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13. ABSTRACT *(Maximum 200*

The growth suppressor protein p21WAF1 (p21) is a cyclin-dependent kinase inhibitor (CKI) that promotes growth arrest by tight-binding to several cyclin/CDK complexes and the DNA replication/repair protein PCNA. Loss of p21 from such complexes is known to occur in cancer cells, including several mammary tumors we have surveyed. We have also recently uncovered and investigated interactions between p21 and a transcription factor, E2F, known to promote cell cycle progression. First, we detected a specific interaction between these proteins in vivo and in vitro. We observe that only a single member of the E2F family forms complexes with p21 in vivo, although there are at least six known members of this family. We have detected the E2F-p21 interaction in vitro by transfection and by capturing native complexes on promoter fragments bearing E2F consensus binding sites. Using this approach, we have mapped the region of p21 needed for association with E2F. In addition, we have mapped the region in E2F required for association. Interestingly, this region corresponds to one conserved between the entire family, suggesting that there are subtle differences in this domain that confer binding to p21. Having established this, we are now testing the effects of p21 on E2F-mediated transcription.

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4. TABLE OF CONTENTS

INTRODUCTION

Progress through the cell cycle requires a concerted interplay between the growth promoting cyclin-dependent kinases (CDKs) and inhibitors such as p21/WAF1 that suppress the CDKs (CKIs). The CDKs are a family of mammalian protein kinases containing at least nine members (CDK1-9) related by several structural features. Regulation of CDK activity occurs at several levels. First, activation of each requires binding to a distinct cyclin regulatory subunit. Thus, CDC2 pairs specifically with cyclins A and B, while CDK2 combines with cyclins A and E, and the D type cyclins pair exclusively with CDK4 and CDK6. Cyclin/CDK complexes are thought to carry out their central function by phosphorylating critical downstream targets. Among the most important targets of CDK activity is the retinoblastoma tumor suppressor protein, pRB. pRB is thought to restrain growth in part through its ability to negatively regulate the E2F transcription factor, which is itself required to activate many genes needed for S phase entry (reviewed in Dyson, 1998). CDK activity overcomes the growth suppressive potential of pRB.

This CDK growth-promoting activity is opposed by another class of regulators, the CDK inhibitors (CKIs) that suppress growth. At least seven distinct CKIs have been identified. CKIs can be divided into those that include the p15, p16, p18, and p19 proteins that bind to, and inhbit, the cyclin D-associated kinases, CDK4 and CDK6 and second set that consists of p21 (known also as WAF1/Cip1/Sdi1/CAP20), p27Kip1, and p57Kip2. The latter proteins are able to inhibit the activity of all CDKs in vitro. The fundamental importance of several CKIs has been determined through gene knock-out experiments which indicate that these proteins play an inhibitory role in cell proliferation (reviewed in Sherr and Roberts, 1999). Furthermore, p21 plays a pivotal role in the DNA damage response, since this stimulus results in p53 activation and enhancement of p21 levels that diminish CDK activity and arrest the cell cycle. This p53-dependent checkpoint is defective in most human cancers.

Given this central role for the p21 family of inhibitors in restraining the cell cycle, we have begun to investigate the composition and function of complexes that contain p21. Our previous studies suggested that normal cells contain the full complement of cyclins (A, B, D, and E) and CDKs (CDC2, CDK2, CDK4, CDK6) as well as PCNA, but that several transformed cell lines and at least one breast cancer cell line lacked certain p21 complexes. Furthermore, through experiments supported by the BCRP, we have shown that two members of the retinoblastoma (pRB) tumor suppressor family, p107 and p130, are both able to bind and repress E2F trans-activation as well as inhibit associated cyclin/CDK2 kinase activity. Interestingly, p107 and p130 appear to bind cyclin/CDK2 complexes through a motif (termed the 'LFG', 'Cy', or 'RXL' motif) similar to one found in p21. Based on this apparent structural and functional similarity between p107/p130 and p21 proteins, we investigated whether a direct regulatory connection exists between the E2F and p21 proteins. We have found in previous studies that in normal cells, p21 forms a complex with E2F-4, but the function of this complex is not yet known. Our research efforts have been directed toward understanding the biochemical consequences of p21 association with this transcription factor during cell cycle progression in human cells.

Body

1. Detection of p21-E2F complexes in normal and breast cancer cell lines

In our previous studies, we were able to detect a complex in normal human WI-38 fibroblasts that contained both p21 and one specific E2F family member, E2F-4. However, we were only able to detect these complexes through a gel mobility shift assay in

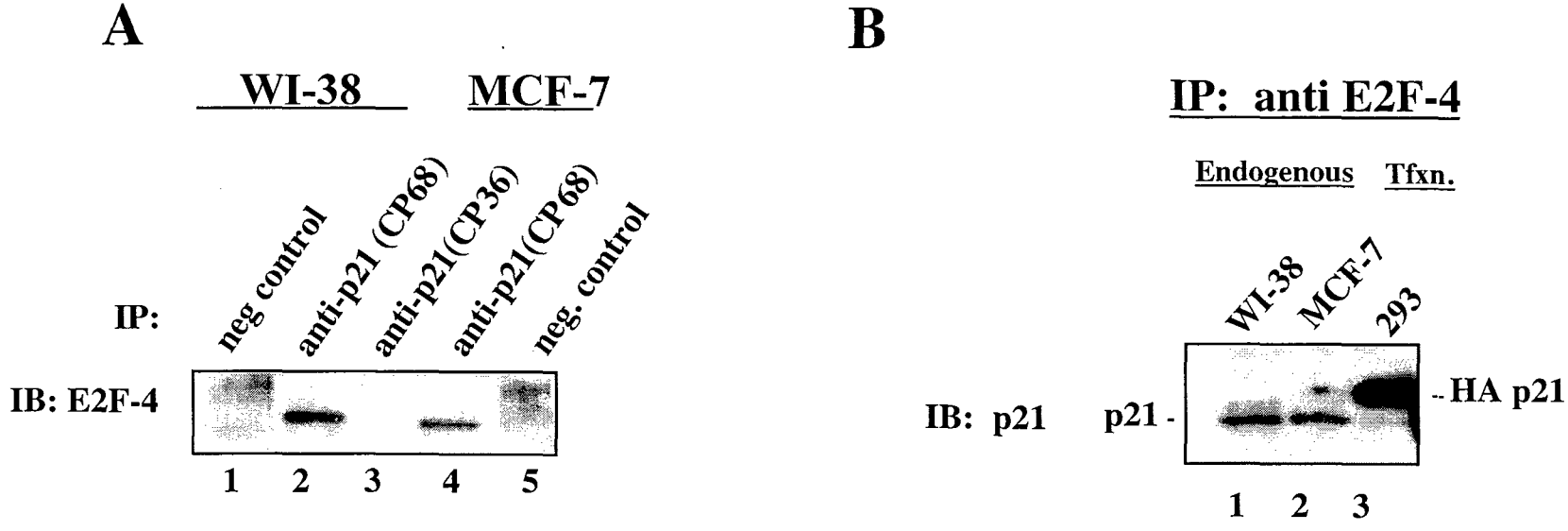

Figure 1.

(**A**) WI-38 (lanes 1 -3) and MCF-7 lysates (lanes 4-5) were **immunoprecipitated** with irrelevant antibody (lanes 1, 5) or p21 mAbs CP68 (lanes 2,3) or CP36 (lane 4). E2F-4 was detected by **immunoblotting** with anti-E2F-4 Ab.

(**B**) WI-38 (1), MCF-7 (2) or 293 cell (lane 3, transfected with E2F-4, DP-1, and HA-p21) lysates were **immunoprecipitated** with anti-E2F-4 Ab, and p21 was detected by **immunoblotting** with CP36.

which we immunoprecipitated total cell protein using certain antibodies against p21, releasing associated proteins with detergent (deoxycholate), and detecting released E2F protein by gel shifts with ^{32}P-labeled oligonucleotides. We were concerned, however, that this assay could be misleading, given its extreme sensitivity and that such complexes might only be detectable using this single assay. Also, since the technique relies on high salt extraction to remove proteins from cellular chromatin, we wished to exclude the possibility that it could allow for mixing and matching of proteins that might not exist in the cell. To rule out this possibility, we performed immunoprecipitations with antibodies against both E2F-4 and p21 using normal human cells (WI-38) and breast cancer cells (MCF-7).

As shown in Figure 1, we were able to detect E2F-4 after immunoblotting specific anti-p21 immunoprecipitates from both types of cell extract. The complete panel of anti-p21 monoclonal antibodies has been described in a previous report to the BCRP. As a control, irrelevant antibodies do not precipitate E2F-4 from either cell line. Importantly, a second p21 monoclonal antibody (CP36) fails to immunoprecipitate E2F-4, although it is capable of immunoprecipitating p21 complexes that contain cyclin A and CDK2. This is consistent with our gel mobility shift assay in which we were able to detect p21-E2F-4 complexes using CP68 but not CP36. This might suggest that the p21-E2F-4 complexes do not contain cyclin A/CDK2, although these observations do not allow a definitive conclusion regarding the presence of additional proteins in the complex. Interestingly, we also detected this complex in MCF-7 breast cancer cells, despite the fact that these cells contain diminished amounts of p21 and cyclin/CDK complexes associated with this CKI, as documented in our 1999 Annual report. We were unable to detect E2F-4 in immunoprecipitates obtained from transformed human and murine cells lacking the p21 protein, confirming the specificity of our antibodies (data not shown).

We were also able to detect the in vivo association of p21 and E2F-4 in the reverse direction. Namely, we could detect p21 in immunoprecipitations with anti-E2F-4 antibodies, but not with antibodies against other E2F family members. Furthermore, we have not reproducibly observed an interaction between endogenous E2F-4 and the other two p21-related CKIs, p27 and p57. Taken together, these data confirm the existence of cellular complexes that specifically contain E2F-4 and p21 and prompted us to further characterize the interaction between p21 and E2F-4 biochemically.

2. The interaction between p21 and E2F-4 is restricted during the cell cycle

i) Cell cycle specific interactions in human fibroblasts

We sought to determine whether the interactions between E2F-4 and p21 were cell cycle-dependent. This proved to be quite a challenge, since normal mammalian cells are notoriously difficult to synchronize without the use of drugs that block the cell cycle (such drugs have been shown in some cases to cause an elevation in p21 levels). We have succeeded in obtaining highly synchronized cultures of human T98G glioblastoma cells. However, these cells have extremely low levels of p21, preventing us from using this cell line (data not shown). We therefore made use of two normal human fibroblast cell lines, WI-38 and IMR-90, which could be growth arrested using by serum deprivation. Such treatment causes 80-90% of the cells to enter a quiescent state (Figure 2A) as determined using flow cytometry with a FACScan Analyzer. Addition of 20% serum allows a synchronous progression through G1 phase and entry into the DNA replicative phase (Figure 2A). Thus, cells harvested at 4-8, 12-16, 20, and 24 hours after serum addition are considered to be in early G1 phase, mid-G1 phase, at the G1/S transition, and in S phase, respectively. After entry into S phase, cells lose synchronicity.

A

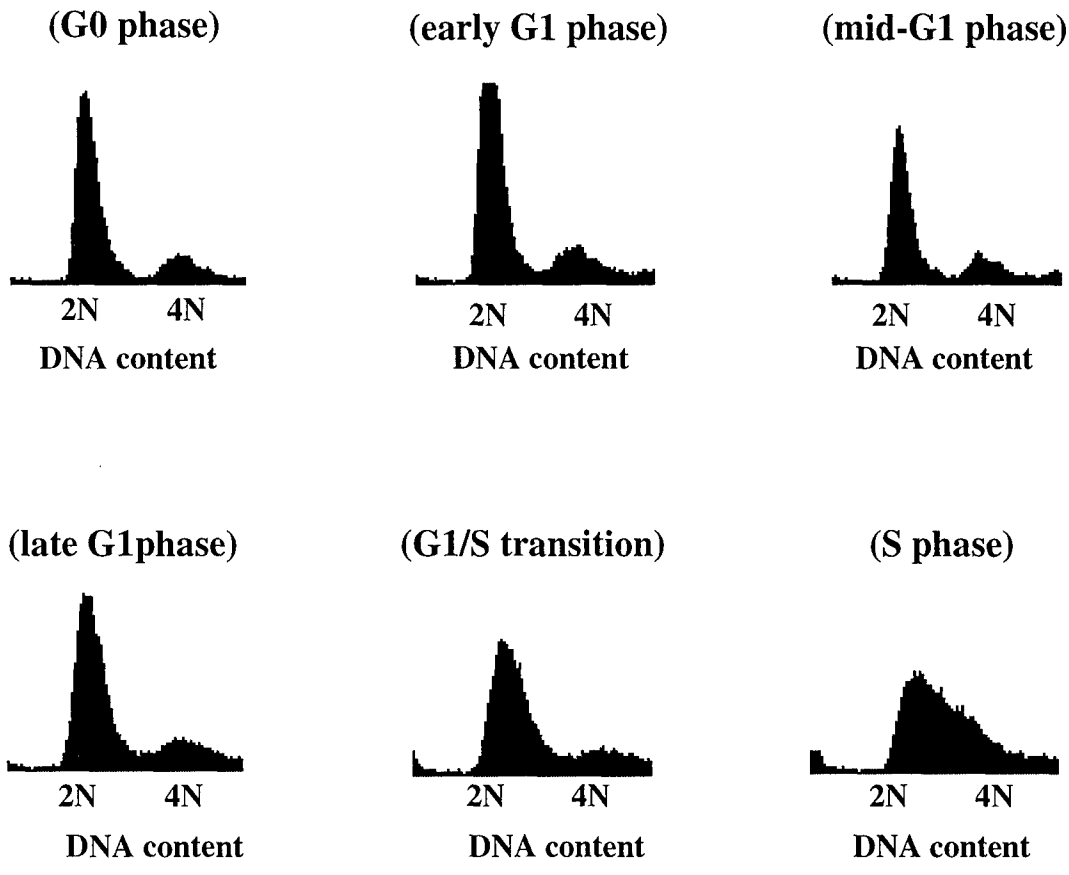

Figure 2A. WI-38 and IMR-90 cells (shown) were synchronized by serum starvation for 3 days and were released back into the cell cycle by addition of 20% serum.

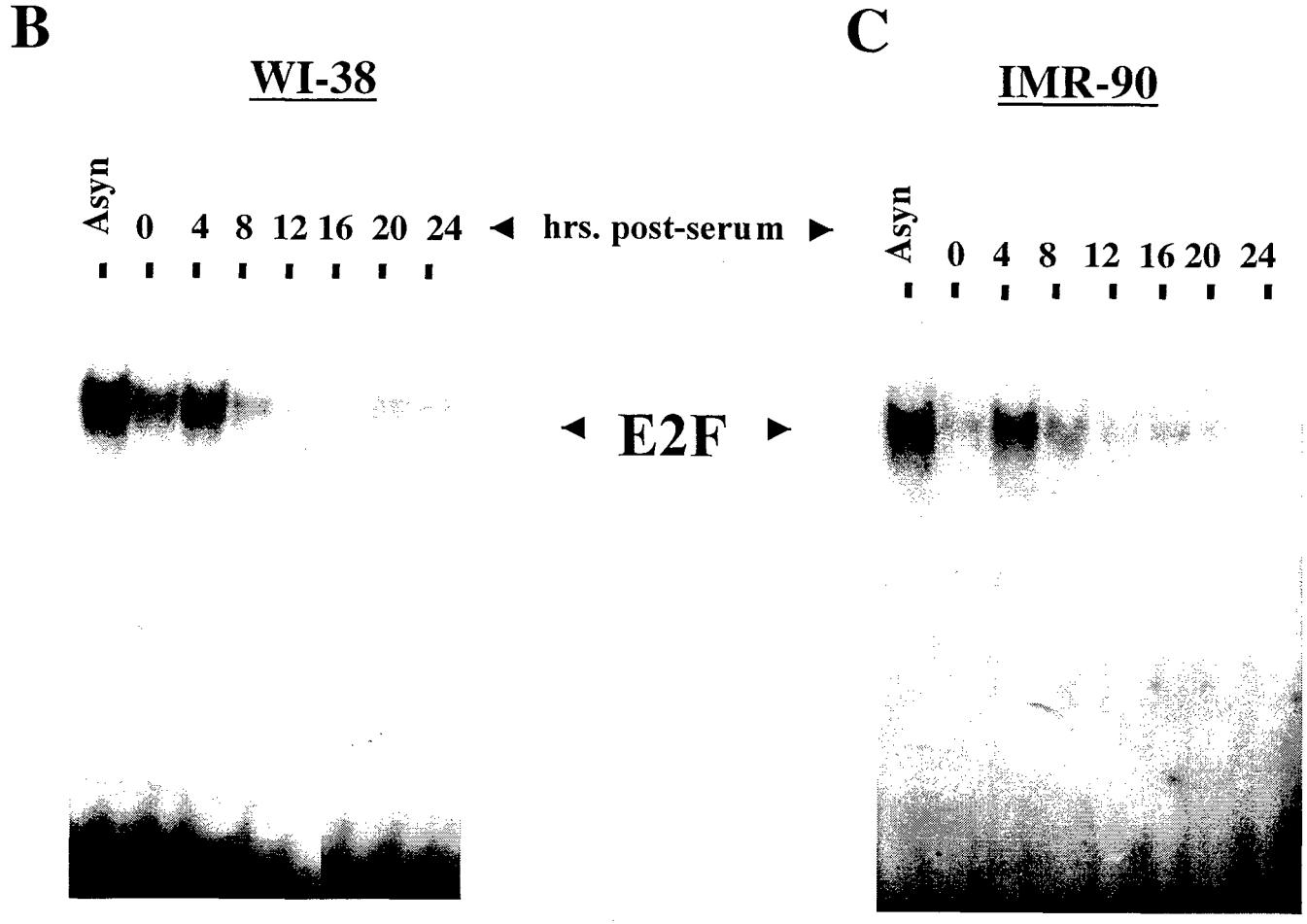

Figure 2 B, C. Abundance of the p21/E2F complex peaks 4 hours after serum addition. WI-38 (**B**) and IMR-90 (**C**) cells synchronized as in Figure 2A were harvested at the indicated times and subsequently examined by immunoprecipitation with anti-p21 mAb (CP68) followed by release of associated proteins with detergent (deoxycholate) and gel shift analysis.

Next, we tested the appearance of p21-E2F-4 complexes during the cell cycle using synchronized IMR-90 fibroblasts. Because our gel mobility shift assays detecting E2F released by deoxycholate from anti-p21 immunoprecipitates closely mirrored our immunoprecipitation-immunoblotting experiments (see section 1 above), we performed the former assay on cells at each cell cycle stage. Interestingly, the p21-E2F-4 complex was most abundant in the early G1 phase of the cell cycle, approximately 4 hours after serum addition (Figure 2C). As cells progressed through G1 and entered S phase, this complex disappeared. We observed a nearly identical profile in WI-38 cells as well (Figure 2B).

This observation could be explained if p21 levels were elevated dramatically as cells entered G1 phase after leaving quiescence. A two-fold increase in p21 levels has been documented in mouse embryo fibroblasts (Macleod et al., 1995). In addition, we have observed that p21 levels increase by approximately two-fold in WI-38 cells released from serum deprivation (Cai et al., 1998), and thereafter, p21 levels do not change whatsoever. We believe this is significant, because we have detected an approximate 5-10-fold increase in the abundance of p21-E2F complex at four hours post-serum addition and a striking reduction four hours later. Western blotting of extracts from these synchronized cells indicates that E2F-4 levels do not change appreciably, further ruling out this possible explanation for the increased levels of p21-E2F-4 complex that we detect. Because the apparent increase in the p21-E2F-4 complex does not wholly reflect p21 or E2F-4 protein levels themselves, it is possible either that (1) post-translation modifications regulate the activity of E2F-4 associated with p21 during this time or (2) another protein plays a limiting role in promoting the association between E2F-4 and p21 specifically during this early period of G1. We are currently investigating both exciting possibilities.

We have also investigated the sub-cellular localization of the p21-E2F-4 complex. We fractionated extracts into nuclear and cytoplasmic compartments and then performed western blotting and gel mobility shift experiments similar to the ones described above. Strikingly, although p21 protein were present at nearly equivalent levels in both nuclear and cytoplasmic compartments, we detected the p21-E2F-4 exclusively in the nuclear fraction. From previous studies by other groups, E2F-4 is known to reside in the nucleus in early-mid G1 phase and to accumulate in the cytoplasm as cells approach S phase. It is possible that p21 may help anchor E2F-4 in the nucleus, since it has a nuclear localization domain but E2F-4 does not.

ii) We have used a second system to address a potential role for p21 in regulation of cell growth. C2C12 cells are a well characterized murine myoblast cell line that undergoes differentiation to myotubes upon depletion of serum growth factors. Several groups have shown a linkage between the upregulation of p21 and the ability to undergo muscle differentiation (see for example Halevy et al., 1995; Zhang et al., 1999). However, the downstream effects of p21 induction are not completely known, and although the effects of p21 are thought to be mediated through inhibition of CDKs, other mechanisms have not been ruled out.

Therefore, we examined p21 expression in growing C2C12 cells and in cells allowed to differentiate between several hours and several days by depletion of serum. Interestingly, in preliminary experiments, we showed that although p21 levels increased slightly, there was a much more robust increase (5-10-fold) in the abundance of the p21-E2F-4 complex as detected by E2F gel mobility shift assays after DOC treatment of anti-p21 immunoprecipitates. Importantly, there was no increase in the levels of E2F-4 or RB. In addition, levels of the p107-E2F-4 complex diminished. One other complex that contains p130 and E2F-4 increased dramatically, however. These data pertaining to increased levels of the p21-E2F-4 complex without concomitant increases in the amount of either protein are

highly reminiscent of those obtained with human fibroblasts (see above) in the early G1 phase of the cell cycle. We are therefore most enthusiastic about continuing these experiments to determine whether p21 might influence differentiation of cells (such as muscle) by regulating the activity of transcription factors to which it binds and transcription levels of downstream target genes involved in cell cycle control. Indeed, in preliminary experiments, we have demonstrated that cyclin A protein levels diminish significantly as the p21-E2F-4 complex appears upon differentiation of C2C12 cells. Atlhough it will be important to examine the cyclin A transcript levels during differentiation, additional experiments described below (see sections 4 and 5), suggest that cyclin A could be a direct target of p21 function.

Collectively, these data suggest that the p21-E2F-4 complex resides in the nucleus and is specifically up-regulated during the early G1 phase of normal human cells. Moreover, our preliminary data suggest a potential role for the interaction between p21 and E2F during the differentiation of certain cell types.

3. Regions of p21 and E2F-4 necessary for association

We used an in vitro assay to map the regions of p21 and E2F-4 necessary for interaction. This assay is described schematically in Figure 3A. Briefly, a biotinylated promoter fragment containing four consensus E2F binding sites is synthesized by PCR and incubated with lysates from cells transfected with plasmids encoding E2F-4 (or derivatives thereof), DP-1, and p21 (and various mutants). After incubation, the biotinylated promoter fragments are captured with streptavidin-coated magnetic beads. After pelleting, the beads are washed and associated proteins are released by addition of sample buffer and samples electrophoresed. Proteins bound to the magnetic beads are detected by western blotting.

Using this approach (Figure 3), we could show that the amino-terminal region of p21 was necessary and sufficient for its association with E2F-4 once the E2F-4/DP-1 heterodimer was bound to DNA. Here, an amino-terminal fragment of p21 is sufficient to bind E2F, as is a flu hemagglutinin (HA)-tagged version of full-length p21 (lanes 1 and 2). This fragment contains the N-terminal half of p21. Interestingly, deletion of the cyclin-binding domain of p21 (Δ17-24) severely diminished the ability of p21 to bind E2F (lane 4), and deletion of the CDK binding domain completely abolished p21 binding to E2F (lane 5). These data indicate that an intact amino-terminal domain containing both cyclin and CDK domains is required for binding to E2F at a promoter. In our 1999 Annual report we suggested that the amino-terminus of p21 was itself not sufficient to bind E2F. However, these in vitro binding experiments were done in the absence of promoter DNA, so it is likely that conformational changes induced by DNA binding altered E2F in such a way as to promote p21 binding. As a control, we used biotinylated promoter fragments lacking E2F sites to show unambiguously that p21 was specifically recruited via interactions with E2F bound to the promoter.

Using a similar approach, we also attempted to map with some precision the region(s) of E2F-4 required for association with p21. E2F proteins have 5 domains that are required for transcriptional activation and for association with other proteins and DNA (Figure 4A). We therefore reasoned that intact proteins should be used in the binding experiments so as to minimally disrupt protein structure. Therefore, we took advantage of the fact that p21 binds tightly to E2F-4 but does not bind strongly to E2F-1 and obtained expression plasmids to produce chimeric E2F-1/E2F-4 proteins in tissue culture cells. These plasmids (a gift of K. Helin, Milan, Italy) produced significant quantities of each protein upon

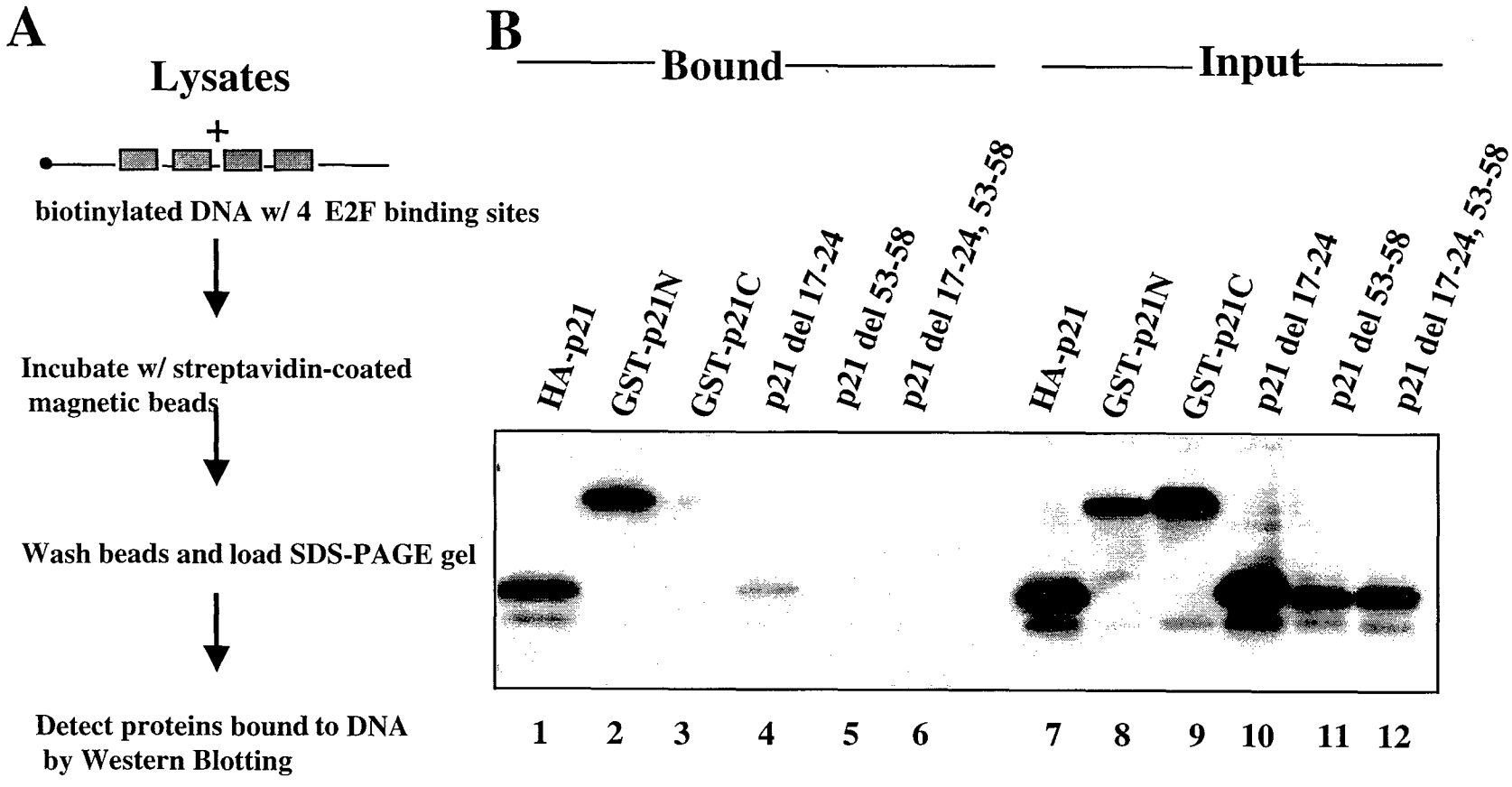

Figure 3. (**A**) Schematic for **magnetic bead** binding experiment. Biotinylated DNA lacking E2F sites was used as a negative control and failed to bind either E2F or p21. (**B**) p21 detected by **Western blotting** with antibodies against N- and C-term. regions of p21.

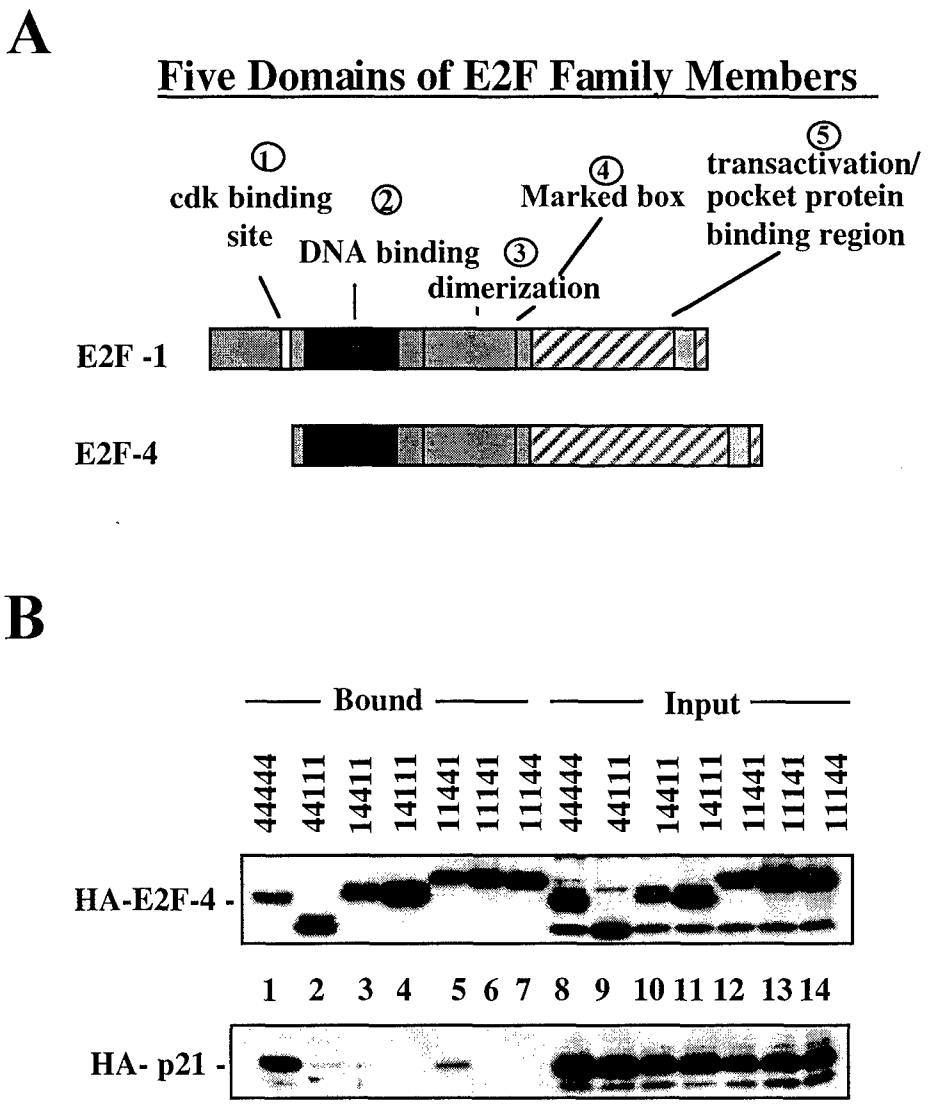

Figure 4. (A) Domains in E2F-1 and E2F-4. (B) Lysates from 293 cells transfected with plasmids encoding HA-p21, DP-1, and each of the indicated E2F-1/E2F-4 chimeras were examined with the magnetic bead binding assay as described in Fig. 3. Lanes 8-14 represent 1% of input lysate.

transfection (Figure 4B). Each plasmid was designated numerically by domain according to which E2F family member was represented. Thus, 44444 represents wild-type E2F-4, while 11441 contains the CDK binding site, DNA binding domain, and trans-activation/'pocket protein' binding domain of E2F-1 and the dimerization domain and 'marked box' of E2F-4. Only wild-type E2F-4 bound p21 significantly, although we observed reproducible and significant binding by the chimera that contained only the dimerization and 'marked box' domains of E2F-4. These data suggest a novel function of the these domains in E2F-4. The function of the 'marked box' is unclear, although it is fairly well conserved in all E2F family members. Our data may be the first indication that this domain plays a role in protein-protein interactions. Additional chimeras are currently being tested; for example, we will test chimeras that include the DNA-binding domain of E2F-4 in addition to the E2F-4 dimerization/'marked box' regions. Addition of these regions may further stabilize the interactions between the chimeric proteins and p21.

4. p21 shows promoter-specific effects on transcription of cell cycle-regulated genes

Given that p21 interacts with E2F-4 via the dimerization domain of the latter protein, we next tested whether co-expression of p21 with E2F-4 and its heterodimeric partner, DP-1, might influence the transcriptional activation properties of this trans-activator. To do so, we performed transient transfection assays and tested the expression of the CAT from a linked reporter. The data are represented in Figure 5.

Expression of E2F-4 in C33A human cervical carcinoma cells leads to a dramatic increase in transcription from the reporter plasmid (Figure 5A). Co-expression of p21 with E2F-4 led to a marked diminution of transcription. We also examined the same E2F-1/E2F-4 chimeras that were tested in our previous in vitro binding assay (Figure 4 and see above). Each chimeric protein was expressed to levels similar to wild-type E2F-4 and potentiated activation of the reporter template. As expected, the chimeric protein containing only the E2F-4 dimerization/'marked box' domains (construct 11441) was weakly repressed (approximately 2-fold) by co-expression of p21. However, we unexpectedly observed similar repression of a second E2F-4/E2F-1 construct (11144) that did not show signficant interactions with p21 in the in vitro binding assay (Figure 4). It is possible that the two proteins interact in vivo in ways that cannot be recapitulated in vitro. Although we are continuing to test additional chimeras to resolve this discrepancy, it is clear that p21 is capable of reproducibly and potently repressing transcription from the cyclin A promoter.

Interestingly, when we tested a second E2F reporter constuct in which the DHFR promoter was placed upstream of CAT, we found that p21 did not repress transcription directed by E2F-4 (Figure 5B). In fact, transcription increased modestly in the presence of p21. These data suggest that the repressive effects of p21 are promoter-specific and may further explain the decreases in cyclin A levels observed upon differentiation of C2C12 cells in which the p21-E2F complex is up-regulated.

5. Studying promoter occupancy by p21 protein

We have recently developed a chromatin immunoprecipitation protocol to study promoter occupancy by a given protein in living mammalian cells (Takahashi et al., 2000). This value of this method lies in its ability to allow examination of physiological occupancy of promoters. Using this protocol, we have performed preliminary experiments to determine whether p21 is bound to selected cell cycle-regulated E2F-responsive promoters. We have examined the cyclin A and E2F-1 promoters, both of which bind to E2F-4 during early G1 phase after serum stimulation of quiescent T98G cells (Takahashi et al., 2000).

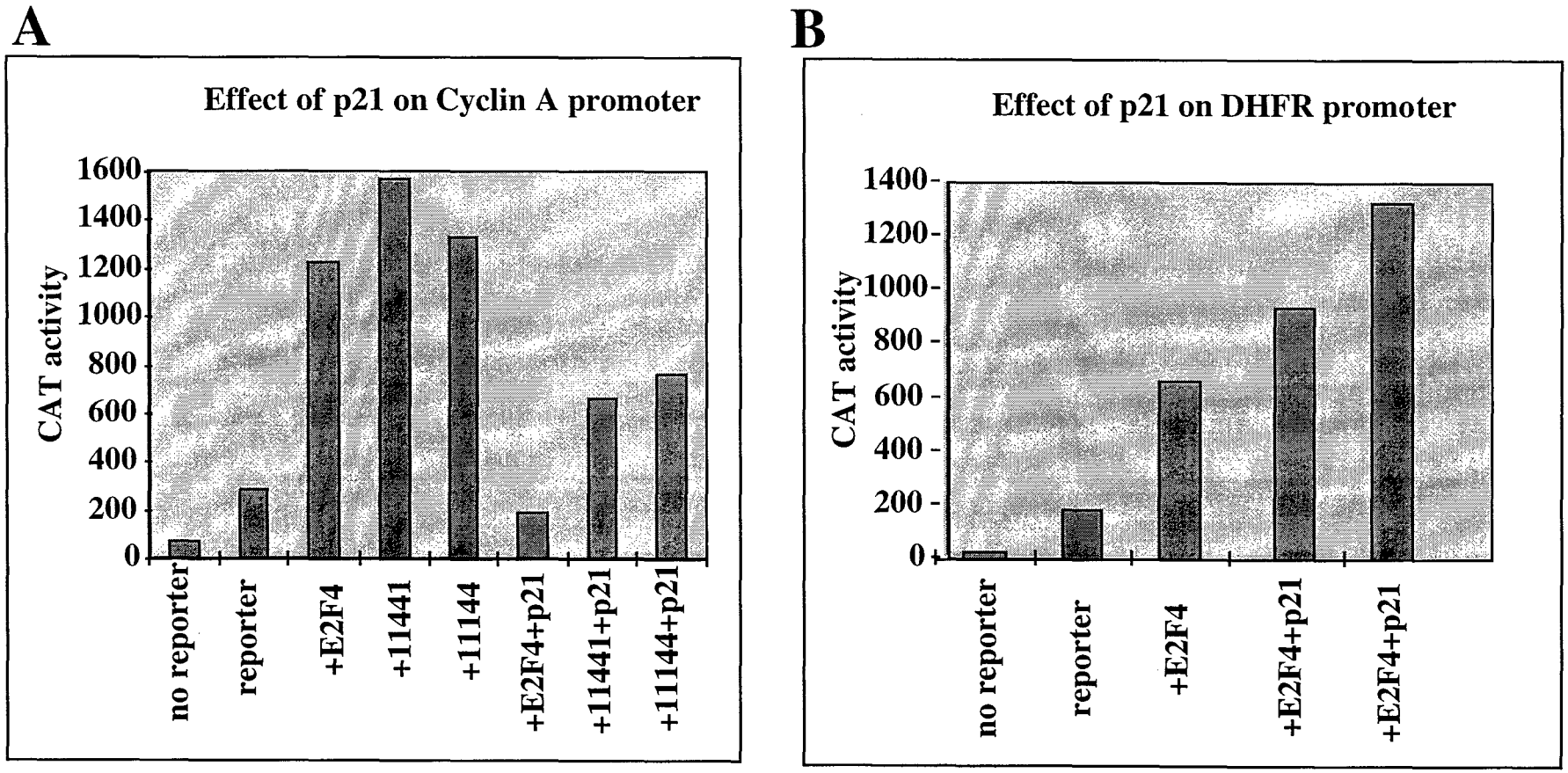

Figure 5. C33A cells were transfected with a CAT reporter plasmid containing either (**A**) the human cyclin A promoter or (**B**) the human DHFR promoter, and transcription was measured from each promoter using CAT assays. E2F-1/E2F-4 chimeras were as described in Figure 5.

Using IMR-90 cells that were made synchronized as described above (Figure 2A), we determined whether p21 bound to each of these promoters at each stage of the cell cycle. As a control, we also determined whether p21 was bound to the actin promoter. Actin serves as an excellent control because it is not under the control of E2F. In preliminary experiments, we detected p21 bound exclusively to the cyclin A promoter but not the E2F-1 or actin promoters (data not shown). Significantly, the appearance of p21 at the cyclin A promoter was cell cycle specific: We observed p21 localization at the promoter in early G1, 4 hours after serum addition, but not in S phase (24 hours after serum addition). These data are consistent with those described in Figure 2C in which the p21-E2F-4 complex was specifically up-regulated 4 hours after serum addition.

We are most enthusiastic about these preliminary results and will continue to pursue these experiments (although the funding period has ended) to a conclusion regarding p21 regulation of cyclin A and other E2F responsive promoters in vivo using both normal and p21-deficient human cells and C2C12 cells.

Key Research Accomplishments

- p21 exists in cellular complexes that contain the E2F transcription factor, in addition to previously characterized complexes that contain cyclins, cdks, and PCNA.

- The p21-E2F-4 complex is most abundant in early G1 phase and can be found in a variety of normal human fibroblasts, but not in their transformed derivatives or other immortal cell lines. This complex is significantly up-regulated in muscle cells undergoing differentiation.

- This complex consists minimally of E2F-4, DP-1, and p21, and we have not ruled out the presence of additional components.

- p21 recognizes a region of E2F-4 overlapping its dimerization and 'marked box' domains.

- p21-E2F-4 complexes may regulate a subset of cell cycle regulated promoters.

Reportable Outcomes

Poster presentation, "Regulation of the E2F transcription factor by the p21 inhibitor," H. Cam and B. Dynlacht, Era of Hope Meeting, June 8-11, 2000.

Conclusions

Our conclusions are summarized in the 'Key Research Accomplishments' section above. Briefly, we believe that we may have identified a novel mechanism whereby p21 arrests cell growth and promotes differentiation by binding to, and negatively regulating, the E2F-4 transcription factor. We have reached this conclusion using a number of in vitro and in vivo methods, and we intend to complete these studies although the funding period for this grant has ended. We will continue our transient transfection studies to examine which promoters are negatively regulated by p21. Simultaneously, we will continue our investigations using chromatin immunoprecipitation to determine whether the promoters that are regulated in transient transfection assays can recruit p21 at a time when p21-E2F-4 complexes are up-regulated.

Final Report: Publications and Personnel

Publications resulting from the research effort

M.S. Woo, I. Sanchez, and **B.D. Dynlacht**. (1997). p130 and p107 use a conserved domain to inhibit cellular cyclin-dependent kinase activity. *Mol. Cell. Biol.* **17**, 3566-3579.

E. Castano, Y. Kleyner, and **B. D. Dynlacht**. (1998). Dual cyclin-binding domains are required for p107 to function as a kinase inhibitor. *Mol. Cell. Biol.* **18**, 5380-5391.

K. Cai and **B.D. Dynlacht**. (1998). Activity and nature of $p21^{WAF1}$ complexes during the cell cycle. *Proc. Natl. Acad. Sci.* **95**, 12254-12259.

H. Cam and **B.D. Dynlacht**. (2000). Characterization of a novel complex containing $p21^{WAF1}$ and an E2F family member. In preparation.

Personnel receiving salary in the period 1999-2000

Leyu Wang
Brian Dynlacht, Principal Investigator

Personnel from previous years receiving salary

Enrique Castano
Kang Cai
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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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