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Cyclin A2 Transcriptional Regulation: Modulation of Cell Cycle Control at the G1/S Transition by Peripheral Cues

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ABSTRACT. Several types of cyclins have been identified and among these, cyclin A2 is synthesized in somatic cells at the onset of DNA synthesis as well as during the G2/M transition associated with cyclin-dependent protein kinases 1 and 2. Modulation of cyclin A transcription is due to the interplay between a cell cycle-dependent periodic relief of a transcriptional repression and signals transduced through adenosine 3′,5′-cyclic monophosphate, transforming growth factor-β, and the integrin-mediated pathways. Using primary mouse embryonic fibroblasts from embryos where the genes coding for the protein responsible for susceptibility to retinoblastoma (pRB) and the related p107 and p130 proteins had been individually inactivated, we showed that cyclin A is a functional target of pRB-mediated cell cycle arrest. The factors involved are discussed. BIOCHEM PHARMACOL **60**;8:1179–1184, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cyclin A; transcription; CREB/ATF; adhesion

Progression through the different phases of the cell division cycle is controlled by a cdk† family (for a review see [1]). As regulatory subunits of cdks, cyclins control the temporal transitions between the various stages of the cell cycle. Several types of cyclins have been identified, with the mammalian A-type cyclin family consisting of two members: cyclin A1 and A2. The latter is synthesized at the onset of DNA synthesis as well as during the G2/M transition in somatic cells. Targeted deletion of its gene in the mouse is embryonic lethal [2]. Cyclin A1 is expressed in mice exclusively in the germ cell lineage, whereas it is expressed at high levels in the testis and certain myeloid leukemia cells in humans. Targeted disruption of the gene encoding cyclin A1 leads to male sterility due to an early block of spermatogenesis, whereas females are normal [3]. We will focus this analysis on cyclin A2 and refer to it as cyclin A throughout the rest of the text.

Cell cycle modulation of cyclin A expression is due in part to the periodic relief of a transcriptional repression when cells enter S phase. We previously analyzed the expression of cyclin A in primary cells as well as in several human and rodent cell lines [4–9], and delineated functional DNA sequences present in the mouse cyclin A promoter. *In vivo* genomic dimethyl sulfate footprinting has revealed the presence of several putative protein-binding elements within a short region containing the major transcription initiation sites of this TATA-less gene. Within 70 bp, three sites are found occupied *in vivo* in most cell types analyzed thus far; two of these which bind *in vitro* proteins of the CREB/ATF and NF-Y families, respectively, are constitutively occupied. The third site, proposed to be involved in the down-regulation of the promoter in quiescent (G0) and early G1 cells, is occupied only when cyclin A transcriptional activity is shut off (Fig. 1). The latter element has therefore been termed the CCRE [6] or the CDE [10].

Finally, we will briefly discuss the interactions occurring at the CRE, the CAAT box, and the CCRE/CDE of cyclin A promoter, respectively, and how they relate to the modulation of cell cycle progression by extracellular stimuli.

THE CCRE/CDE CONNECTION

Functional assays where cyclin A promoter directed the expression of a reporter gene revealed that mutation of the CCRE/CDE resulted in a complete deregulation of the promoter in G0/G1. This led to the notion that a repressor was binding to this element [6, 10, 11]. Another element whose mutation also abolished cyclin A repression in G0/G1 resides 3′ to the CCRE/CDE [8, 12, 13]. This element, together with the CCRE/CDE, was found to be

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† *Abbreviations:* ATF, activating transcription factor; cdk, cyclin-dependent kinase; CREB, cyclic adenosine monophosphate response element binding protein; CCRE, cell cycle responsive element; CDE, cell cycle-dependent element; CRE, cyclic adenosine monophosphate response element; CHR, cell cycle gene homology region; TGF, transforming growth factor; cAMP, cyclic adenosine monophosphate; pRB, retinoblastoma susceptibility gene product; CREM, cyclic adenosine monophosphate response element modulator; ICER, inducible cyclic adenosine monophosphate early repressor; and CHF, cyclin A CHR binding factor.

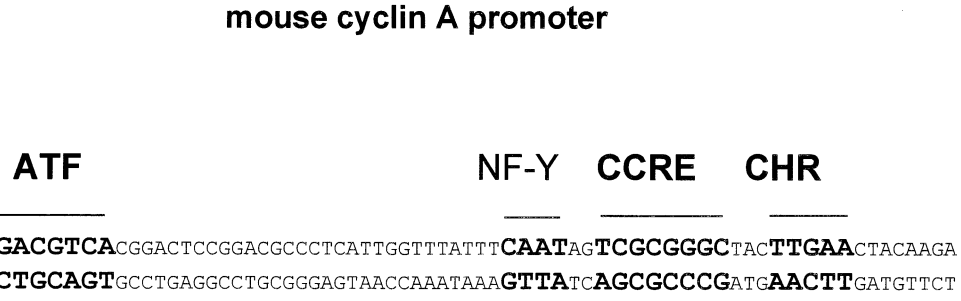

FIG. 1. Schematic representation of the murine cyclin A promoter. ATF (CREB/ATF-binding site), NF-Y (CAAT box), and CCRE correspond to previously identified *in vivo* footprints [6, 10]; CHR [12, 22].

shared by other genes such as *B-myb*, *cdc2*, and *cdc25*C and was therefore termed CHR (Fig. 2).

Transcription of genes involved in cell cycle control requires coordinate activation and repression of specific sets of transcription factors, among which members of the E2F/DP family constitute a thoroughly studied archetype (for reviews, see [14, 15]). Activity of E2F/DP transcription factors is repressed by their association with pocket proteins such as pRB and the two closely related proteins p107 and p130, initially described as adenovirus E1A binding proteins. pRB becomes hyperphosphorylated as cells transit G1 and dissociate from the E2F/DP complex, leading to the activation of E2F-regulated genes. Interestingly, cyclin A belongs to a group of genes whose transcription is repressed in G0/early G1 and de-repressed either in late G1 (*B-myb*, *E2F-1*), during S phase (*cyclin A*, *cdc2*), or as cells transit into G2 (*cdc25*C). These genes are therefore potential target candidates for E2F/DP-mediated repression processes. Whereas some groups reported that the E2F-containing complex interacted with the CCRE/CDE [11, 16, 17], others did not observe such a binding [6]. Moreover, no CHR-binding protein has yet been purified. However, an *in vitro* binding activity termed CDF-1, which is not related to E2F, was characterized and proposed to interact with both the CDE and CHR of *cdc25*C [18, 19]. Electromobility shift assays and oligonucleotide competition experiments suggested the presence of a general factor that was not able to discriminate between the CCRE/CDE–CHR modules present in the genes encoding *cdc25*C, *cdc2*, and *cyclin A*. Neither further characterization of the complex nor determination of the number and molecular properties of its components were undertaken.

The high sequence conservation of the diverse CCRE/CDE–CHR modules together with the apparent lack of specificity observed *in vitro* for the binding of CDF-1 are at odds with the observed *in vivo* sequential timing of promoter activity during the cell cycle. Differential binding of E2Fs and CDF could contribute to this timing [19, 20]. Mutations introduced into the cyclin A promoter interestingly led to either a loss or a tightening of E2F binding [19],

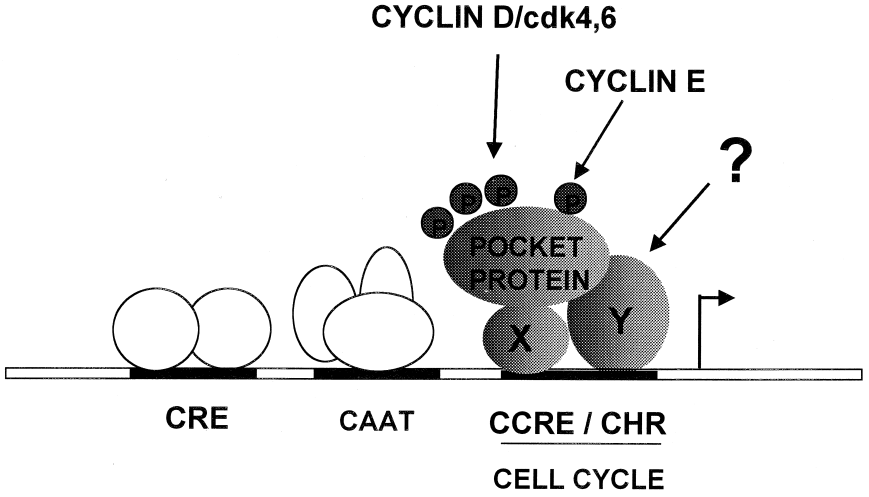

FIG. 2. Proteins present at the level of the bipartite repressor element. X: protein(s) binding to the CCRE/CDE element. Some data suggest that the E2F/DP complex could be a modulator of cyclin A promoter activity [19, 20]. However, the *in vitro* affinity measured by electomobility shift experiments is very low and suggests that protein–protein interactions should stabilize the complex formation *in vivo* [6, 9]. Whatever the protein present on the CCRE/CDE, it probably tethers a pocket protein (most likely pRB), which is the target of inactivating phosphorylation events catalyzed by the cyclin D/cdck4,6 complex. Accordingly, cyclin A is probably a primary target of pRB and cyclin E/cdk2 a modulator of this pathway. Y: protein binding to the CHR element and dominant over X. It is not purified yet, and the question remains whether its action is either limited to cyclin A or if it is a general repressor of a specific subset of genes repressed in G0/G1 and expressed later in the cell cycle.

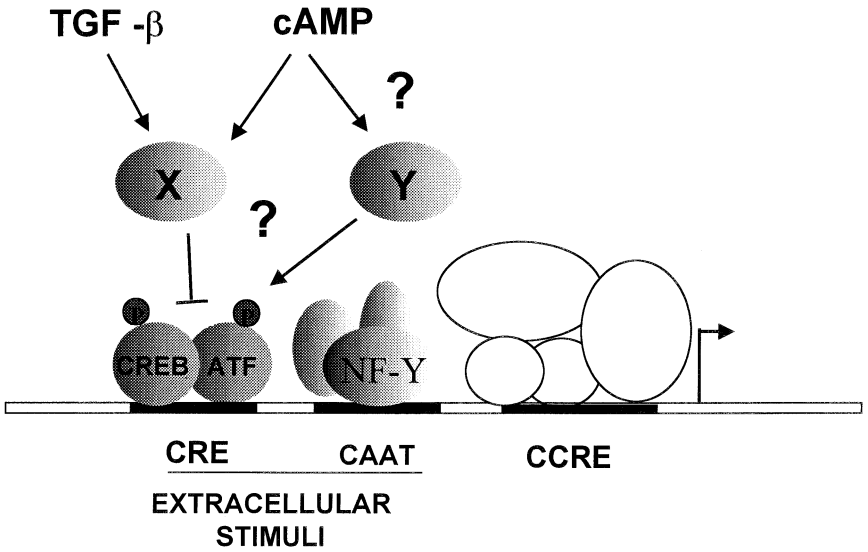

FIG. 3. Proteins binding to the cyclin A CRE and CAAT box elements. The CAAT box and the CRE element bind NF-Y and proteins from the CREB/ATF family, respectively. NF-Y probably acts as an architectural protein, organizing the binding of the other surrounding factors. CREB/ATF proteins are activated by phosphorylation events and could both control the basal activity of the promoter, as well as its modulation by extracellular factors such as TGF-β [5, 35], or stimuli which modulate the intracellular cAMP level [5, 33].

with only a minor effect on CDF-1 binding. Mutants deficient for E2F binding, while still repressed in quiescent cells, harbored a delayed expression pattern. Conversely, E2F high-affinity mutants resulted in premature up-regulation of cyclin A promoter. Whereas these experiments pointed to the CHR-binding factor as the major repressor of *cyclin A* gene, they also suggested that E2F/DP could only contribute to the fine timing of its transcription. This is consistent with the selective *in vitro* binding of the cyclin A CDE–CHR with E2F-1 and E2F-3-glutathione synthase fusions and not with *E2F-4*, which is the major E2F member expressed in quiescent cells when cyclin A is actually repressed. These data suggest that E2F cannot be the repressor of cyclin A in G0 cells, and could explain the observed up-regulation of cyclin A promoter following an ectopic expression of E2F-1 [12, 21]. Characterizing the CHR-binding factor is therefore an important issue. We recently isolated a 90–95 kDa protein present in quiescent cells and absent in cells blocked in S phase that binds specifically to *cyclin A* CHR, but not to *B-myb*, *cdc25C*, or *cdc2* CHRs [22]. Both *in vitro* transcription and *in vivo* microinjection experiments demonstrate that this protein, termed CHF, is able to discriminate between the *cyclin A* and *cdc25C* CHRs, thereby establishing a functional difference between these two repressor elements.

Whatever the nature of the proteins binding to the CCRE/CDE–CHR, overexpression of viral proteins such as adenovirus E1A, simian virus 40 and polyoma T antigens, or papilloma E7 has been shown to increase cyclin A expression, thus providing some support to the notion that pocket proteins may modulate cyclin A transcription [4, 7, 23–25]. Mice have been derived where the various RB-related genes were inactivated through homologous recombination [26–30]. This demonstrated that cyclin A expression is deregulated in primary mouse embryonic fibroblasts from pRB(−/−), but not from p130(−/−), p107(−/−), or even p130(−/−)/p107(−/−) double mutant embryos [7, 31]. That cyclin A is a downstream target of pRB-mediated cell cycle arrest was further substantiated by experiments whereby dominant pRB or $p16^{INK4A}$ were able to regulate the cyclin A promoter through the CCRE, while dominant E2F was not [32]. Consistent with this, ectopic expression of cyclin A reversed pRB-mediated G1 arrest. Interestingly, in the same experiment, ectopic expression of cyclin E was able to antagonize pRB-mediated repression of cyclin A promoter, but not that of two other genuine E2F-regulated promoters. This points to cyclin E as a modulator of pRB-mediated G1/S inhibition [30].

THE CRE CONNECTION

As mentioned earlier, while the CCRE is protected *in vivo* from dimethyl sulfate reactivity only in G0/G1 cells, the CRE and CAAT elements are protected in quiescent, stimulated, and exponentially growing cells [6, 9] (Fig. 3). Mutation of these sites, although reducing the overall activity of cyclin A promoter (an effect whose extent depends upon the cell type), did not abolish its cell cycle dependence. *In vitro* translated CREB and ATF-1 proteins, as well as bacterially generated CREMα, CREMβ, and ICER proteins, bound equally well to the cyclin A CRE [5, 33]. In contrast, antibodies directed at ATF-1, while supershifting some of the complexes formed with extracts from hamster CCL39 cells, either almost completely abolished the formation of complexes prepared from murine NIH3T3 cells or supershifted most complexes from human

T lymphocytes [5, 6, 9]. This reflects some cell specificity and suggests either the presence of some redundancy or that overall cyclin A transcriptional activity can be modulated according to the cell type. The variation of the level of CREM and CREB family members throughout the cell cycle was determined in synchronized Hs 27 primary human fibroblasts [33]. Whereas the level of ICER transcriptional repressors dropped as cells progressed though G1 to S, that of the CREMβ repressor or CREB and CREMτ activators remained constant. In contrast, phosphorylation of CREB serine 133 or CREMτ serine 117, known to determine their transactivation potential, was observed in G0 and G1, but was not detectable in S and G2. However, no functional experiments were conducted which could have established a firm link between these observations and cyclin A promoter activation.

Cell cycle progression is prevented by TGF-β through the inhibition of several regulators including cyclin A (for a review see [34]). We have shown that in the latter case the effect appeared to be mediated in hamster CCL39 fibroblasts at the transcriptional level by the cyclin A CRE [5]. Activation of the cAMP pathway in these cells gave rise to a strong inhibition of proliferation but, surprisingly, TGF-β-mediated arrest did not require a functional cAMP-dependent protein kinase. However, in mink lung epithelial cells, CRE-mediated cyclin A down-regulation by TGF-β was associated with a strong reduction in CREB and ATF phosphorylation, which was reverted by okadaic acid, overexpression of protein kinase A catalytic subunit, or of the simian virus 40 small tumor antigen known to inhibit PP2A phosphatase [35].

The CAAT box element turned out to be an *in vitro* high-affinity binding site for NF-Y. The latter protein belongs to a family of CAAT box-binding proteins involved in the control of the expression of many eukaryotic genes. It consists of three subunits of 43 (Y_A), 32 (Y_B), and 90 (Y_C) kDa, respectively. Antibodies directed against both the A and B subunits were able to supershift the complexes formed with many cell extracts in a quantitative manner [6, 9]. Utilization of large mutated DNA fragments containing the three binding sites in order to probe for factor binding in extracts from activated lymphocytes led to the suggestion that NF-Y factor could organize the binding of nearby factors [9]. Stable expression of a tetracycline-controlled dominant negative form of NF-Y gave rise to a delayed entry into S phase and a prolonged cell division time, consistent with this factor's playing a general role in the control of cell proliferation [36].

CONCLUDING REMARKS

When quiescent cells are stimulated to proliferate, D-type cyclins are synthesized and assembled with cdk4 and cdk6 to produce active kinases that target pRB and inactivate its growth suppressive function. Active cyclin D-dependent kinases are not required for S phase entry in cells lacking a functional pRB. Moreover, expression of D cyclins depends upon continuous stimulation of cells by growth factors throughout the cell cycle, and serum withdrawal leads to a rapid cyclin D destruction via the ubiquitin–proteasome pathway and, as a consequence, to an accumulation of hypophosphorylated pRB. Interestingly, Ras-mediated sig-

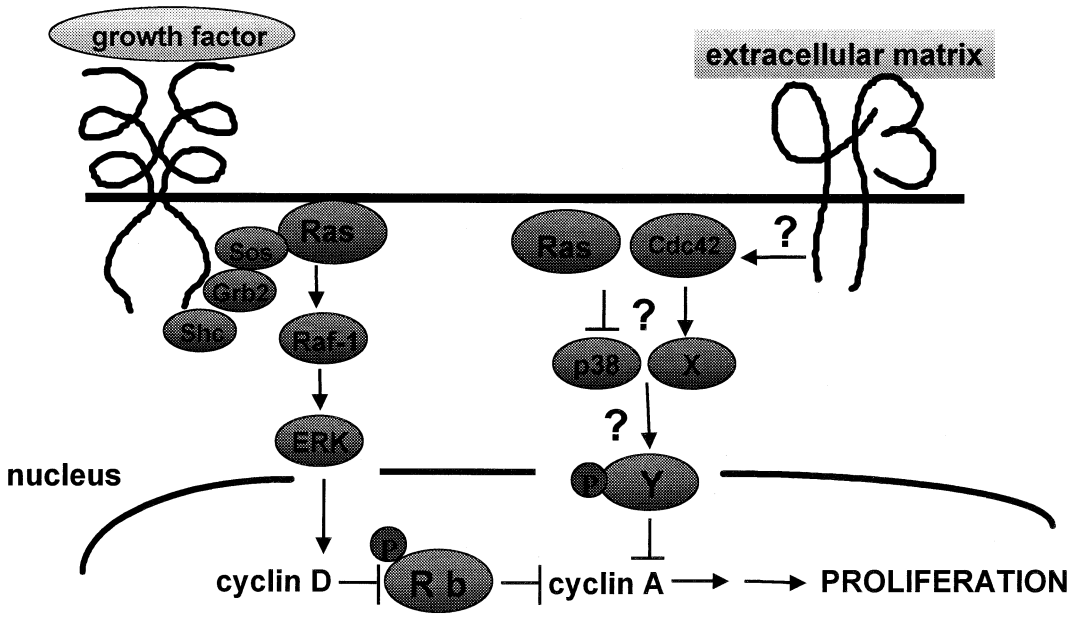

FIG. 4. Cyclin A expression is controlled by both growth factors and extracellular matrix. The Ras/Raf/extracellular signal-regulated kinase cascade is indirectly involved in the induction of cyclin A transcription through the activation of cyclin D-type-activated kinases. However, signals generated by integrins are likely to be required for entry into S phase. p38 mitogen-activated protein kinase exerts an inhibitory function on cell cycle progression, one probably controlled by the coordinated action of Ras and Cdc42. The link between integrin recruitment and the activation of the GTPases is not yet understood, nor is the link between GTPase effectors such as p38 and their downstream transcription factor targets.

naling has been proposed to control both synthesis and degradation of D cyclins, thereby underscoring the role of this network as a growth factor sensor. Because its gene is a downstream target of pRB, cyclin A transcriptional induction is indirectly dependent upon mitogenic stimulation through cyclin D activation. However, cyclin E can modulate pRB inhibition of S phase entry and therefore cyclin A induction. This activity is also controlled by the degradation of cyclin E via the ubiquitin–proteasome pathway, but the signal is provided internally through cdk2, the kinase partner of cyclin E.

As was discussed above, cell cycle progression can still be halted in exponentially growing cells if the intracellular cAMP level is increased or if cells are exposed to TGF-β. This can be mediated by many cell cycle regulators including cdk4, $p15^{INK4B}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$, Cdc25A, or cyclin A. Moreover, proliferation of mammalian cells is under the control of both growth factors and interactions with the extracellular matrix, and cyclin A expression is clearly linked to cell adhesion (Fig. 4). A working model has been proposed whereby both growth factors and the extracellular matrix are required for the induction of cyclin D1–cdk4,6 and cyclin E–cdk2 activities (for a review, see [37]), which then lead to the hyperphosphorylation of pRB. However, depending on the cell type, the steady state of the cdks and their cognate cyclins do not always vary as a function of the anchorage properties of the cells. It is noteworthy, however, that cyclin A down-regulation under anchorage-free conditions is a constant in most published studies, and its forced expression allows cells to proliferate in the absence of adhesion. Interestingly, this adhesion-dependent cyclin A expression is mediated at the transcriptional level by the CCRE/CDE–CHR and its cognate factors. Characterizing CDF/CHF will probably shed some light on how integrin-mediated signaling impinges on cell cycle control. A first clue was uncovered by the use of several mutants of Ras and its related Rho GTPases such as Rac and Cdc42. It was shown that activated forms of Ras or Cdc42 can alleviate the anchorage requirement for cell cycle progression [38–41]. In mouse embryo fibroblasts, we showed that Ras and Cdc42 act in strong synergy through parallel pathways, which probably leads to p38 kinase down-regulation [41]. The challenge now is to establish the links between integrin receptors and Cdc42 activation on the one hand and downstream effectors such as p38 kinase and transcription factors binding to cyclin A promoter on the other.

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