MOLECULAR AND CELLULAR BIOLOGY, July 1996, p. 3789–3798 Vol. 16, No. 7
0270-7306/96/$04.00+0

Cyclin A Expression Is under Negative Transcriptional Control during the Cell Cycle

X. HUET, J. RECH, A. PLET, A. VIÉ, AND J. M. BLANCHARD*

Institut de Génétique Moléculaire de Montpellier, CNRS UMR 5535, 34033 Montpellier cedex 1, France

Received 29 February 1996/Accepted 11 April 1996

Transcription of the gene coding for cyclin A, a protein required for S-phase transit, is cell cycle regulated and is restricted to proliferating cells. To further explore transcriptional regulation linked to cell division cycle control, a genomic clone containing 5′ flanking sequences of the murine cyclin A gene was isolated. When it was fused to a luciferase reporter gene, it was shown to function as a proliferation-regulated promoter in NIH 3T3 cells. Transcription of the mouse cyclin A gene is negatively regulated by arrest of cell proliferation. A mutation of a GC-rich sequence conserved between mice and humans is sufficient to relieve transcriptional repression, resulting in a promoter with constitutively high activity. In agreement with this result, in vivo footprinting reveals a protection of the cell cycle-responsive element in G_0/early G_1 cells which is not observed at later stages of the cell cycle. Moreover, the footprint is present in dimethyl sulfoxide-induced differentiating and not in proliferating Friend erythroleukemia cells. Conversely, two other sites, which in vitro bind ATF-1 and NF-Y, respectively, are constitutively occupied throughout cell cycle progression.

Cyclins are a family of proteins involved in the control of the eukaryotic cell division cycle. As regulatory subunits of a particular subset of periodically activated protein kinases, they control the temporal transition between the various stages of the cell cycle (for reviews, see references 18, 38, 48, 49, 59, and 60). Their dysregulation has also been proposed to be associated with the genesis of some human cancers (8, 23, 26, 31, 32, 52, 56, 67, 68). The A-type cyclin has been implicated in the control of the entry into S phase, as well as in the G_2/M transition (22, 47, 53, 66), by binding to the cdk2 and cdc2 kinases, respectively (51, 65). Cyclin A mutants of *Drosophila* embryos arrest in G_2 (36, 37). In addition to its role in the dependence of mitosis on S-phase completion, it may have a role in mitotic control (37, 63, 66). Cyclin A is found in cellular activities which promote both replication (10, 19, 47, 50, 53, 62) and transcription (for reviews, see references 35, 45, 69). In the former case, it has been shown to be associated with replication complexes and is likely to directly participate in the phosphorylation of replication protein A (RPA) (20, 70). In the latter case, its association with the E2F/DRTF1 family of transcription factors has been proposed to play a central role in cell cycle transcriptional control. A large body of evidence suggests that cyclin-dependent kinases participate in the regulation of the activity of this transcription complex. This is believed to occur either directly, by the phosphorylation of E2F by cyclin A-cdk2 kinase, or indirectly through the phosphorylation of a group of proteins structurally related to RB, the retinoblastoma susceptibility gene product, by cyclins D, E, and A, which are associated with cdk4, cdk6, and cdk2 kinases. In agreement with these ideas, cyclin A is found complexed with E2F in late G_1 phase and during S phase, recruiting the catalytic kinase subunit to the DNA-binding complex (9, 17, 24, 35, 39, 46, 61, 71).

Cyclin A mRNA and protein accumulate at the end of G_1 phase and rapidly reach a plateau during S phase, after a transcriptional activation of its gene (4, 5, 25). We describe here studies aimed at understanding the mechanisms of cell cycle regulation of cyclin A gene transcription. We have isolated a genomic DNA fragment containing the 5′ flanking portion of the mouse cyclin A gene and show that it directs the transcription of a luciferase reporter activity in a fashion similar to that of the endogenous gene. A sequence conserved between mice and humans has an in vivo occupancy which varies according to the proliferative status of the cells. Its mutation abolishes the repression of the cyclin A promoter in quiescent cells, thus resulting in a constitutive activity.

* Corresponding author. Mailing address: Institut de Génétique Moléculaire de Montpellier, CNRS UMR 5535, BP 5051, 1919 route de Mende, 34033 Montpellier cedex 1, France. Phone: (33) 67 61 36 49. Fax: (33) 67 61 36 01, (33) 67 04 02 31. Electronic mail address: blanchard@igm.cnrs-mop.fr.

MATERIALS AND METHODS

Isolation of the mouse cyclin A promoter and construction of reporter plasmids. The mouse genomic library (a generous gift from J. P. Rouault) was obtained by inserting DNA from mouse CGR8 embryonic stem cells into the *Bam*HI sites of phage λEMBL3, after partial digestion with *Mbo*I. After plating on nylon filters, phages were screened with a full-length human cDNA probe (68), and positive clones were rescreened first with a small restriction fragment corresponding to the 5′ part of the cDNA and then with diverse 5′ flanking sequences at low stringency. Two clones gave positive scores and were used for further analysis. The phage insert was cut with several restriction enzymes, and the resulting fragments were subcloned into pBluescript KS plasmid (Stratagene). Sequencing of double-stranded DNA fragments was carried out by the dideoxynucleotide chain termination method with T7 DNA polymerase (Pharmacia) and primers derived from plasmid and insert sequences. Selected fragments were inserted into the promoterless plasmid pGL2-Basic, containing the firefly luciferase gene as a reporter (Promega). The two oligonucleotides corresponding to the cyclin A cell cycle-responsive element (CCRE), 5′-CGCGTAG TCGCGGGCTAC and 5′-TCGAGTAGCCCGCGACTA, were annealed, and the resulting double-stranded oligonucleotide was cloned into the *Mlu*I and *Xho*I sites of pGL3-Promoter (Promega). The latter plasmid transcribes the luciferase gene from a minimal, enhancerless early simian virus 40 (SV40) promoter.

Mutagenesis. A splice overlap extension PCR technique was used for the genesis of the different mutations with the following oligonucleotides which introduce an *Eco*RI site at the selected position: (i) GC-rich (CCRE) site, 5′-GTAGAATTCGACTATTGAAATAAACCAATGAGGG and 5′-GTCGAA TTCTACTTGAACTACAAGACCAG; (ii) NF-1/NF-Y 5′ half, 5′-CCCGAATT CGTTTATTTCAATAGTCGCGG and 5′-AACGAATTCGGGCGTCCGGAG TC; and (iii) NF-1/NF-Y 3′ half, 5′-GGGAATTCTAGTCGCGGCTACTTG and 5′-CTAGAATTCTAAACCAATGAGGGCG. The reconstituted full-length fragments were all amplified with the following oligonucleotides, which allow cloning into the *Kpn*I and *Hin*dIII sites of pGL2-Basic: 5′-CGCGGTACCTATG AGGCTACAGATC and 5′-GGGAAGCTTCTGCAGGCGGGAGGA. All mutations were verified by sequencing.

RNase protection and primer extention assays. A 278-bp *Bam*HI-*Pst*I mouse DNA fragment containing the putative transcription initiation site(s) subcloned

into pBluescript SK$^+$ was used to synthesize a uniformly labeled probe with [α-^{32}P]UTP (400 Ci/mmol; Amersham) and T3 RNA polymerase (Boehringer) as indicated by the suppliers. After digestion of the template with 15 U of RNase-free DNase (Bethesda Research Laboratories) for 15 min at 37°C, RNA was extracted with phenol-chloroform and precipitated with 2 volumes of ethanol in the presence of 10 μg of yeast tRNA. The radioactive RNA was fractionated by electrophoresis through a 5% polyacrylamide–8 M urea gel, hybridized to 50 μg of total RNA prepared from exponentially growing NIH 3T3 cells, and digested with RNase A. The resulting protected bands were extracted with solvent, ethanol precipitated in the presence of carrier tRNA, and resolved on a 5% polyacrylamide sequencing gel in parallel with labeled size markers and a sequence ladder from the same region.

The synthetic oligonucleotide 5′-GGGAAGCTTCTGCAGGCGCCAGGA (50 ng) was labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) and polynucleotide kinase. Primer extention was performed with avian myeloblastosis virus reverse transcriptase after hybridization to poly(A)$^+$ RNA purified from 200 μg of total RNA. Elongated products were analyzed on the type of sequencing gels described above.

Cell culture and transfections. Mouse NIH 3T3 cells and human fibroblasts from skin biopsies were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. A total of 2×10^5 cells per 3.5-cm-diameter dish were transfected by the calcium phosphate method with 4 μg of total DNA as described elsewhere (6). The cells were synchronized with a 48-h culture in 0.5% serum and then by refeeding with complete medium. After cell lysis at the indicated times, luciferase activities were measured with a Lumat LB9501 Berthold luminometer. When necessary, [^{3}H]thymidine incorporation into acid-precipitable radioactivity was monitored by standard procedures.

Friend leukemia cells derived from the 745A clone were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and were differentiated by the addition of 1.5% (vol/vol) dimethyl sulfoxide (DMSO) as described elsewhere (43).

In vivo genomic footprint analysis. Cells from 9-cm petri dishes were exposed for 2 min to 1 μl of dimethyl sulfate (DMS) per ml, and the reaction was quenched with 2% β-mercaptoethanol and then by washing in phosphate-buffered saline (PBS). After extraction of genomic DNA and piperidine cleavage of methylated guanines, methylation interference was monitored by a ligation-mediated PCR as described elsewhere (6, 21). The following oligonucleotides were used for the sense-strand analysis: 5′-GCGGGAGGAGCGTAGAGCCC, 5′-AGCCCAGGAGCCGCGAGCTG, and 5′-CAGGAGCCGCGAGCTGCG CG. For the antisense strand, the following oligonucleotides were used: 5′-CCT CAGGCTCCCGCCCTGTAAGATT, 5′-CTGTAAGATTCCCGTCGGGCCT TCG, and 5′-AAGATTCCCGTCGGGCCTTCGCTCG. The last oligonucleotide was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP as described above, and radioactive elongated DNA was fractionated on a 6% polyacrylamide sequencing gel.

Flow cytometry and cell cycle analysis. Quantitative fluorescence analysis was performed with a fluorescence-activated cell sorter (FACS) Ventage (Becton Dickinson, Mountain View, Calif.) equipped with an argon ion 90.3 laser (Coherent, Palo Alto, Calif.) tuned at 488 nm (250 mW). Cells were treated for 2 min at room temperature with 1 μl of DMS per ml and then with 2% β-mercaptoethanol prior to cell sorting. The cells were stained by propidium iodide (65 μg/ml) after permeabilization in PBS containing 0.1% Triton X-100. Each selected population (1×10^6 to 2×10^6 cells) was reanalyzed with a FACScan (Becton Dickinson) to evaluate its purity (92 to 99% within the gated region), and the DNA was processed as described above.

Gel mobility shifts. The following double-stranded oligonucleotides corresponding, respectively, to the Sp1 and E2F consensus sites, as well as the cyclin A cyclic AMP (cAMP)-responsive element (CRE), the NF-Y, and the CCRE sites, were used: SP1, 5′-ATTCGATCGGGGCGGGGCGAGC; E2F, 5′-ATTT AAGTTTCGCGCCCTTTCTCAA; CRE, 5′-GGGTCGCCTTGAATGACGTC AAGGCCGCGA; NF-Y, 5′-GAGCGCTTTCATTGGTCCATTT; CCRE, 5′-G GGATTTCAATAGTCGCGGGCTACTTGAA.

When indicated, the following mutated oligonucleotides were also used: mE2F, 5′-ATTTAAGTTTCGATCCCTTTCTCAA; mCRE, 5′-GGGTCGCCT TCAACGACCTCGAGGCCGCGA; mNF-Y, 5′-GAGCGCTTGAATTCGTC CATTT; mCCRE, 5′-GGGATTTCAATAGTCGAATTCTACTTGAA. The annealed oligonucleotides described above were either end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) or were labeled by filling in of the recessive ends with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham). Nuclear proteins were extracted by a 0.45 M KCl salt washing of isolated nuclei, dialyzed against 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–20% (vol/vol) glycerol–0.1 M KCl–0.2 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride–0.5 mM dithiothreitol, and used in the presence of either 1 μg of poly(dI-dC) (CRE and NF-Y) or dA-dT (E2F and CCRE) and radioactive probe (0.1 to 1 ng) in 20 μl of the same buffer. The complexes were resolved in a 6% polyacrylamide (acrylamide-bisacrylamide, 29:1) gel run in 0.25× Tris-borate-EDTA (TBE) at 4°C. Anti-NF-YA and B antibodies were generous gifts from C. Benoist and D. Mathis. All other antibodies were from Santa Cruz Biotechnology and were as follows: SP1, rabbit polyclonal immunoglobulin G (IgG) PEP2; ATF-1, mouse monoclonal IgA C41-5.1; CREB-1, rabbit polyclonal IgG C-21; CREM-1, rabbit polyclonal IgG X-12; E2F-1, rabbit polyclonal IgG C-20; E2F-4, rabbit polyclonal IgG C-108; E2F-5, mouse monoclonal IgG MH-5. Human recombinant DP-1, E2F-1, -4, and -5 were synthesized in vitro in the rabbit reticulocyte lysate (Promega), starting from the appropriate cDNA vectors, and were generous gifts from C. Sardet.

RESULTS

Isolation of the mouse cyclin A promoter. A mouse genomic library was screened with a full-length human cyclin A cDNA probe (68). Positive clones were selected for further study by hybridization at low stringency with probes derived from the 5′ flanking sequence of the human gene (25). A phage clone characterized during this analysis contained a 15-kb insert whose sequence revealed regions of similarity leading to its identification as a genomic copy of the mouse cyclin A gene (data not shown). Sequencing upstream of the 5′-most region of homology with the human cDNA sequence revealed the presence of a highly conserved nucleotide stretch (underlined in Fig. 1A). Like its human counterpart, the murine sequence showed no canonical TATA box. To map precisely the transcription initiation site(s), an RNase protection experiment was carried out on total RNA extracted from exponentially growing NIH 3T3 cells. At variance with the highly heterogeneous initiation described for the human promoter (25), three major initiation sites and a fourth, weaker initiation site were clearly visible for the mouse promoter (Fig. 1B). The positions were confirmed by primer extension with a set of two internal oligonucleotides (data not shown).

A closer inspection of the DNA sequence within the conserved fragment pointed to the existence of three motifs, which were previously described for the human promoter: CRE, an NF-1/NF-Y site, and a GC-rich E2F/SP1-like sequence, which are exactly conserved in the mouse promoter (Fig. 1C). Surprisingly, these elements are positioned downstream from the first three initiation sites.

An *Eco*RI-*Pst*I DNA fragment spanning nucleotides −795 to +100 relative to the 3′-most initiation site was linked to the firefly luciferase gene to check if the isolated putative promoter-proximal fragment was functional after transient transfection into NIH 3T3 cells. As shown in Fig. 2, only very low levels of luciferase activity were detected in cycling cells transfected with the promoter-less pGL2-Basic vector. In contrast, the fragment from −795 to +100 fragment gave rise to a 280-fold stimulation of luciferase activity when it was inserted in the sense orientation. The same fragment inserted in the antisense orientation was totally inactive in the same test. A shorter construct (nucleotides −177 to +100), which was derived by recutting the former DNA fragment with *Bam*HI, elicited an even stronger activation (1,170-fold). However, reduction down to +20 almost completely abolished promoter activity, indicating the presence of important elements controlling cyclin A transcription within the DNA fragment from nucleotides −177 to +20.

An in vivo footprint is present on the GC-rich motif only in quiescent cells. To analyze in more detail the mechanism involved in the serum inducibility of cyclin A gene transcription, we performed an in vivo footprint analysis of the region containing the conserved upstream promoter sequence. Cells were rendered quiescent by serum starvation and were then restimulated with fresh serum. At each indicated time of treatment, cells were exposed to DMS and genomic DNA was processed for the detection of hypomethylated guanine residues as described elsewhere (6, 21). A clear protection was visible on both the CRE and the GC-rich (CCRE [see below]) motifs in quiescent cells, together with a weaker but significant protection of the NF-1/NF-Y site (Fig. 3, left panel). However, whereas the CRE and the NF-1/NF-Y sites were protected

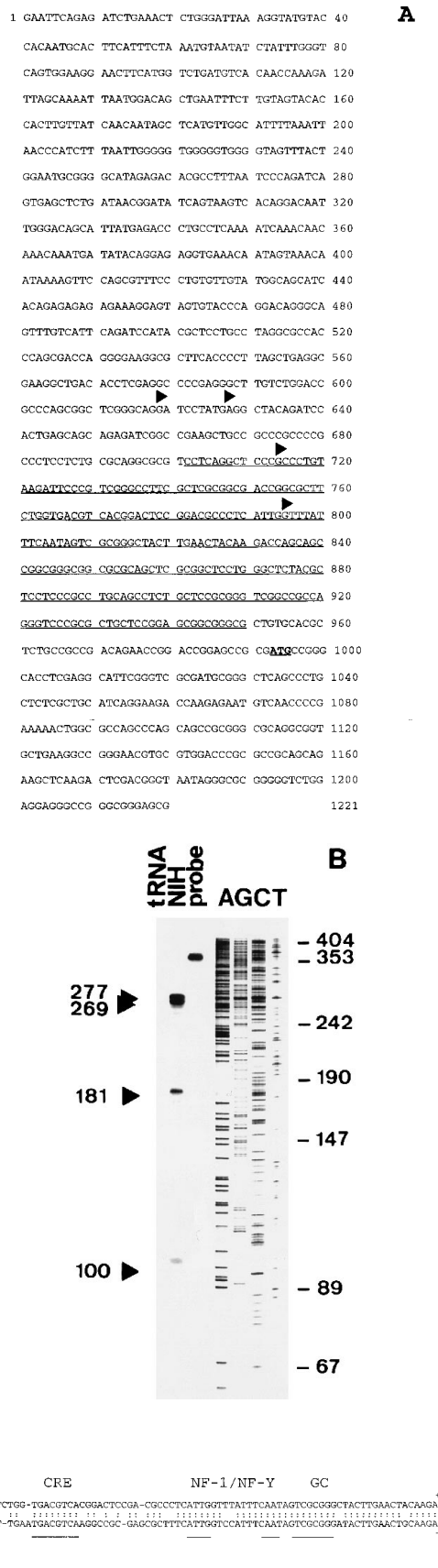

FIG. 1. Characterization of the mouse cyclin A promoter. (A) DNA sequence of the mouse cyclin A 5′ flanking region. A region conserved between mice and humans is underlined, and the ATG translational initiation site is indicated by boldface characters. Arrows indicate the major mapped transcription initiation sites. (B) Determination of transcription initiation sites by RNase protection with a ^{32}P-labeled, 377-nucleotide-long antisense RNA probe. Numbers on the left, sizes of the protected RNA fragments indicated by arrows; numbers on the right, lengths of *Hpa*II-cut pUC-labeled DNA fragments run in the same gel, together with a sequence ladder obtained after sequencing of the region including the putative initiation sites. The probe was degraded after hybridization to either 50 μg of tRNA (tRNA) or 50 μg of RNA extracted from cycling NIH 3T3 cells (NIH) or was incubated alone (probe). Protected fragments were separated on a standard 5% polyacrylamide sequencing gel and autoradiographed as indicated. (C) Comparison of the sequences of mouse and human 5′-flanking DNA regions. The numbers for the human sequence refer to those used by Henglein et al. (25). Sequences for a CRE and a putative NF-1/NF-Y site and a GC-rich conserved sequence are underlined.

whatever the time point selected for the stimulated cells, the GC-rich motif became progressively exposed as cells progressed toward S phase. The footprint was no longer visible in exponentially growing cells.

The good conservation of the sequence between the human and mouse promoters prompted us to carry out the same type of analysis on normal human fibroblasts which were growth arrested either by serum starvation or by treatment with transforming growth factor β1 (TGF-β1). TGF-β1 belongs to a family of cytokines with multifunctional properties as diverse as the control of cell proliferation, cell differentiation, or cell adhesion and migration (for reviews, see references 1, 33, 42, 54). It inhibits the proliferation of many cell lines by negatively regulating progression through the G_1 phase of the cell cycle

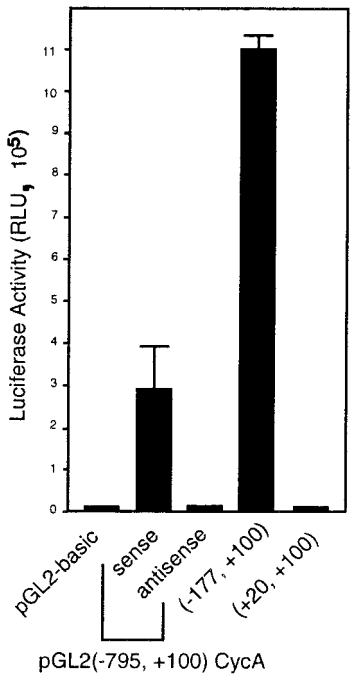

FIG. 2. Promoter activity directed by mouse cyclin A genomic DNA fragments. Growing NIH 3T3 cells were transfected as indicated, and luciferase activity was monitored 24 h later. An *Eco*RI-*Pst*I (−795 to +100) genomic fragment was inserted in both sense and antisense orientations into the pGL2-Basic promoterless vector [pGL2(−795,+100) CycA]. (−177,+100) and (+20,+100), constructs in which deletions were introduced starting from the 5′ side of the initial (−795 to +100) insert cloned in the sense orientation. Values are the averages of three independent experiments, and standard deviations are shown. RLU, relative luciferase units (normalized for β-galactosidase activity).

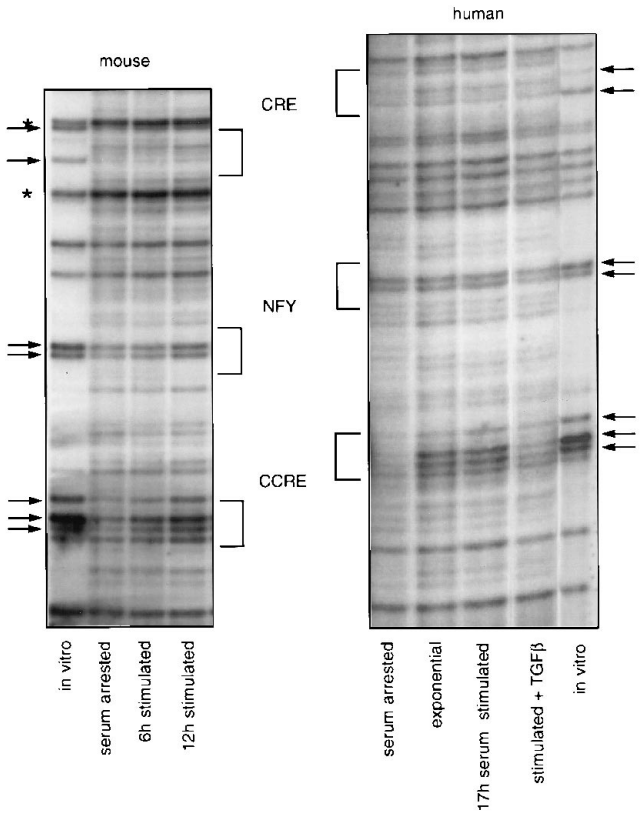

FIG. 3. In vivo DMS footprinting of the conserved region present in mouse and human cyclin A promoters. (Left panel) Serum-starved, growth-arrested NIH 3T3 cells restimulated by the addition of 10% calf serum. After exposure of cells to DMS at the indicated times, methylated DNA was analyzed as indicated in Materials and Methods. in vitro, naked DNA methylated in vitro. The sequence of the GC-rich element (CCRE [see below]) and the positions of the putative NF-Y and CRE DNA-binding sites are indicated by brackets on the right side of the sequence ladders. (Right panel) The same type of analysis was performed on human primary fibroblasts that were either serum starved (serum arrested), exponentially growing (exponential), or serum restimulated either alone (17 h serum stimulated) or in the presence of 10 ng of TGF-β per ml (stimulated + TGF-β). Arrows, protected G sites; asterisks, hypersensitive sites. Note that the amount of in vitro-methylated DNA is slightly less in the right panel, so that the protection appears weaker for this particular set of experiments.

(11, 42, 54). A specific protection was observed for the GC-rich motif in quiescent cells, while no protection of this site was detectable after 17 h of serum refeeding the same cells or in normally cycling cells (Fig. 3, right panel). In contrast, the CRE and NF-1/NF-Y sites were always found protected in quiescent, stimulated, and normally cycling cells, in agreement with our previous observation (6).

To rule out any artifacts arising from the starvation-based synchronization procedure, we repeated this analysis on cells selected by preparative cell sorting of an exponentially growing cell population. While the preliminary experiments were carried out on NIH 3T3 cells, we finally selected mouse Friend erythroleukemic cells, which grow in suspension, in order to avoid any artifacts potentially generated by the trypsinization step. The results obtained with cells which were, respectively, in the G_0, G_1, S, and G_2/M phases of the cell division cycle are shown in Fig. 4. A maximal protection of the GC-rich motif was observed only for cells in G_0 on both the sense and antisense strands. Interestingly, in the latter case, a strong hypersensitive site was also present next to the GC-rich motif only in quiescent cells and vanished progressively when the cells reached S phase. To get a better estimate of the period during which the footprint present on the GC-rich element is released when cycling cells transit through the G_1 phase of the cell cycle, cells were blocked with demecolcine at the G_2/M border. After a 9.5-h treatment with 100 nM demecolcine, Friend cells were extensively washed and their progression through the cell cycle was monitored by FACS analysis (Fig. 5). Cells were processed for genomic footprinting at various time intervals, and the protected bands within the GC-rich sequence were submitted to a densitometric analysis. The quantitative result, integrating 13 different experiments, is shown in Fig. 5 for the band labeled A in Fig. 4. The footprint is prominent in early G_1 (1.5 h post-demecolcine block) and is released in mid-G_1 (4 h post-demecolcine block). Another advantage in using Friend cells is that as erythroid precursor cells, they can undergo upon addition of inducers such as DMSO, a differentiation program closely resembling the final stage of normal erythrocytes (43). When Friend cells were treated with 1.5% (vol/vol) DMSO, their growth rate was not affected for at least 4 days and then reached a plateau after 5 days of culture in the presence of the inducer (data not shown [43]). In a parallel way, a large proportion of the cells differentiated, as revealed by the increase in hemoglobin content, which was measured by benzimidine staining (Fig. 6). As a consequence of the DMSO-induced cell growth arrest, the footprint on the GC-rich element, which was absent in growing cells, built up progressively as cells entered a differentiated, quiescent state.

These data are consistent with the existence of a negative factor mediating the inhibition of the cyclin A promoter in quiescent and early G_1 cells through its binding to the upstream GC-rich motif.

The cyclin A promoter is under negative control in quiescent cells. Previous work carried out on the human promoter had shown that the late G_1/S accumulation of cyclin A mRNA upon release from G_0 is the consequence of transcriptional activation of the gene (6, 25). To delineate sequences important for cell cycle control of cyclin A expression, we have used promoter-luciferase gene fusions in transient transfection assays carried out in mouse NIH 3T3 growth-arrested cells. Because both the fragments from −795 to +100 and from −177 to +100 gave rise to the same induction kinetics upon serum addition (data not shown), the shorter fragment was selected for the subsequent part of this analysis. We have already shown that the cyclin A promoter is down-regulated by TGF-β1 and cAMP in human and hamster fibroblasts through the CRE. However, a CRE mutant was still able to respond, albeit with a lower efficiency, to serum stimulation (5, 6). This observation, in conjunction with the in vivo footprint data reported above, suggests that the region next to the CRE, and likely the GC-rich motif, could be the CCRE. To resolve this issue, NIH 3T3 cells grown for 36 h in the presence of 0.5% calf serum were transfected with vectors containing either a wild-type (pGL2-CycA) promoter or a mutated (pGL2-CycAm) promoter, in which the GCGGGG had been changed into GAATTC. After exposure to 10% serum, luciferase activities were monitored for the indicated times and were normalized to β-galactosidase expressed from a cell cycle-insensitive promoter. The entry of stimulated cells into S phase was monitored by [^{3}H]thymidine incorporation in parallel plates (Fig. 7A). For the wild-type construct, luciferase activity began to increase significantly 10 h after refeeding the cells with serum, with a maximum of 18- to 20-fold stimulation at 12 h (Fig. 7B). In contrast, an almost constitutive activity, with variations which remained within a factor of 2, was observed for the mutant promoter (Fig. 7C). Moreover, luciferase activity directed by the mutant promoter was approximately 10-fold

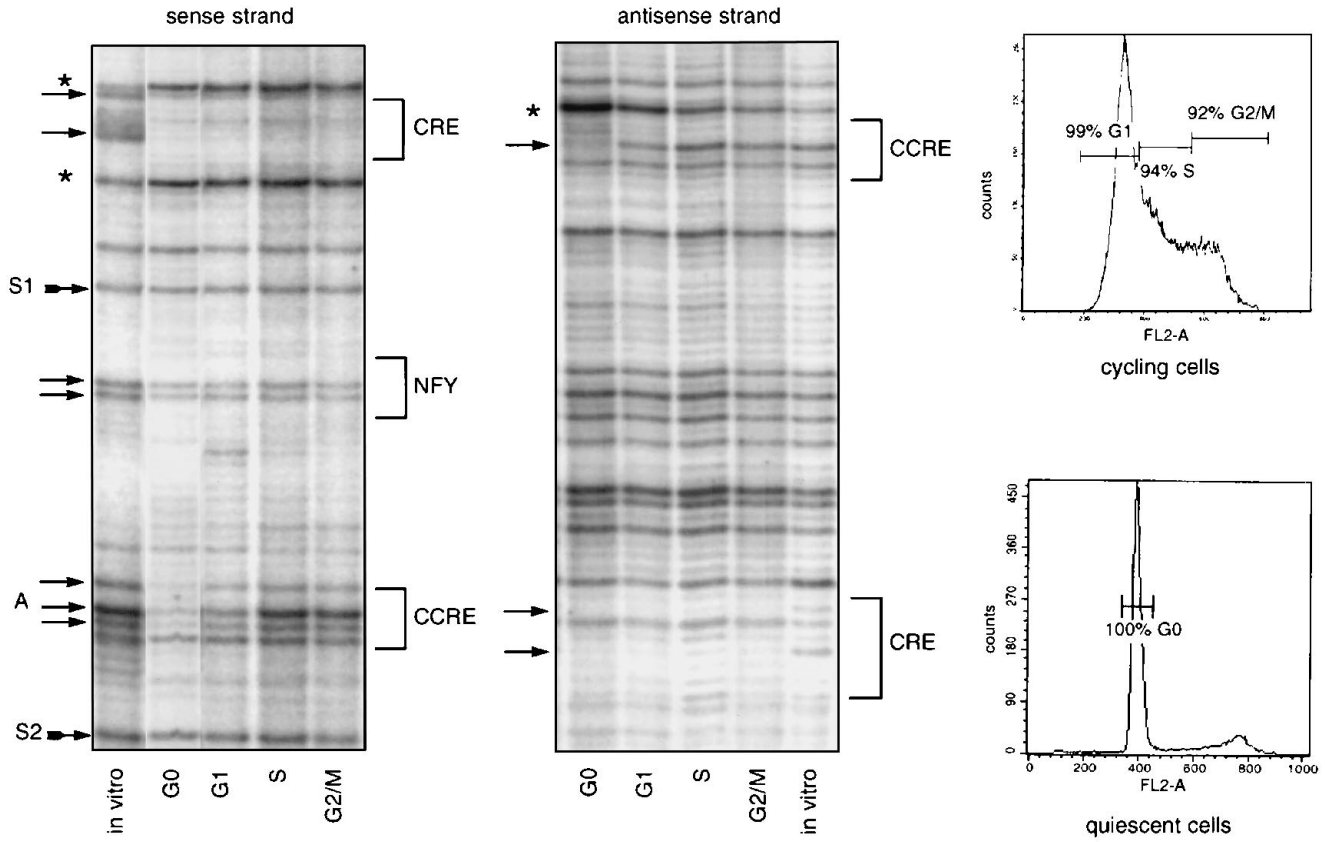

FIG. 4. Identification of in vivo protein-binding sites in exponentially growing and density-arrested Friend mouse cells isolated by preparative cell sorting. FACS analyses of both populations of Friend cells were done before and after cell sorting. The results are plotted at the right. Numbers refer to the proportions of each cell subpopulation in the indicated phases of the cell cycle, after cell sorting. In vivo DMS footprinting analyses for both the sense and the antisense strands were carried out as indicated in the legend to Fig. 3. Bands taken either as internal controls (S1 and S2) or as an indicator of site occupancy (A) in the quantitative analysis shown in Fig. 5 are indicated.

higher than that observed for the wild-type promoter (Fig. 7D). Mutation of the putative NF-1/NF-Y site located 5′ relative to the GC-rich element did not abolish the response to serum and only mildly affected its amplitude (data not shown). We will use the abbreviation CCRE to describe the GC-rich site in the rest of this work.

The cyclin A CCRE functions as an inhibitory element in a heterologous promoter. The absence of variation upon release from quiescence after serum refeeding, as well as the 10-fold-higher transcriptional activity supported by the mutated promoter, prompted us to test the inhibitory effect of the CCRE on a heterologous promoter. To this end, we inserted an oligonucleotide containing the sequence TAGT**CGCGGG**ATAC into the *Mlu*I and *Xho*I sites of pGL3-Promoter vector (Promega), in which the luciferase gene is transcribed from an early SV40 promoter. The SV40 promoter is not cell cycle regulated and was stimulated to a modest level upon serum addition (1.5-2 fold; Fig. 8A). Introduction of the CCRE sequence resulted in a dramatic decrease in promoter activity (8- to 10-fold), confirming its inhibitory effect. However, the observed inhibition was no longer cell cycle dependent, suggesting that other elements participate in the cell cycle activity of the cyclin A CCRE. In order to further analyze the contribution of sequences surrounding the CCRE to its activity, we inserted the *Hae*III-*Nae*I fragment spanning nucleotides −60 to +45, in both orientations, into the *Sma*I site of pGL3-Promoter. In the antisense orientation, the fragment from −60 to +45 did not give rise to a strong inhibition of the SV40 promoter, and it did not restore a cell cycle-dependent behavior (Fig. 8B). However, the same fragment in the sense orientation led to a 7- to 10-fold reduction of promoter activity in quiescent cells (data not shown) and a 6- to 8-fold induction upon serum refeeding (Fig. 8B). The same DNA fragment containing a mutated CCRE was ineffective in restoring the cell cycle-regulated phenotype.

Proteins binding in vitro to the various elements present within the cyclin A promoter. Nuclear extracts were prepared from quiescent NIH 3T3 cells and NIH 3T3 cells that were serum refed for 16 h and were used in mobility shift assays carried out with ^{32}P-labeled, double-stranded oligonucleotides containing either the CRE, the NF-1/NF-Y, or the cyclin A CCRE motifs.

It has previously been shown that the cyclin A CRE was able to bind equally well in vitro synthesized CREB and ATF-1 proteins (6, 16). We show here that in the presence of a specific anti-ATF-1 antibody, specific complexes formed by nuclear extracts prepared from quiescent NIH 3T3 cells can be almost completely inhibited, together with the appearance of a faint supershift (Fig. 9, left panel). The same result was obtained with extracts from serum-restimulated cells (data not shown). Anti-CREB-1 or anti-CREM antibodies were ineffective in reproducibly displacing the same complexes, thus suggesting that ATF-1 is a major effector for this element.

As far as the NF-1/NF-Y element is concerned, it turned out

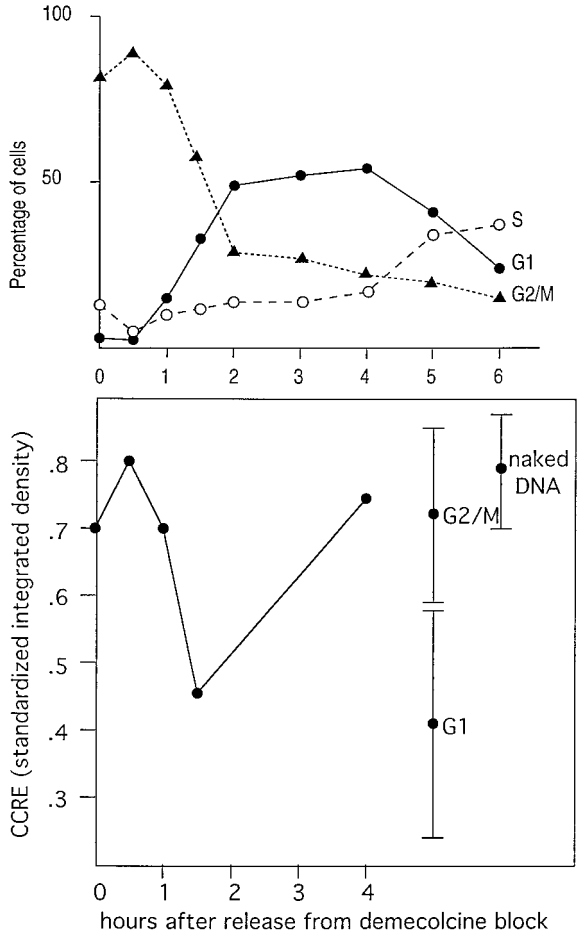

FIG. 5. Differential occupancy of the GC-rich site in early versus late G_1 phase of the cell cycle. Friend cells were blocked prior to mitosis by a 9.5-h treatment with 100 nM demecolcine and were then released by extensive washing. Progression in the cell cycle was monitored by FACS analysis (upper panel). Cells were methylated with DMS at the indicated times after release and were sorted according to their DNA contents to focus on the G_1 population. The cells were then processed for genomic footprinting as described in Materials and Methods. The autoradiograms were scanned for densitometry, and the integrated intensities of the bands labeled A in Fig. 4 were normalized for each point by using the integrated densities of bands S1 and S2 surrounding the GC-rich site and taken as internal standards. These values which were plotted in the lower panel, can be compared with the means and 95% confidence limits obtained for 3 to 13 different ligation-mediated PCRs carried out on naked DNA as well as for the DNAs prepared from G_1 and G_2/M cells.

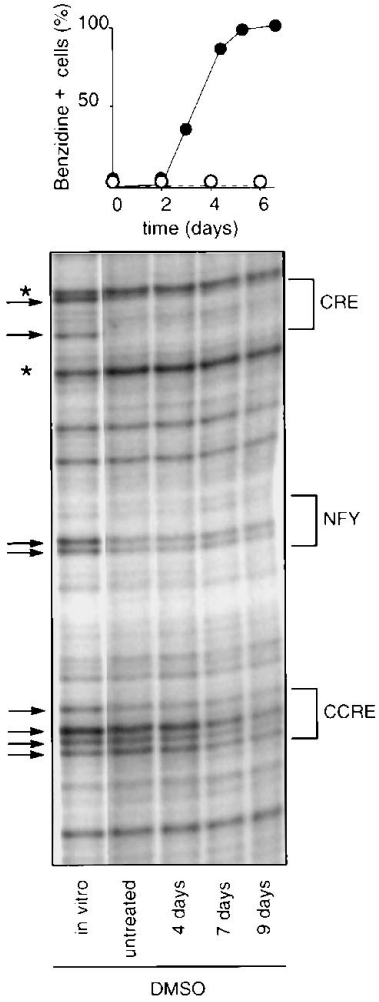

FIG. 6. Differential occupancy of the GC-rich site in proliferating versus differentiating Friend cells. Cells were exposed to 1.5% (vol/vol) DMSO, and the percentages of hemoglobin-producing cells, counted as benzidine positive (plotted at the top), were evaluated daily (●) and were compared with those of cells grown without the inducer (○). Cells were harvested at the indicated times, treated with DMS, and processed for genomic footprinting as described in the legend to Fig. 4.

to be an in vitro high-affinity binding site for NF-Y. The latter protein belongs to a family of CCAAT box-binding proteins involved in the expression of diverse eukaryotic genes. It consists of two subunits of 40 to 43 kDa (Y_A) and 32 kDa (Y_B), respectively (28). Antibodies directed against both the A and the B subunits were able to supershift the specific complex formed in vitro with the cyclin A NF-1/NF-Y element and extracts from both quiescent (Fig. 9, right panel) and serum-stimulated NIH 3T3 cells (data not shown).

Several DNA protein complexes were clearly visible with the cyclin A CCRE motif and extracts prepared from both cycling and quiescent cells. They were competed out by a 100-fold molar excess of the nonradioactive probe (Fig. 10), and no competition was observed with the mutated oligonucleotide. Complexes with higher mobility were seen with extracts from S-phase-enriched cells (Fig. 10, lanes 1 to 3). Because of its GC richness, the binding of the cyclin A probe to purified Sp1 (generous gift from J. Imbert) was assayed and compared with that of an oligonucleotide containing SV40 Sp1 motifs. Whereas a clearly detectable set of retarded complexes was observed with the SV40 probe, the cyclin A probe did not give rise to appreciable binding (data not shown). This result was confirmed by the use of specific anti-SP1 antibodies. They prevented almost completely the formation of complexes with the Sp1 probe, while leaving complexes formed on the cyclin A probe unaffected (Fig. 10).

The cyclin A CCRE contains within its core the motif TCGCG which is present within the consensus E2F sequence. E2F proteins are an expanding family of transcription factors which form heterodimeric complexes with members of the DRTF1/DP-1 family (30, 35, 55). The E2F/DP complex is functionally regulated by its phosphorylation and by the binding to one or more of the structurally related Rb, p107, and p130 proteins (for reviews, see references 24, 27, 35, 45, 69). E2F-1, E2F-2, and E2F-3 form complexes with Rb, whereas E2F-4 and E2F-5 specifically bind to p107 and p130 (for a

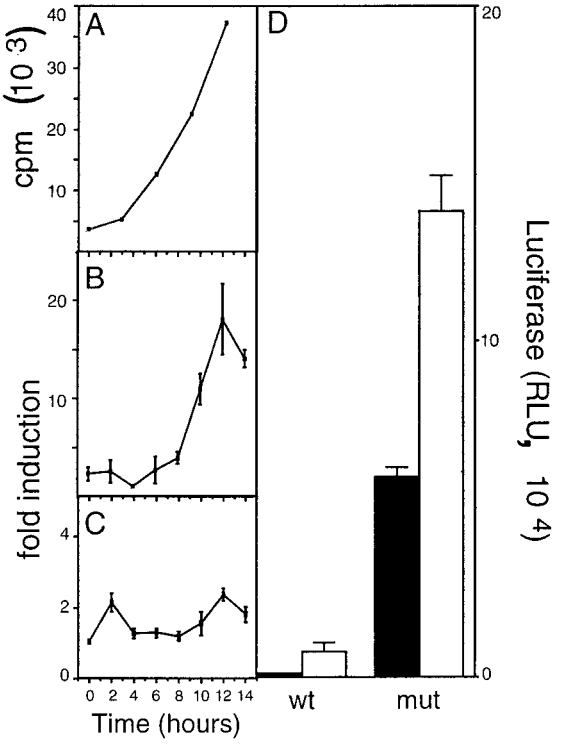

FIG. 7. A GC-rich sequence confers cell cycle regulation on the cyclin A promoter. (A) Serum-starved NIH 3T3 cells were restimulated with serum and labeled with [^{3}H]thymidine for 15 min at the indicated times, and trichloroacetic-precipitable radioactivity was monitored. (B and C) Relative luciferase activities in extracts from cells transfected with either the pGL2-CycA or the pGL2-CycAm construct containing, respectively, the wild-type and the mutated promoters. Transfections were carried out prior to serum stimulation, and fold inductions were plotted relative to luciferase activities observed for quiescent cells. The results are the averages of three experiments, and standard deviations are given. (D) Absolute luciferase activities obtained in a representative experiment, showing the increase of promoter activity resulting from the mutation of the GC-rich element. Luciferase activities were measured for quiescent cells (filled rectangles) and for cells that were serum stimulated for 24 h (open rectangles) and transfected with either pGL2-CycA (wt) or pGL2-CycAm (mut). RLU, relative luciferase units.

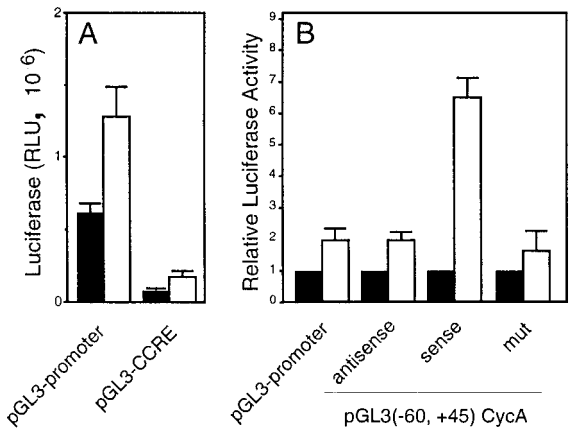

FIG. 8. Repression of early SV40 promoter activity by the cyclin A CCRE. (A) Quiescent cells were transfected with either the pGL3-Promoter, which contains an early SV40 promoter devoid of enhancer, or with the same vector containing the cyclin A CCRE (pGL3-CCRE). Luciferase activities were measured for extracts prepared from either quiescent cells (filled rectangles) or cells that were serum refed for 24 h (open rectangles). (B) Cells processed as described for panel A, but with transfections carried out with the pGL3-Promoter vector containing the fragment from nucleotides −60 to +45) [pGL3(−60,+45) CycA] in either orientation. mut, the fragment from −60 to +45 harboring a mutated CRE and inserted in the sense orientation. Luciferase activities relative to those observed with each vector in quiescent cells are shown. The values are the averages of three independent experiments, and standard deviations are shown. RLU, relative luciferase units.

review, see reference 45). A current model proposes that each of these pocket proteins interacts with the various E2Fs in defined periods of the cell cycle.

Anti-E2F-1, -4, and -5 antibodies were thus used successively without any reproducible effect on the mobilities of the various complexes, regardless of whether the extracts were prepared from quiescent cells (Fig. 10; only the result with anti-E2F-1 is shown) or from serum-stimulated cells (data not shown). Conversely, the same antibodies were able to recognize their cognate in vitro-synthesized E2F proteins (data not shown), and each displaced a specific subset of the complexes formed by a consensus E2F oligonucleotide (Fig. 10). Moreover, a 100-fold molar excess of the consensus E2F oligonucleotide failed to displace the complexes from the cyclin A CCRE. Finally, mobility shift assays were carried out with recombinant proteins synthesized in vitro in the rabbit reticulocyte lysate. No binding on the cyclin A probe was observed, whereas DP-1 in combination with E2F-1, -4, or -5 gave rise to specific retarded bands with the consensus E2F probe (Fig. 11).

DISCUSSION

Numerous studies have shown that cyclin A mRNA accumulation is cell cycle regulated by a transcriptional activation of its gene in the late G_1 phase of the cell division cycle (6, 25). Therefore, we have undertaken the analysis of sequences and factors involved in the control of the expression of both the human and mouse cyclin A genes. In this study, by a combination of RNase protection experiments and transient transfections of luciferase reporters in NIH 3T3 cells, we have

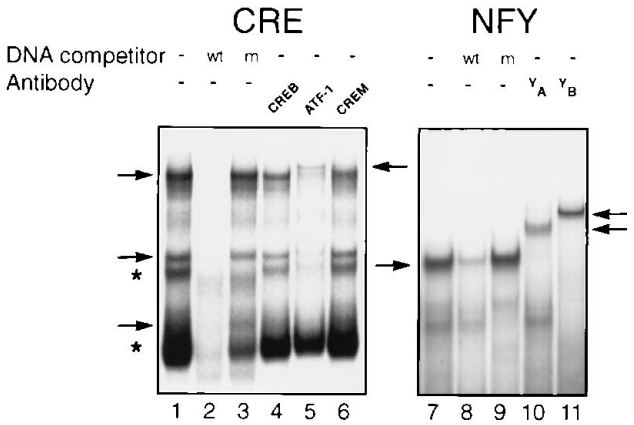

FIG. 9. Protein complexes formed in vitro with oligonucleotides containing the cyclin A CRE or NF-1/NF-Y sites. Gel shifts were carried out with cyclin A CRE (CRE) or NF-1/NF-Y (NFY) oligonucleotides labeled by end filling. Complexes formed with 5 μg of nuclear extracts were resolved in 5% polyacrylamide gels as described in Materials and Methods. The gels were fixed in an acetic acid-ethanol-water mixture (1:1:8), dried, and then exposed for autoradiography. Only retarded bands are shown. wt and m, 100-fold molar excess of, respectively, wild-type and mutated unlabeled corresponding oligonucleotides added in the reaction mixture (2, 3, 8, 9). When indicated, specific antibodies directed against CREB (4), ATF-1 (5), CREM (6), Y_A (10), and Y_B (11) subunits of NF-Y were added 1 h prior to the addition of the probe. Arrows, specific complexes (on the left) and supershifted bands (on the right); asterisk, unspecific complexes. Probes incubated alone or in the presence of antibodies did not produce retarded bands (data not shown).

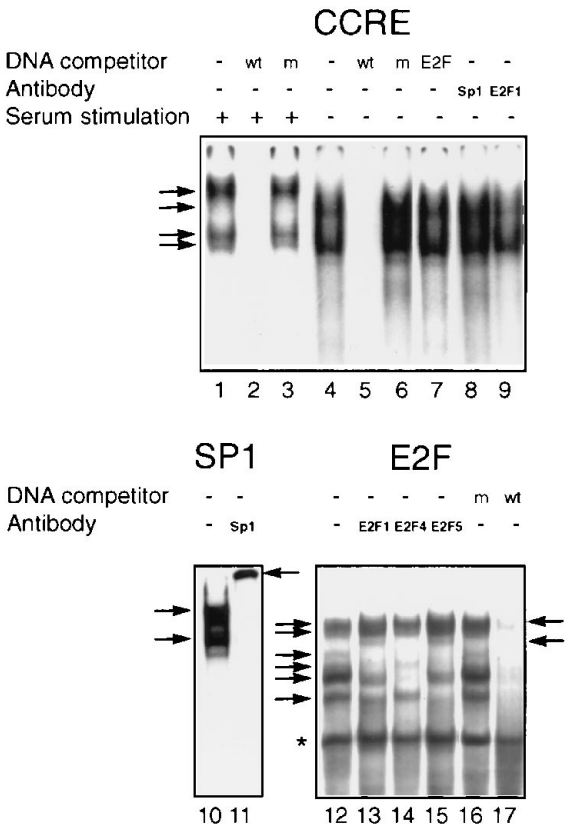

FIG. 10. Protein complexes formed in vitro with the cyclin A CCRE or control SP1 and E2F oligonucleotides. Gel shifts were carried out with cyclin A CCRE or Sp1 and E2F consensus oligonucleotides as indicated in the legend to Fig. 9. Nuclear extracts were prepared from either 18-h serum-stimulated cells (lanes 1 to 3) or quiescent NIH 3T3 cells (lanes 4 to 17). As controls, the probes were incubated in the presence of a 100-fold molar excess of an unlabeled corresponding oligonucleotide harboring a wild-type (wt) (lanes 2, 5, and 17) or a mutated (m) sequence (lanes 3, 6, and 16). In the case of the CCRE, a competition assay with the E2F oligonucleotide was also performed (E2F) (lane 7). When indicated, extracts were incubated with antibodies directed against Sp1 (lanes 8 and 11), E2F-1 (lanes 7 and 13), E2F-4 (lane 14), or E2F-5 (lane 15), which were added at room temperature 1 h prior to electrophoresis. Symbols are as described in the legend to Fig. 9.

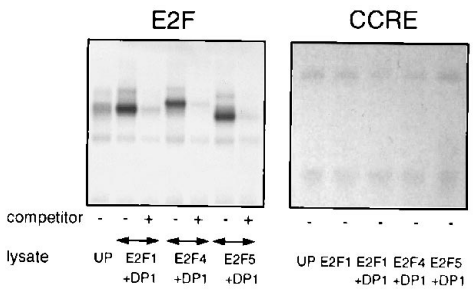

FIG. 11. Comparative binding of in vitro-synthesized E2Fs and DP-1 proteins to the CCRE and E2F oligonucleotides. The consensus E2F and CCRE radioactive probes were incubated with proteins synthesized in the rabbit reticulocyte lysate as indicated. UP, stands for unprogrammed lysate. The specificities of the complexes formed with the E2F probe were assessed by a competition assay with a 100-fold molar excess of unlabeled E2F oligonucleotide (+). Separation of the complexes was as described in the legend to Fig. 9.

characterized the structure of the mouse cyclin A gene. Comparison of the sequences of the human and mouse promoters revealed the presence of a region of striking homology containing a CRE, a NF-Y-binding site, and a GC-rich motif. Our previous work on cyclin A down-regulation by TGF-β had pointed to the CRE as an important mediator of the inhibitory effect of the cytokine (5, 6). However, a promoter which was mutated on the CRE was still able to respond to serum stimulation, albeit at a lower efficiency. A close examination by DMS footprinting of the pattern of protein binding in vivo to this region revealed the presence of complexes in the vicinity of the major transcription initiation sites. Whereas the CRE and the NF-Y sites were occupied throughout the cell cycle, the occupancy of the GC-rich site was specifically observed in quiescent as well as early G_1 cells, when cyclin A gene is transcriptionally silent. Moreover, the same result was obtained in normal human fibroblasts rendered quiescent either by serum starvation or by exposure to TGF-β, which is known to arrest cells in G_1. This raised the possibility of the binding of a repressor type of complex to this region. This prompted us to mutate the conserved GC-rich motif and assess the serum responsiveness of the resulting promoter in quiescent cells restimulated with serum. Mutation of the GC-rich sequence led to a 10-fold increase in reporter activity and almost totally impaired its cell cycle-dependent activation, thus confirming its negative regulatory role. We then transferred the CCRE into the early SV40 enhancerless promoter, which is not naturally cell cycle responsive. Whereas the CCRE alone decreased dramatically the efficiency of the SV40 promoter, it did not endow it with a cell cycle-dependent transcriptional activity. However, the addition of surrounding sequences, spanning the entire conserved region, restored the expected behavior. This result thus suggests a cooperation between neighboring sequences and the CCRE which, in their absence, may function only as a constitutive negative regulatory element. The presence of a constitutive in vivo footprint on both the CRE and the NF-Y sites nearby points to their possible involvement in modulating the CCRE activity. Interestingly, several recent reports have stressed the importance of the CRE in mediating cyclin A gene expression in human Hs27 (16) or hamster CCL39 fibroblasts (6), as well as in bovine aortic endothelial cells (72). Despite the presence of serum, TGF-β1 treatment of CCL39 cells (5, 6) or contact inhibition of bovine aortic endothelial cells (72) led to a down-regulation of cyclin A, which in both cases was mediated by the CRE. This opens an interesting possibility of cooperation between these different motifs, whereby one of the elements (the CRE?) would integrate signals generated at the cell periphery (cell anchorage, contact inhibition, TGF-β, etc.) and the other one (the CCRE?) would control a proper cell cycle-dependent expression of cyclin A.

The data presented in this paper are reminiscent of previous studies conducted with the promoters of the human *cdc2* (14, 64), *E2F-1* (44), and *cdc25C* (40) genes, as well as with the mouse B-*myb* (34) gene. All of these genes are expressed either during the G_1/S or the G_2/M transition of the cell division cycle. Their promoters have also been reported to be negatively regulated in G_0/G_1 through GC-rich motifs. The involvement of the E2F family of transcription factors has been strongly substantiated for the regulation of *cdc2*, *E2F-1*, and B-*myb* genes. However, its participation in the control of cdc25C transcription appears unlikely, since biochemically purified E2F did not show any in vitro affinity for the cdc25C regulatory element (40). The same situation seems to hold in the case of the cyclin A promoter. Antibodies directed against E2F-1, -4, or -5 did not prevent proteins from binding in vitro to a CCRE probe when reacted with nuclear extracts from

either quiescent or serum-stimulated cells. Whereas proteins binding in vitro to the upstream elements have unambiguously been identified as, respectively, ATF-1 and NF-Y, we have not been able to identify those binding in vitro to the cyclin A CCRE.

After completion of this work, two reports (57, 75) reached the same conclusion about the role of the CCRE in controlling the cell cycle behavior of the cyclin A promoter. However, they disagreed about the nature of the protein(s) binding in vitro to this sequence. Therefore, this leaves open the possibility of the presence of either a distantly related member of the E2F family, harboring a very low in vitro affinity for the cyclin A CCRE, or another factor that has not yet been identified. Interestingly, in support of the first hypothesis, cyclin A mRNA accumulation is greatly enhanced in adenovirus E1A-transformed rodent cell lines (7). Overexpression of viral proteins such as adenovirus E1A or SV40 T, which are known to complex RB-like proteins, releases E2F from this inhibitory interaction (12, 29, 41, 58, 73, 74). Moreover, a recent report based on the use of a recombinant adenovirus expressing E2F-1 points to cyclin A as one of the putative cellular targets of this transcription factor (15). Should members of the E2F family be involved either directly or indirectly in the regulation of cyclin A expression, this would provide the cell with an interesting feedback loop. On the one hand, E2F type factors would exchange on the cyclin A promoter as resting cells proceed through the cell cycle. As a result, an inhibitory transcriptional complex would be switched into an active one. On the other hand, cyclin A/cdk2 has been reported to bind directly to E2F-1, inhibiting the DNA-binding activity of E2F-1/DP-1 by phosphorylation.

ACKNOWLEDGMENTS

We are grateful to J. Imbert and C. Sardet for invaluable discussions as well as for their gifts of plasmids and antibodies. The antibodies directed against the NF-YA and B subunits were generous gifts from C. Benoist and D. Mathis. We thank B. Henglein for communicating his data prior to publication.

This work was funded by institutional grants from CNRS and Université Montpellier II, as well as by contracts from the Association pour la Recherche sur le Cancer, the Ligue Nationale Contre le Cancer, and Rhône-Poulenc Rorer. X.H. is supported by a fellowship from the Ligue Nationale contre le Cancer.

The first two authors contributed equally to this work.

REFERENCES

1. **Attisano, L., J. L. Wrana, F. Lopez-Casillas, and J. Massagué.** 1994. TGF-β receptors and actions. Biochem. Biophys. Acta. **1222:**71–80.
2. **Bagchi, S., R. Weinmann, and P. Raychaudhuri.** 1991. The retinoblastoma protein copurifies with E2F-1, an E1A regulated inhibitor of the transcription factor E2F. Cell **65:**1063–1072.
3. **Bandara, L. R., J. P. Adamczewski, T. Hunt, and N. LaThangue.** 1991. Cyclin A and the retinoblastoma gene product complex with a common transcription factor. Nature (London) **352:**249–251.
4. **Bandara, L. R., and N. LaThangue.** 1991. Adenovirus E1A prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature (London) **351:**494–497.
5. **Barlat, I., D. Fesquet, C. Bréchot, B. Henglein, A. Dupuy d'Angeac, A. Vié, and J. M. Blanchard.** 1993. Loss of the G1-S control of cyclin A expression during tumoral progression of Chinese hamster lung fibroblasts. Cell Growth Differ. **4:**105–113.
6. **Barlat, I., B. Henglein, A. Plet, N. Lamb, A. Fernandez, F. McKenzie, J. Pouysségur, A. Vié, and J. M. Blanchard.** 1995. TGF-β and cAMP attenuate cyclin A gene transcription via a cAMP responsive element through independent pathways. Oncogene **11:**1309–1316.
7. **Buchou, T., O. Kranenburg, H. van Dam, D. Roelen, A. Zantema, F. L. Hall, and A. van der Heb.** 1993. Increased cyclin A and decreased cyclin D levels in adenovirus 5 E1A-transformed rodent cell lines. Oncogene **8:**1765–1773.
8. **Buckley, M. F., K. J. E. Sweeney, J. A. Hamilton, R. L. Sini, D. L. Manning, R. I. Nicholson, A. DeFazio, C. K. W. Watts, E. A. Musgrove, and R. L. Sutherland.** 1993. Expression and amplification of cyclin genes in human breast cancer. Oncogene **8:**2127–2133.
9. **Cao, L., B. Faha, M. Dembski, L. H. Tsai, and E. Harlow.** 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature (London) **355:**176–179.
10. **Cardoso, M. C., H. Leonardt, and B. Nadal-Ginard.** 1993. Reversal of terminal differentiation and control of DNA replication: cyclin A and cdk2 specifically localize at subnuclear sites of DNA replication. Cell **74:**979–992.
11. **Chambard, J. C., and J. Pouysségur.** 1988. TGF-β inhibits growth factor induced DNA synthesis in hamster lung fibroblasts without affecting early mitogenic events. J. Cell. Physiol. **135:**101–107.
12. **Chittenden, T., D. Livingston, and W. G. Kaelin.** 1991. The E1A/T binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA binding protein. Cell **65:**1073–1082.
13. **Cobrinik, D., P. Whyte, D. Peeper, T. Jacks, and R. Weinberg.** 1993. Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes Dev. **7:**2392–2404.
14. **Dalton, S.** 1992. Cell cycle regulation of the human cdc2 gene. EMBO J. **11:**1797–1804.
15. **DeGregori, J., T. Kowalik, and J. R. Nevins.** 1995. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G_1/S-regulatory genes. Mol. Cell. Biol. **15:**4215–4224.
16. **Desdouets, C., G. Matesic, C. A. Molina, N. S. Foulkes, P. Sassone-Corsi, C. Brechot, and J. Sobczak.** 1995. Cell cycle regulation of cyclin A gene expression by the cyclic AMP-responsive transcription factors CREB and CREM. Mol. Cell. Biol. **15:**3301–3309.
17. **Devoto, S. H., M. Mudryj, J. Pines, T. Hunter, and J. R. Nevins.** 1992. A cyclin A-specific protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F-cyclin A complex. Cell **68:**167–176.
18. **Dorée, M.** 1990. Control of M-phase by maturation-promoting factor. Curr. Opin. Cell Biol. **2:**269–273.
19. **D'Urso, G., R. L. Marraccino, D. R. Marshak, and J. M. Roberts.** 1990. Cell cycle control of DNA replication by a homologue from human cells of the p34cdc2 protein kinase. Science **250:**786–791.
20. **Dutta, A., and B. Stillmann.** 1992. Cdc2 family kinases phosphorylate a human cell DNA replication factor, RPA, and activate DNA replication. EMBO J. **11:**2189–2199.
21. **Garrity, P. A., and B. J. Wold.** 1992. Effects of different DNA polymerases in ligation-mediated PCR: enhanced genomic sequencing and in vivo footprinting. Proc. Natl. Acad. Sci. USA **89:**1021–1025.
22. **Girard, F., U. Strausfeld, A. Fernandez, and N. J. C. Lamb.** 1991. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell **67:**1169–1179.
23. **Gong, J., B. Ardelt, F. Traganos, and Z. Darzynkiewicz.** 1994. Unscheduled expression of cyclin B1 and cyclin E in several leukemic and solid tumor lines. Cancer Res. **54:**4285–4288.
24. **Helin, K., and E. Harlow.** 1993. The retinoblastoma proteins as a transcriptional repressor. Trends Cell Biol. **3:**43–46.
25. **Henglein, B., X. Chenivesse, J. Wang, D. Eick, and C. Bréchot.** 1994. Structure and cell cycle regulated transcription of the human cyclin A gene. Proc. Natl. Acad. Sci. USA **91:**5490–5494.
26. **Hinds, P. W., S. F. Dowdy, E. N. Eaton, A. Arnold, and R. A. Weinberg.** 1994. Function of a human cyclin gene as an oncogene. Proc. Natl. Acad. Sci. USA **91:**709–713.
27. **Hinds, P. W., and R. A. Weinberg.** 1994. Tumor suppressor genes. Curr. Opin. Genet. Dev. **4:**135–141.
28. **Hooft van Huijsduijnen, R., X. Y. Li, D. Black, H. Matthes, C. Benoist, and D. Mathis.** 1990. Co-evolution from yeast to mouse: cDNA cloning of the two NF-Y (CP-1/CBF) subunits. EMBO J. **9:**3119–3127.
29. **Huang, S., E. Shin, K. A. Sheppard, L. Chokoroverty, B. Shan, Y. W. Qian, E. Y. H. P. Lee, and A. S. Yee.** 1992. The retinoblastoma protein region required for interaction with the E2F transcription factor includes the T/E1A binding and carboxy-terminal sequences. DNA Cell Biol. **11:**7802–7812.
30. **Ivey-Hoyle, M., R. Conroy, H. E. Huber, P. J. Goodhart, A. Oliff, and D. C. Heimbrook.** 1993. Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. Mol. Cell. Biol. **13:**3301–3309.
31. **Jiang, W., S. M. Kahn, P. Zhou, Y. J. Zhang, A. M. Cacace, A. S. Infante, S. Doi, R. M. Santella, I. B. Weinstein.** 1993. Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression, and gene expression. Oncogene **8:**3447–3457.
32. **Keyomarsi, K., N. O'Leary, G. Molnar, E. Lees, H. J. Fingert, and A. B. Pardee.** 1994. Cyclin E, a potential prognostic marker for breast cancer. Cancer Res. **54:**380–385.
33. **Kingsley, D. M.** 1994. The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev. **8:**133–146.
34. **Lam, E. W. F., and J. Watson.** 1993. An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. EMBO J. **12:**2705–2713.
35. **LaThangue, N.** 1994. DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell cycle control. Trends Biochem. Sci. **19:**108–114.

36. **Lerner, C. F., and P. H. O'Farrell.** 1989. Expression and function of *Drosophila* cyclin A during embryonic cell cycle progression. Cell **56:**957–968.
37. **Lerner, C. F., and P. H. O'Farrell.** 1990. The role of *Drosophila* cyclins A and B in mitotic control. Cell **61:**535–547.
38. **Lew, D. J., and L. S. Reed.** 1992. A proliferation of cyclins. Trends Cell Biol. **2:**77–80.
39. **Li, Y., C. Graham, S. Lacy, A. Duncan, and P. Whyte.** 1993. The adenovirus E1A-associated 130 kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes Dev. **7:**2366–2377.
40. **Lucibello, F. C., M. Truss, J. Zwicker, F. Ehlert, M. Beato, and R. Müller.** 1995. Periodic cdc25C transcription is mediated by a novel cell cycle regulated element (CDE). EMBO J. **14:**132–142.
41. **Ludlow, J. W., J. Shon, J. M. Pipas, D. M. Livingston, and J. A. Decaprio.** 1990. The retinoblastoma susceptibility gene product undergoes cell cycle dependent dephosphorylation and binding to and release from SV40 large T. Cell **60:**387–396.
42. **Massagué, J.** 1990. The transforming factor beta family. Annu. Rev. Cell Biol. **6:**597–641.
43. **Mechti, N., M. Piechaczyk, J. M. Blanchard, L. Marty, A. Bonnieu, P. Jeanteur, and B. Lebleu.** 1986. Transcriptional and post-transcriptional regulation of c-myc expression during the differentiation of murine erythroleukemia Friend cells. Nucleic Acids Res. **14:**9653–9666.
44. **Neuman, E., E. K. Flemington, W. R. Sellers, and W. G. Kaelin, Jr.** 1994. Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. Mol. Cell. Biol. **14:**6607–6615.
45. **Nevins, J. R.** 1992. A link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
46. **Pagano, M., G. Draetta, and P. Jansen-Durr.** 1992. Association of cdk-2 kinase with the transcription factor E2F during S-phase. Science **255:**1144–1147.
47. **Pagano, M., R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta.** 1992. Cyclin A is required at two points in the human cell cycle. EMBO J. **11:**961–971.
48. **Peter, M., and I. Herskowitz.** 1994. Joining the complex: cyclin-dependent kinase inhibitory proteins and the cell cycle. Cell **79:**181–184.
49. **Pines, J.** 1993. Cyclins and their associated cyclin-dependent kinases in the human cell cycle. Biochem. Soc. Trans. **18:**921–925.
50. **Pines, J., and T. Hunter.** 1990. p34cdc2, the S and M kinase? New Biol. **2:**389–401.
51. **Pines, J., and T. Hunter.** 1990. Human cyclin A is adenovirus E1A associated protein p60 and behaves differently from cyclin B. Nature (London) **346:**760–763.
52. **Quelle, D. E., R. A. Ashmun, S. A. Shurieff, J. Y. Kato, D. Barsagi, M. F. Roussel, and C. J. Sherr.** 1993. Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev. **7:**1559–1571.
53. **Resnitzky, D., L. Hengst, and S. I. Reed.** 1995. Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G_1 by $p27^{Kip1}$. Mol. Cell. Biol. **15:**4347–4352.
54. **Roberts, A. B., and M. B. Sporn.** 1990. The transforming growth factor betas, p. 419–471. *In* M. B. Sporn, and A. B. Roberts (ed.), Peptide growth factors and their receptors. Springer-Verlag, Heidelberg.
55. **Sardet, C., M. Vidal, D. Cobrinik, Y. Geng, C. Onufryk, A. Chen, and R. A. Weinberg.** 1995. E2F-4 and E2F-5, two novel members of the E2F family, are expressed in the early phases of the cell cycle. Proc. Natl. Acad. Sci. USA **92:**2403–2407.
56. **Schauer, L. E., S. Siriwardana, T. A. Langan, and R. A. Sclafani.** 1994. Cyclin D1 overexpression versus retinoblastoma inactivation: implications for growth control evasion in no-small cell and small cell lung cancer. Proc. Natl. Acad. Sci. USA **91:**7827–7831.
57. **Schulze, A., K. Zerfass, D. Spitkovsky, S. Middendorp, J. Bergès, K. Helin, P. Jansen-Dürr, and B. Henglein.** 1995. Cell cycle regulation of the cyclin A gene promoter is mediated by a variant E2F site. Proc. Natl. Acad. Sci. USA **92:**11264–11268.
58. **Schwarz, J. K., S. H. Devoto, E. J. Smith, S. P. Chellapan, L. Jakoi, and J. R. Nevins.** 1993. Interaction of the p107 and Rb proteins with E2F during the cell proliferation response. EMBO J. **12:**1013–1020.
59. **Sherr, C. J.** 1993. Mammalian G1 cyclins. Cell **73:**1059–1065.
60. **Sherr, C. J.** 1994. G1 phase progression: cycling on cue. Cell **79:**551–555.
61. **Shirodkar, S., M. Ewen, J. DeCaprio, D. Morgan, D. M. Livingston, and T. Chittenden.** 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle regulated manner. Cell **68:**157–166.
62. **Sobczak-Thepot, J., F. Harper, Y. Florentin, F. Zindy, C. Brechot, and E. Puvion.** 1993. Localization of cyclin A at the sites of cellular DNA replication. Exp. Cell Res. **206:**43–48.
63. **Swenson, K. I., K. M. Farrell, and J. V. Ruderman.** 1986. The clam embryo protein cyclin A induces entry into M phase and the resumption of meiosis in *Xenopus* oocytes. Cell **47:**861–870.
64. **Tommasi, S., and G. P. Pfeifer.** 1995. In vivo structure of the human cdc2 promoter: release of a p130-E2F-4 complex from sequences immediately upstream of the transcription initiation site coincides with induction of cdc2 expression. Mol. Cell. Biol. **15:**6901–6913.
65. **Tsai, L. H., E. Harlow, and M. Meyerson.** 1991. Isolation of the human cdk2 gene that encodes the cyclin A and adenovirus E1A-associated p33 kinase. Nature (London) **353:**174–177.
66. **Walker, D. H., and J. L. Maller.** 1991. Role for cyclin A in the dependence of mitosis on completion of DNA replication. Nature (London) **354:**314–317.
67. **Wang, T. C., R. D. Cardiff, L. Zukerberg, E. Lees, A. Arnold, and E. V. Schmidt.** 1994. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature (London) **369:**669–671.
68. **Wang, J., X. Chenivesse, B. Henglein, and C. Bréchot.** 1990. Hepatitis B virus integration in a cyclin A gene in a hepatocellular carcinoma. Nature (London) **343:**555–557.
69. **Weinberg, R. A.** 1995. The retinoblastoma protein and cell cycle control. Cell **81:**323–330.
70. **Xiong, Y., H. Zhang, and D. Beach.** 1993. Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. Genes Dev. **7:**1572–1583.
71. **Xu, M., K.-A. Sheppard, C.-Y. Peng, A. S. Yee, and H. Piwnica-Worms.** 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol. Biol. Cell **14:**8420–8431.
72. **Yoshizumi, M., C. M. Hsieh, F. Zhou, J. C. Tsai, C. Patterson, M. A. Perrella, and M. E. Lee.** 1995. The ATF site mediates downregulation of the cyclin A gene during contact inhibition in vascular endothelial cells. Mol. Biol. Cell. **15:**3266–3272.
73. **Zamanian, M., and N. LaThangue.** 1993. Transcriptional repression by the Rb-related protein p107. Mol. Biol. Cell. **4:**389–396.
74. **Zhu, L., K. Van den Heuvel, A. Fattaey, M. Ewen, D. M. Livingston, N. Dyson, and E. Harlow.** 1993. Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes Dev. **7:**1111–1125.
75. **Zwicker, J., F. C. Lucibello, L. A. Wolfraim, C. Gross, M. Truss, K. Engeland, and R. Müller.** 1995. Cell cycle regulation of the cyclin A, cdc25C and cdc2 genes is based on a common mechanism of transcriptional repression. EMBO J. **14:**4514–4522.