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ATF/CREB site mediated transcriptional activation and p53 dependent repression of the cyclin A promoter

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Abstract **Cyclin A is a pivotal regulatory protein which, in mammalian cells, is involved in the S phase of the cell cycle. Transcription of the human cyclin A gene is cell cycle regulated through tight control of its promoter. We have previously shown that the ATF/CREB site, present in the cyclin A promoter, mediates transcriptional regulation by cAMP responsive element binding proteins. The main goal of the present study was to investigate whether this site is involved in transcriptional regulation of the gene. We have constructed stable NIH-3T3 cell lines that express the luciferase reporter gene under the control of normal or mutated versions of the cyclin A promoter. We show that the ATF/CREB is required to achieve maximal levels of transcription from the cyclin A promoter starting in late G1. We also show that down-regulation of the cyclin A promoter by p53 does not implicate a direct binding of p53 to its cognate consensus sequence but occurs probably by interference with trans-activating factors. This result suggests that p53 can interfere with transcription of the cyclin A gene, in the absence of a TATA sequence in the promoter.**

Key words: Cell cycle; cAMP responsive element; Gene regulation

1. Introduction

The cyclins are a group of proteins periodically synthesized and degraded during the cell cycle. This enables them to activate, at the appropriate time, the cyclin-dependent kinases (cdks), whose activity is needed to drive cells through the cell-cycle (for recent reviews, see [1-3]).

Mammalian cyclin A binds and activates cdk2 in S phase and cdk1 (cdc2) in G2 and M phase [4-6]. In vertebrates, following synthesis in late G1, cyclin A accumulates in the nucleus [7], a process which appears to depend on its association with cdk2 [8]. The cyclin A/cdk2 kinase activity is present during S phase and is necessary for S phase entry; direct inhibition of S phase entry was observed in tissue culture cells following microinjection of antisense cDNA or anti-cyclin A antibodies [9-11]. The same phenomenon occurred after microinjection of antibodies directed against cdk2 [12]. Localization of cyclin A at the replication sites in vivo [13,14], the interaction of the cyclin A/cdk 2 kinase with the proliferating cell nuclear antigen (PCNA) [15] and the phosphorylation of the DNA replication factor RPA by cyclin A/cdk2 kinase [16,17] further support the hypothesis that cyclin A is involved in DNA replication. In addition, deregulated expression of cyclin A can disturb the normal regulation of the G1-to-S transition [18,19].

The cyclin A gene contains eight exons spanning 8 kb of DNA with a TATA-less promoter and multiple transcriptional start sites have been identified by S1 nuclease protection analysis [20]. A number of different studies have examined the mechanisms controlling the synthesis of cyclin A. Basal transcription of the cyclin A gene appears to require TAF_{II} 250, the largest subunit of TFIID [21]. Another study has demonstrated that both cyclin A mRNA levels and E2F activity are increased in cells overexpressing c-*myc*, suggesting that E2F may be involved in the positive regulation of cyclin A expression [22]. A variant E2F site was recently shown to mediate this positive regulation at S phase entry [23]. Cell cycle dependent elements have also been recently identified. Transcriptional repression of the cyclin A gene during the G0 and G1 phases is mediated by two distinct elements: a cell cycle-dependent element (CDE) and a cell cycle gene homology region (CHR) [24]. The CDE-mediated repression is also the principle underlying the periodic transcription of the human *cdc25*C and *cdc*2 genes. However, identification of positive and negative cis-acting sequences implicated in the promoter regulation during the cell cycle is still incomplete.

The main goal of the present study was to investigate whether the ATF/CREB and p53 sites might be involved in the periodic transcription of the cyclin A gene. We have recently shown that the ATF/CREB site located in the cyclin A promoter shows a cell cycle-regulated response to stimulation by the cAMP signal transduction pathway [25]. Moreover, the same site has been also implicated in cyclin A-promoter down-regulation in contact-inhibited endothelial cells [26] and in chinese hamster lung fibroblasts upon treatment with TGF-β [27]. Altogether, these results identified the ATF/CREB site as a central element of the cyclin A promoter.

Negative transcriptional regulation of the cyclin A gene by p53 was also recently reported in human and rat cells, but the significance of this regulation during the cell cycle was unclear [28]. It has been demonstrated that wild type p53 is able to down-regulate transcription of promoters containing a TATA box element [29,30]. This repression is believed to be due to the capacity of p53 to interfere with the assembly of the transcriptional complex on the TATA site. However, p53 is a transactivating protein that is able to activate transcription of genes containing a specific p53 DNA binding element such as WAF/CIP1, mdm-2, GADD45, Bax, cyclin G or IGFBP3 [31-36].

In this study, we show that the ATF/CREB is required to achieve maximal levels of transcription from the cyclin A promoter starting in late G1. In contrast, down-regulation

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of the cyclin A promoter by p53 did not involve a direct binding of p53 to its cognate consensus sequence but occurred probably through interference with trans-activating factors.

2. Materials and methods

2.1. Plasmid construction

The cyclin A promoter region (a 1292 bp *Bgl*II-*Sma*I fragment) was cloned in a luciferase reporter plasmid [37] to generate the pWT-LUC plasmid [20]. pdelATF/CREB-LUC, pdelp53-LUC were obtained by site-directed mutagenesis of the ATF/CREB, p53 sequences of pWT-LUC (ATF/CREG: GAATGACGTCA mutated to AAATGAATTCA; p53: TGCCTGCC mutated to TGGATCCC) (Clontech). One copy of the cyclin A ATF/CREG or p53 site was cloned in the sense orientation upstream the TK promoter [38] in the pTK-LUC reporter plasmid (clone CRE1TK-LUC [25], clone p53.TK-LUC). The PG13-LUC reporter plasmid contains the RGC p53 DNA binding sequence linked to a minimal polyoma early promoter [39]. Wild type p53 and mutant (A143) expression vectors were as described [40].

2.2. Antibody

Human cyclin A purified from HeLa cells was used to immunize mice and raise monoclonal antibodies. Clone 11B2 was found to specifically recognize human cyclin A in ELISA. It also recognized mouse cyclin A.

2.3. Cell culture, DNA transfection and luciferase assays

Stable transfections were performed into the mouse cell line NIH-3T3. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 UI ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin in a humidified atmosphere with 5% CO_2/95% air. Different constructs (pWt-LUC, pdelATF/CREB-LUC, pdelp53-LUC, pTK-LUC, pCRE1TK-LUC) were cotransfected with the pSV-neo vector (Promega) used as a selectable marker into NIH-3T3 cells using the calcium phosphate precipitation technique [41]. 20 μg of total plasmid DNA per 100 mm dish were used. 24 h after transfection, the cells were selected for 2 weeks in complete medium with 0.8 mg/ml of G418 (Geneticin, Gibco-BRL). G418-resistant colonies of the different clones were pooled and expanded. These clones were analysed respectively for luciferase content per cell, which remained constant during a 2-week observation period. These different populations were synchronized by serum deprivation (0.2% FCS) for 72 h and then refed with 10% FCS. We recorded cell cycle stage and cell number by flow cytometry (see below) and the luciferase activity was measured by a standard assay using a Lumat LB9501 Berthold luminometer. Proteins were quantified using the BCA assay (Pierce). Results were expressed as relative luciferase units per μg total proteins.

Transient transfections, for p53 experiments, were performed into Saos-2 cells, maintained as described for NIH-3T3 cells. 10^6 cells were plated on 60 mm dishes and transfected on the following day by the calcium phosphate procedure [41]. The amount of DNA used for transfection varied according to the type of analysis performed and is shown in the figure legend. Assays were performed 48 h after transfection.

2.4. Flow cytometry

10^6 cells were washed twice in PBS and fixed with 75% ethanol in PBS for a minimum of 1 h at 4°C. After washing in PBS, cells were resuspended in 0.7 ml H_2O containing 40 μg/ml propidium iodide and 50 μg/ml DNase-free RNase A. 20 000 event characteristics were acquired using a FACStar Plus (Becton Dickinson). Cell cycle phases were analysed using the Cell Fit program. For measurement of relative cell size, ethanol-fixed and/or living cells were analysed by forward angle light scatter (FSC).

2.5. Northern blotting

Total RNA was prepared using standard procedures [42] and analysed by Northern blot. The blots were probed with total human cyclin A cDNA probe [43]. The probe was labelled with the Multiprime system (Amersham). The same blots were dehybridized and probed subsequently with a 28S rRNA oligonucleotide probe to normalize the amounts of mRNAs loaded [44].

2.6. Western blot analysis

NIH-3T3 cells ($3–6\times10^6$ cells) were washed twice with PBS, lysed in 150 μl Laemmli buffer, and boiled for 5 min [45]. 20 μg of protein were fractioned on 12.5% SDS-PAGE and then transferred onto PVDF membranes (BioRad). Membranes were blocked in Tris-buffered saline (20 mM Tris-HCl, pH 7.5/137 mM NaCl) containing 5% non-fat dry milk. The blots were then probed with a monoclonal antibody directed against purified human cyclin A (1:1000 in blocking solution) overnight at 4°C, washed in Tris-buffered saline, and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000) for 1 h at 25°C. Peroxidase activity was visualized by the enhanced chemiluminescence (ECL) detection system (Amersham).

3. Results

The most relevant part of the cyclin A promoter is shown in Fig. 1. The promoter region contains several DNA sequences that are known to participate in the transcriptional control of the gene (ATF/CREB, E2F, CDE-CHR). In addition, several putative recognition sites (p53 and Sp1) may participate to the cell-cycle dependent transcriptional regulation of the gene. We performed site directed mutagenesis in order to evaluate the respective roles of p53 and ATF/CREB binding sites in the periodic transcription of the cyclin A gene. Each site was mutated independently by nucleotide substitution in the cyclin A promoter-luciferase construct pWt-LUC, which extends from positions −925 to +245 relative to the major transcription start site (Fig. 1). Both wild type and mutant constructs were stably transfected into NIH-3T3 cells and luciferase ac-

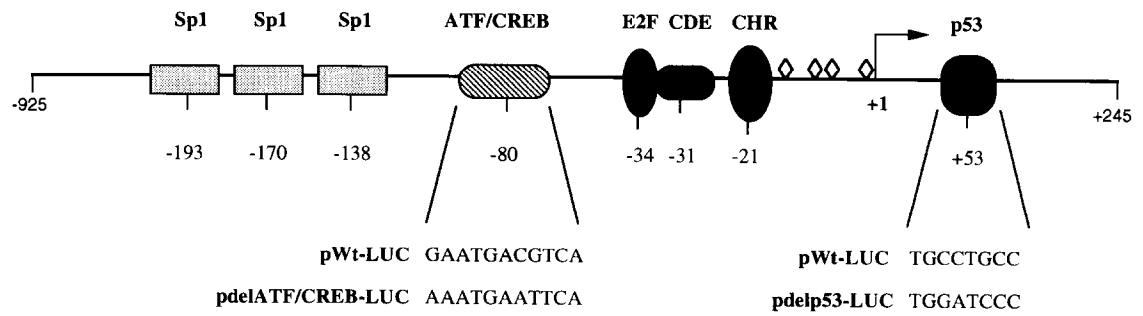

Fig. 1. Map of human cyclin A proximal promoter. A schematic diagram of the human cyclin A promoter indicating the position of consensus binding sites for transcription factors: Sp1, ATF/CREB, E2F, CDE, CHR and p53. Diamonds indicate minor transcription start sites (located between positions −48 and +1). The major transcription start site is indicated by the arrow (+1). Translation (ATG codon) starts at position 256. Sequence of the mutant promoter in plasmids pdelATF/CREB-LUC and pdelp53-LUC is also shown, and compared to the wild type sequence (pWt-LUC).

tivity was measured after synchronization in G0 by serum deprivation and re-stimulation by fetal calf serum. Under these conditions, cells entered S phase 14 h after stimulation and reached G2/M after 22 h (Fig. 2).

We first monitored the cell cycle regulation of the wild type promoter in cells containing pWt-LUC. As shown in Fig. 3, the luciferase activity was increased as cells entered S phase. This activity precisely reflects the promoter activity, as the turn-over of luciferase is rapid in these cells, with a half-life of 2 h (data not shown). Luciferase activity increased in parallel with the endogenous cyclin A mRNA in late G1 (10 h after serum stimulation) and remained high as cells proceeded to G2/M (22 h). In addition, cyclin A protein was not detectable in G0 cells and during the first 10 h after stimulation, i.e. during most G1, and dramatically increased at 18 h, when most cells had entered S phase. These data demonstrate that luciferase activity in these stable cell lines closely mirrors the physiological regulation of the cyclin A gene, i.e. a repression of the promoter in G0/G1 and a transcriptional activation in late G1 followed by a plateau in S and G2.

We then compared kinetics of expression driven by p53 or ATF/CREB mutant cyclin A promoter to that driven by the wild type construct. First, we monitored cyclin A promoter down-regulation during serum deprivation (Fig. 4A). Luciferase activity under control of the wild type cyclin A promoter, decreased to basal levels within 72 h. Mutation of either ATF/CREB or p53 sites did not impair this down-regulation. Second, we analysed cyclin A promoter activation after serum stimulation. We observed no significant deregulation when the p53 site was mutated. The timing of activation as well as the level of luciferase activity produced from the mutant

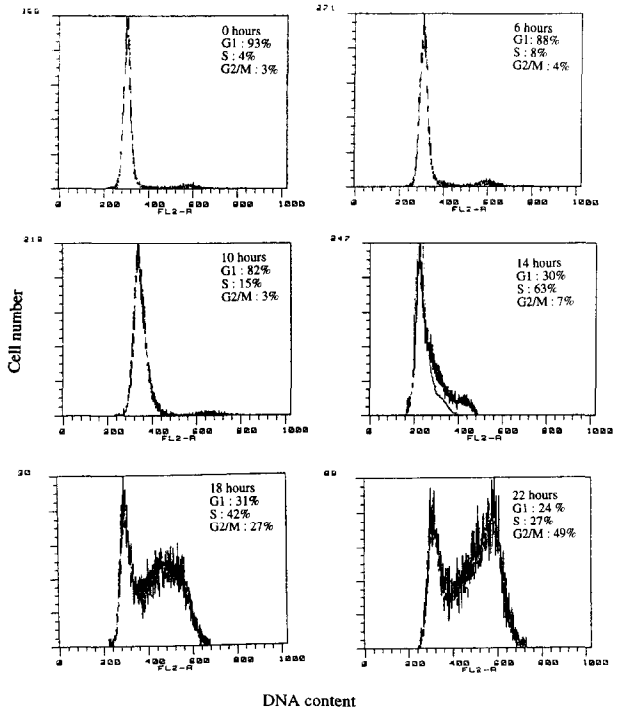

Fig. 2. Analysis of NIH-3T3 cell synchronization by flow cytometry after propidium iodide staining. Growth-arrested NIH-3T3 cells were obtained by serum deprivation (0.2% FCS) for 48 h, and then refed with 10% FCS. They were allowed to proceed through one cell cycle, S phase starting at 14 h. The two peaks represent G1 phase cells (2*n* DNA contents) and G2/M phase cells (4*n* DNA contents).

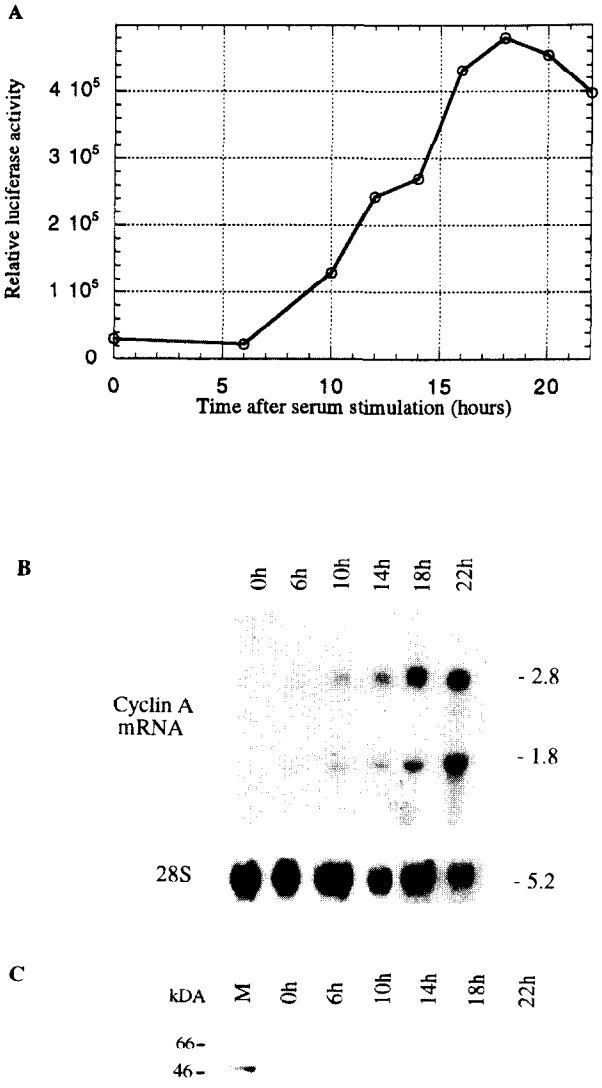

Fig. 3. Cell cycle regulation of cyclin A in NIH-3T3 cells after serum stimulation of cells synchronized in G0 by serum deprivation. (A) Luciferase activity driven by the cyclin A promoter. (B) Northern blot showing cyclin A mRNA accumulation at the indicated time after serum stimulation. The blot was then hybridized to a 28S oligonucleotide probe for normalization size in kilobases is indicated on the right. (C) Western blot showing cyclin A protein accumulation at the indicated time after serum stimulation. The two bands correspond probably to phosphorylated isoforms as described [47]. Molecular weight markers are shown on the left (M).

construct paralleled that of the wild type (Fig. 4B). In contrast, promoter activity was impaired when the ATF/CREB binding site was mutated (Fig. 4C). As cells proceeded to S and G2 phases, the level of luciferase activity produced from the mutant construct increased to a value only 6–7-fold greater than that of G0 cells as compared to 19-fold greater in the case of the wild type promoter. In a parallel experiment, we established two other cell lines containing either zero or one copy of the human cyclin A ATF/CREB site linked to the minimal HSV-TK promoter. Neither construct gave rise to the increase of luciferase activity in late G1 and S phases that is observed with the Wt-cyclin A promoter construct (Fig. 4D). These data demonstrate that the ATF/CREB site

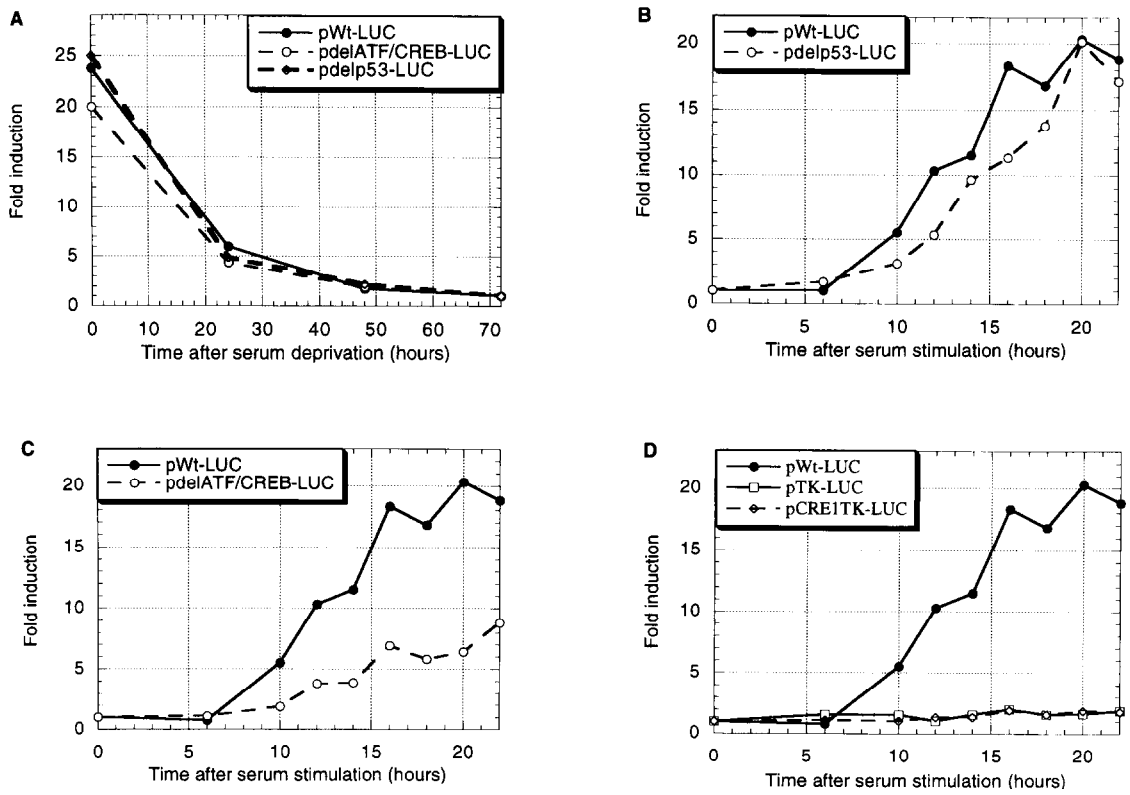

Fig. 4. Kinetics of luciferase activity in synchronised NIH-3T3 cells stably transfected with wild type or mutated cyclin A promoter-luciferase constructs. First, the activity from the three constructs was monitored during serum deprivation (A). Second, the activity was monitored after serum stimulation: (B) pWt-LUC and pdelp53-LUC, (C) pWt-LUC and pdelATF/CREB-LUC, (D) pWt-LUC, pTK-LUC and pCRE1.TK-LUC. The level of induction for each construct tested correspond to the values relative to activity in G0 cells. Results are the mean of three independent determinations.

is involved in the transactivation of the cyclin A gene but is not sufficient to confer a cell cycle dependent regulation to an heterologous promoter.

It has been suggested that p53 may repress the cyclin A promoter in neuroblastoma cells [28]. In order to identify the sequences in the cyclin A promoter that may be responsible for p53-mediated repression, we first analysed a series of 5′-deletion constructs (A–F, [20]) in transient transfection assays. Saos-2 cells, which carry a deletion in the p53 gene, were cotransfected with the selected cyclin A-promoter luciferase constructs and normal/mutant p53 expression vectors. We used the Ala143 mutant, which is found in numerous human cancers, as a control devoid of transactivating activity [40]. As shown in Fig. 5A, wild type p53 repressed the cyclin A promoter in a dose dependent manner whereas mutant p53 had no repressing activity. 5′-terminal deletion to position −79 (pWt/F-LUC) in the cyclin A promoter reduced the promoter activity by 20-fold [20] but did not abolish the repression effect of p53 (Fig. 5A). Similar results were found with intermediate deletion constructs (data not shown). Thus, there is no p53 specific element upstream of −79 which is responsible for p53-mediated promoter repression. These experiments mapped the minimal region sufficient to observe the repression effect to −79/+245. In a second series of experiments, we investigated the functionality of the putative p53 DNA binding element located at position +53 (see Fig. 1). p53 is known to activate transcription of target genes through its binding to a specific DNA binding element. Mutation of the p53 binding site in the cyclin A promoter did not alter the promoter activity (data not shown) or impair p53-mediated repression (Fig. 5A). In addition, the human cyclin A p53 site was unable to confer p53 responsiveness to the minimal HSV-TK promoter (Fig. 5A) in contrast to the RGC p53 binding sequence (Fig. 5B). In fact, we observed repression of the HSV-TK promoter by wild type p53 as previously described for other promoters containing a TATA sequence [31-36]. All

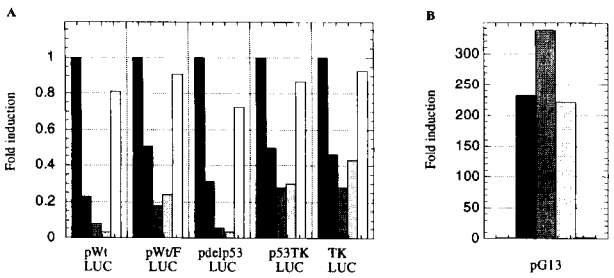

Fig. 5. Repression of cyclin A promoter upon p53 expression. Saos-2 cells were transfected with 2 μg reporter plasmids (closed bar), or together with increasing amounts of Wt p53 expression plasmids (0.1, 0.5, 2 μg: dotted bars) or 2 μg of Ala143 mutant p53 expression plasmid (open bar). (A) The reporter plasmids are indicated (see Fig. 1 and section 2). (B) pG13 reporter plasmid was used to monitor the transactivation activity of p53 [39]. Results are expressed as fold induction relative to the activity of the reporter plasmid alone (referred to as 1).

these data demonstrate that the p53 site is not implicated in the regulation of the cyclin A gene.

4. Discussion

The human cyclin A gene is transcribed in a cell-cycle dependent manner, starting in late G1 and increasing until G2 phase. The main goal of the present study was to investigate whether the ATF/CREB and p53 sites located in the promoter might be involved in the transcriptional regulation of the gene.

Our experiments demonstrate that the ATF/CREB site is necessary for a high level of cyclin A expression from late G1 until G2 phase. We have previously shown that transcription factors of the CREB/CREM family are able to repress and transactivate the cyclin A promoter through their binding to the ATF/CREB site. The finding that the activity of CREB/CREM factors is cell cycle dependent led to the hypothesis that they might be involved in cyclin A promoter transactivation in late G1 [25]. While our present data indicate that the ATF/CREB site is not responsible for cell-cycle specific activation, we identify this site as a key sequence operating to upregulate the cyclin A promoter at the G1/S transition when cyclin A protein is required for the entry into S phase. Interestingly, the same site was recently implicated in cyclin A promoter down-regulation in contact-inhibited endothelial cells [26] and in chinese hamster lung fibroblasts upon treatment with TGF-β [27]. These findings are in contrast to our observation that this site is not involved in repression observed in serum-deprived cells. It is likely, the effects of multiple pathways converge on the ATF/CREB site which thus acts as the final target of both positive and negative membrane derived signals.

In a separate set of experiments, we investigated the mechanism underlying the p53-mediated regulation of cyclin A gene. Initial experiments had suggested that p53 might repress cyclin A expression [28]. p53 is a transactivating protein that is able to activate transcription of genes containing a specific p53 DNA binding element. The cyclin A gene contains a putative p53 binding site located in the 5′ untranslated region. Our experiments show that this site is unable to mediate transactivation by p53; in addition careful examination of this sequence revealed that the first part of the site has only 7/10 conserved nucleotides and the second part 5/10 when compared to the latest concensus p53 binding sequence [46]. p53 is also able to down-regulate transcription of promoters containing a TATA box element, this was shown for several early genes such as c-*fos*, c-*jun* and c-*myc* [29,30]. In the case of the cyclin A gene, our results show that repression upon p53 expression occurs with a minimal cyclin A promoter, suggesting an interference with the transcription complex. The originality of this phenomenon reside in the absence of a TATA sequence in the cyclin A promoter. Our results thus extend interference by p53 to a TATA-less promoter.

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