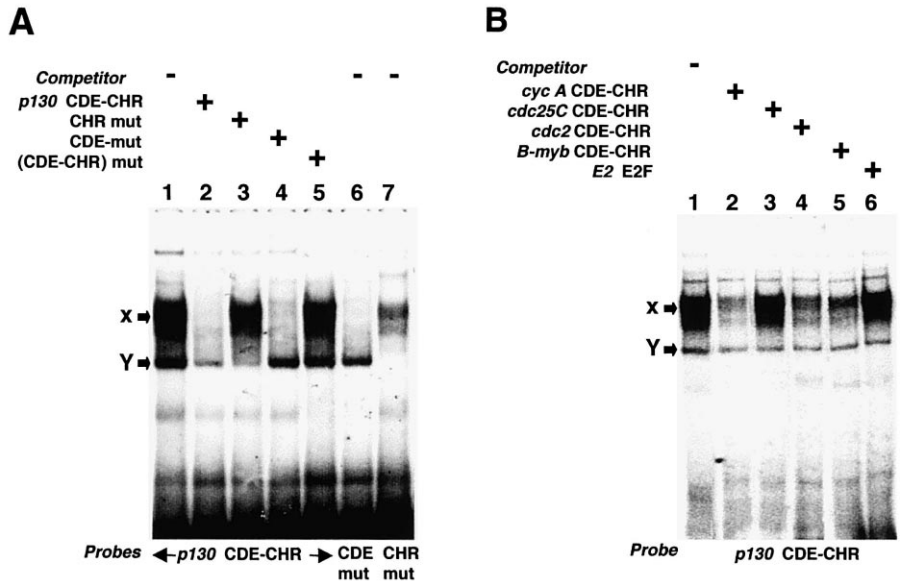

Fig. 3. EMSAs showing a specific binding of protein complexes to the *p130* CDE/CHR element. A: Two different protein complexes bind the CDE and CHR sites of the *p130* promoter. Nuclear extracts from exponentially growing NIH 3T3 cells were incubated with the p130 CDE–CHR radiolabelled oligonucleotide (lanes 1–5). Lanes 2–5 represent competition experiments with the wild-type cold oligonucleotide (lane 2) or with oligonucleotides containing a mutation either in the CDE (lane 3) or in the CHR (lane 4) or in both the CDE and CHR (lane 5). Complexes formed on either the CDE (X) or the CHR (Y) are indicated. The same nuclear extracts were also incubated with radiolabelled probes containing a mutation in either the CDE (lane 6) or the CHR (lane 7). B: Similar protein complex(es) bind the CDE of several cell cycle-regulated genes. Competition experiments using the *p130* CDE–CHR probe with the same nuclear extracts as in A and a 50-fold molar excess of the indicated cold oligonucleotides (CDE–CHR sequences of the *cyclin A, cdc25C, cdc2, b-myb* promoters or the consensus E2F site of the adenovirus *E2* promoter).

their in vivo occupation by proteins in the major groove of DNA, strongly suggest that expression of the *p130* gene is actively repressed in growing cells by a bipartite CDE–CHR-like regulatory element.

However, while it is clear that the periodic occupation of the CDE–CHR element in the cdc2, cdc25C, cyclin A and B-myb promoters is responsible for the cell cycle-dependent modulation of expression of these genes (at the G_1/S or G_2/M transitions), the biological function of this element in the context of the *p130* gene remains to be explored. Indeed, the measurement of the luciferase activity in cells synchronized at various stages of the cell cycle (G_0 by serum starvation or confluency, G_1/S using hydroxyurea, G_2/M using nocodazole) did not reveal any significant cell cycle-dependent modulation of activity of either the wild-type *p130* promoter or CDE–CHR-mutated versions of the promoter, the later being consistently more active than the former constructs (Fig. 2B). Consistent with this is the fact that very little oscillation of the level of *p130* mRNA transcripts is observed in quiescent and cycling rodent fibroblasts ([22]; Fajas et al., unpublished observation). Also consistent with our results is the fact that *p130* mRNAs, p130 proteins and E2F complexes containing p130 are almost undetectable in exponentially growing cells [1,3,15,16,22]. Thus, a possible explanation for the presence of a permanently active repressor element in the *p130* promoter might be to maintain a low level of expression of p130 in cycling cells. In contrast, E2F complexes containing p130 predominate in the G_0 state of many differentiated cells and tissues where they are thought to play a major role in the maintenance of the quiescent state of the cells [1,3,13]. This correlates with a large increase in the amount of p130 protein in these cells which might result from post-transcriptional changes in protein stability [22] and/or from large variations of *p130* mRNA level, such as in testis during sexual maturation [23] or in p19 embryonal carcinoma (EC) upon their retinoic acid-induced differentiation [17]. Although we did not address this question in this report, our results provide the basis and tools warranting further studies to investigate *p130* gene regulation during cell differentiation and in particular to test whether this increased expression of p130 upon differentiation results from a release of the CDE–CHR-mediated repression.

3.4. Characterization of the DNA–protein complexes formed on the p130 CDE–CHR-like element

The exact composition of the protein complex(es) that bind the various CDE–CHR repressor elements in vivo is still highly controversial. Although it has been reported that the CDE of the *cdc2* and *cyclin A* promoters can bind cellular E2F ternary complexes in vitro with low efficiency [8,25] other reports disagree with this [6,26]. Moreover, the CDE–CHR sequence of the cdc25C promoter [19] is clearly unable to bind E2Fs in vitro. These data appear consistent with those suggesting that the whole CDE–CHR module in the *cdc2*, *cdc25C* and *cyclin A* promoters bind efficiently an unidentified factor, named CDF1, that is clearly distinct from E2F complexes and which does not associate directly with pocket proteins [19,27]. More recently, it was also shown that a novel cellular activity termed CHF (cyclin A CHR binding factor), independent from CDF- and E2F-activities which has been shown at present to bind only the CHR element of the *cyclin A* promoter [28]. However, in the cases of CHF and CDF, protein species carrying these activities remain to be identified.

Here, we report a preliminary characterization of the DNA–protein complexes formed on the *p130* CDE–CHR element. EMSA analyses were performed using nuclear extracts

from exponentially growing NIH-3T3 cells and a DNA probe spanning the CDE–CHR region of the mouse *p130* promoter. At least two CDE–CHR-specific retarded DNA–protein complexes were detectable (Fig. 3A). Competition experiments using a 100-fold molar excess of unlabelled oligonucleotides containing a mutation in either the CDE or the CHR sequence demonstrated that the fastest and slowest migrating complexes are specific for the CHR and CDE, respectively (Fig. 3A). The specificity of each of these two complexes for either the CDE or the CHR sequence was confirmed by performing EMSAs with DNA probes bearing mutations either in the CDE or the contiguous CHR sequence (Fig. 3A).

We then tested whether these DNA–protein complexes could be similar to those detected on other CDE–CHR elements. The complex formed on the CDE turned out to be competed out by an 100-fold excess of unlabelled double stranded oligonucleotides bearing the CDE–CHR sequence of either the *cyclin A* [2,5,7] and *cdc2* [2,8] promoters and, although to a lesser extend, by the variant CDE–CHR/E2F-DRS site of the *b-myb* promoter [2,9,10] (Fig. 3B). Notably, this competing effect of the *cyclin A* CDE–CHR for the *p130* CDE-specific complex depends only on the CDE sequence since competition was still observed when the *cyclin A* oligonucleotide was mutated within the CHR, but not if it was mutated within its CDE sequence (Fig. 3B).

In contrast, neither an oligonucleotide bearing the CDE–CHR of the *cdc25C* promoter [2,5] nor the consensus E2F binding site of the *E2* promoter [1,4] were able to interfere with the formation of this CDE complex (Fig. 3B). Moreover, none of these oligonucleotides was able to compete out the CHR-specific complex formed on the *p130* probe (Fig. 3B). Altogether, these EMSAs suggest not only that two different complexes bind independently to the CDE and the CHR elements of the *p130* promoter but also that the protein(s) bound on the CDEs of *p130*, *cyclin A* and *cdc2* genes might be related, whereas different protein complexes might bind to the CHR element.

Finally, since it has been reported that the CDE/CCRE of the *cdc2* and *cyclin A* promoters can bind cellular E2F ternary complexes in vitro with low efficiency [8,25] we also tested whether E2Fs and E2F-associated proteins could be present in the cellular complexes formed on the *p130* probe. However, neither antibodies directed against -E2F1, -E2F4, -E2F5, -DP1, nor those directed against -pocket proteins (p107, pRB and p130) were able to interfere with the *p130* CDE complex in EMSA (data not shown, reagents described in reference [4]). Moreover, recombinant E2F1/DP1 and E2F4/DP1 complexes [4] were unable to bind that probe (data not shown) whereas they bound avidly a probe containing the E2F site of the E2 promoter [4]. This strongly suggests that E2F-complexes are not directly involved in the regulation of the CDE–CHR repressor element of the *p130* promoter.

The precise characterization of the protein complexes that bind and repress these various CDE–CHR elements will be required to further understand the molecular basis of CDE–CHR-mediated repression.

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