Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation

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In normal human diploid fibroblasts, cyclins of the A, B, and D classes each associate with cyclin-dependent kinases (CDKs), proliferating cell nuclear antigen (PCNA), and p21, thereby forming multiple independent quaternary complexes. Upon transformation of diploid fibroblasts with the DNA tumor virus SV40, or its transforming tumor antigen (T), the cyclin D/p21/CDK/PCNA complexes are disrupted. In transformed cells, CDK4 totally dissociates from cyclin D, PCNA, and p21 and, instead, associates exclusively with a polypeptide of 16 kD (p16). Quaternary complexes containing cyclins A or B1 and p21/CDK/PCNA also undergo subunit rearrangement in transformed cells. Both PCNA and p21 are no longer associated with CDC2–cyclin B1 binary complexes. Cyclin A complexes no longer contain p21, and a new 19-kD polypeptide (p19) is found in association with cyclin A. The pattern of subunit rearrangement of cyclin–CDK complexes in SV40-transformed cells is also shared in those containing adeno- or papilloma viral oncoproteins. Rearrangement also occurs in p53-deficient cells derived from Li–Fraumeni patients that carry no known DNA tumor virus. These findings suggest a mechanism by which oncogenic proteins alter the cell cycle of transformed cells.

[*Key Words*: Cyclin; cyclin-dependent kinase (CDK); cell cycle; cellular transformation; p53; PCNA]

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Neoplasia is characterized by deregulated cell growth and division. Inevitably, molecular pathways controlling cell growth must interact with those regulating cell division. It was not until very recently, however, that experimental evidence became available to bring such connection to light. Cyclin A was found in association with the adenovirus oncoprotein E1A in virally transformed cells (Giordano et al. 1989; Pines and Hunter 1990). In an early hepatocellular carcinoma, the human cyclin A gene was found to be the integration site of a fragment of the hepatitis B virus, which leads to activation of cyclin A transcription and a chimeric viral cyclin A protein that is not degradable in vitro (Wang et al. 1990, 1992). The cell cycle gene implicated most strongly in oncogenesis thus far is the human cyclin D1. It was originally isolated through genetic complementation of yeast G_1 cyclin deficiency (Lew et al. 1991; Xiong et al. 1991) as cellular genes whose transcription is stimulated by CSF-1 (colony-stimulating factor-1) in murine macrophages (Matsushime et al. 1991) and as the putative oncogene *PRAD1* rearranged in parathyroid tumors (Motokura et al. 1991). Two additional human D-type cyclins, cyclin D2 and D3, were subsequently isolated using PCR and low-stringency hybridization techniques (Inaba et al. 1992; Xiong et al. 1992a). Cyclin D1 is genetically linked to the *bcl-1* oncogene, a locus activated by translocation to an immunoglobulin gene enhancer in some B-cell lymphomas and leukemias, and located at a site of gene amplification in 15–20% of human breast cancers and 25–48% of squamous cell cancers of head and neck origin (Withers et al. 1991, for review, see Hunter and Pines 1991; Lammie and Peters 1991). The biochemical role played by the cell cycle regulators in these tumors is not yet understood.

Cyclins were originally discovered in invertebrate eggs as proteins whose abundance after fertilization oscillated during the early cleavage divisions owing to abrupt proteolytic degradation at mitosis (Rosenthal 1980; Evans et al. 1983; Swenson et al. 1986). Subsequently, cyclin genes have been isolated from virtually all eukaryotic species and constitute a multigene family (for review, see Xiong and Beach 1991). It is now well established that cyclins function by serving as regulatory subunits of the cdc2/CDC28 protein kinase (for review, see Draetta 1990). In yeast cells, a single cdc2/CDC28 kinase controls both the G_2/M and G_1/S transitions through its association with different types of cyclins (Booher and Beach 1987; Wittenberg et al. 1990; Bueno et al. 1991; Ghiara et al. 1991; Surana et al. 1991). In vertebrates, however, cdc2 is a representative of a multi-gene family.

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Through genetic complementation testing and identification of associated cyclin regulatory subunits, at least five cyclin-dependent kinases have been identified (Fang and Newport 1991; Paris et al. 1991; Tsai et al. 1991; Elledge et al. 1992; Matsushime et al. 1992; Meyerson et al. 1992; Pagano et al. 1992a,b; Rosenblatt et al. 1992; Xiong et al. 1992b). By use of PCR and low-stringency hybridization techniques, further potential members of the cdc2 family have been identified on the basis of their protein sequence similarities to cdc2 (Meyerson et al. 1992; Okuda et al. 1992). Involvement of most of these protein kinases in cell cycle regulation, especially the possible association with cyclins, has yet to be established.

To elucidate the mechanism by which the cyclin-dependent kinase (CDK) complexes control the cell cycle and cell growth, cellular proteins that interact with cyclin–CDK complexes must be identified. Cyclin A and CDK2 have been found to form a quaternary complex with a retinoblastoma gene product (Rb1)-related protein (p107) and the transcription factor E2F in a cell cycle-regulated manner, suggesting that cyclin A–CDK2 kinase might be controlling gene expression during G_1 and S phase of the cell cycle (Cao et al. 1992; Devoto et al. 1992; Ewen et al. 1992; Faha et al. 1992; Pagano et al. 1992b; Shirodkar et al. 1992; for review, see Nevins 1992). Similarly, cyclin E was also found to form a quaternary complex with CDK2, p107, and E2F but in a temporally distinct manner from the cyclin A quaternary complex (Lees et al. 1992). D-type cyclins associate with multiple protein kinases and form quaternary complexes in normal human diploid fibroblast cells with proliferating cell nuclear antigen (PCNA) and a polypeptide of 21 kD, p21. Many combinatorial variations involving cyclin D1, D3, and CDK2, CDK4, and CDK5 may assemble in vivo (Xiong et al. 1992b). As reported elsewhere (H. Zhang, Y. Xiong, and D. Beach, in prep.), PCNA and p21 serve as universal components of cyclin–CDK complexes. Here, we report that cellular transformation is associated with selective subunit rearrangement of cyclin–CDK complexes.

Results

Disruption of cyclin D/p21/CDK/PCNA complexes in SV40-transformed cells

During attempts to identify cyclin D-associated catalytic subunits and other associated cellular proteins, we noticed that cyclin D/CDK/p21/PCNA quaternary complexes were detected in WI-38 and Hs68 cells, both of which are normal human diploid fibroblast cells, but such complexes were not detected in several other cell lines that are transformed (Xiong et al. 1992b). The absence of the cyclin D quaternary complexes in these cells might be attributed to cell type specificity or to the consequence of cellular transformation. To directly test the latter possibility, we compared the subunit composition of cyclin D complexes in normal human diploid fibroblasts and their transformed derivatives. WI-38 VA13, subline 2RA (hereafter referred to as VA13), is an SV40 virus-transformed derivative of the WI-38 cell line (see Materials and methods). Expression of SV40-encoded large T antigen in VA13 cells, in addition to other SV40-transformed cells used in this study (see below), has been confirmed by immunoblotting (data not shown). Anti-cyclin D1 immunoprecipitates of [^{35}S]methionine-labeled WI-38 and VA13 lysates were examined. As shown previously in WI-38 cells, the anti-cyclin D1 antiserum precipitates a dominant 35-kD band corresponding to cyclin D1 and three major associated proteins, $p36^{PCNA}$, $p33^{CDK4}$, and p21 (Fig. 1, lane 10, note that the molecular identity of these proteins has been formally confirmed in Xiong et al. 1992b). In SV40-transformed VA13 cells, the level of cyclin D1 is decreased by two- to threefold as determined by autoradiography of [^{35}]methionine-labeled immunoprecipitates (Fig. 1, lanes 10,11), and by Western blotting (data not shown). Strikingly, none of the three major cyclin D1-associated proteins was visibly associated with cyclin D1 in SV40-transformed VA13 cells. Reciprocal immunoprecipitations were carried out with anti-CDK4 and anti-CDK2 antisera to confirm the observed disruption of cyclin D complexes in SV40-transformed cells. In WI-38 cells, anti-CDK4 antibody precipitated CDK4 in addition to three major associated proteins, $p36^{PCNA}$, $p35^{cyclin\ D1}$, and p21 (Xiong et al. 1992b; Fig. 1, lane 6). The specificity of these associations has been established previously and was confirmed further here by peptide competition (Fig. 1, lane 7). In SV40-transformed VA13 cells, whereas CDK4 is present at a similar level compared with untransformed WI-38 cells (cf. lanes 6 and 8 of Fig. 1 with lanes 3 and 4 of Fig. 3A, below), no detectable PCNA, cyclin D1, or p21 was present in the anti-CDK4 immunoprecipitates (Fig. 1, lane 8). Similar results were also observed in immunoprecipitates with anti-CDK2, another cyclin D-associated kinase catalytic subunit. Cyclin D1 is one of the major CDK2-associated proteins in WI-38 cells (Xiong et al. 1992b; Fig. 1, lane 3), but the association is undetectable in SV40-transformed VA13 cells, even though the level of CDK2 is similar or slightly higher in the transformed cells (Fig. 1, lane 5). Also, the level of CDK2-associated p21 was reduced in transformed cells but is present at a detectable level (Fig. 1, lanes 4,5). As shown below, the p21 present in anti-cdk2 precipitates probably consists of p21 present in p21/cyclin A/CDK2 complexes. As reported recently (Zhang et al. 1993), CDC2 associates with cyclin D1, although at a much lower level compared with CDK4 and CDK2. The association between cyclin D1 and CDC2 also declines to undetectable levels in SV40-transformed VA13 cells (Fig. 1, lanes 1,2; note that the molecular identity of PCNA, cyclin D1, and p21 in CDC2 complexes is formally demonstrated in H. Zhang, Y. Xiong, and D. Beach, in prep.). Reciprocal experiments examining anti-PCNA and anti-p21 immunoprecipitates were not performed because of the lack of suitable immunological reagents that can precipitate PCNA–cyclin D complexes and the unknown molecular identity of p21 at the present time.

To seek further evidence concerning the subunit com-

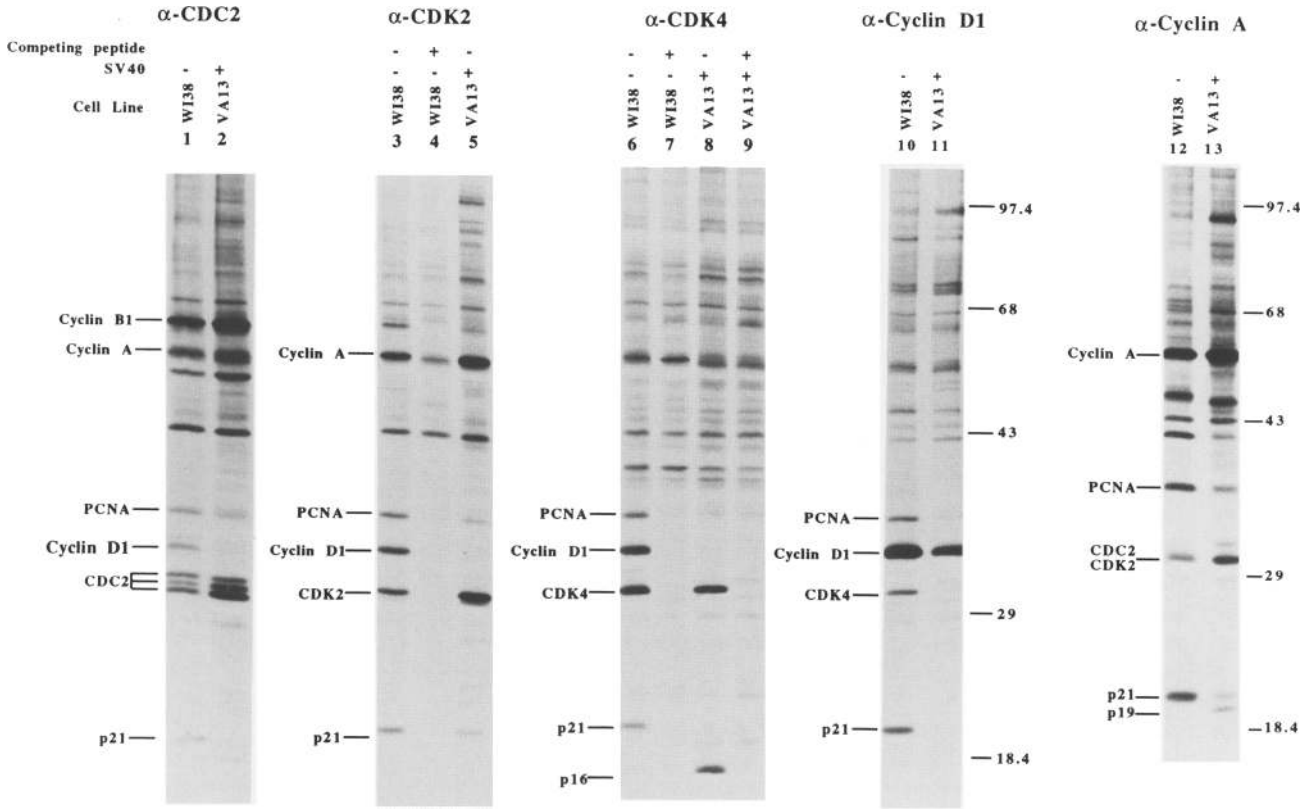

Figure 1. [35]Methionine-labeled immunoprecipitates of normal and transformed fibroblasts. [35]Methionine-labeled immunoprecipitates were prepared from normal human diploid fibroblast WI-38 cells (lanes *1,4,5,7,8,11,13*) and SV40-transformed VA13 cells (lanes *2,6,9,10,12,14*) as indicated by SV40 − or + at the top of each lane. Conditions for immunoprecipitations and electrophoresis are described in Materials and methods. Proteins in each immunoprecipitate whose identity has been established are marked at *left*, and molecular mass markers are indicated at *right*. Molecular masss markers next to lanes *11* and *12* also apply to lanes *1–10*. The presence and absence of competing peptide in anti-CDK2 and anti-CDK4 immunoprecipitations are indicated by + and −, respectively, at the *top* of each lane.

position of cyclin D complexes in transformed cells, several additional normal human diploid fibroblast cell lines and their SV40-transformed derivatives were examined. HSF43, a diploid fibroblast cell line derived from neonatal foreskin and CT10-2C-T1 (hereafter referred to as CT10), was obtained from HSF43 by introduction of a plasmid containing the SV40 large tumor antigen gene (T) driven by a Rous sarcoma virus (RSV) promoter (Ray et al. 1990). The pattern of subunit composition and dissociation of the normal and transformed cells was very similar to that observed in the WI-38 and VA13 cells. No cyclin D1 was seen in anti-CDK4 (Fig. 2, lane 6), anti-CDK2 (lane 4), and anti-CDC2 (lane 2) immunoprecipitates prepared from T-transformed CT10 cells, but it was apparent in the HSF43 cells. (Fig. 2, lanes 1,3,5). Reciprocally, CDK4 was not seen in anti-cyclin D1 precipitates in CT10 cells (data not shown). CDK4-associated PCNA and p21 were also not detected in CT10 cells (Fig. 2, lane 6). p21 in the T-transformed cells appeared to be replaced by a newly identified p16 protein (Fig. 2, lane 6) as in the VA13 cell line (Fig. 1, lane 8). In addition, essentially identical results were also obtained from other pairs of human fibroblast cell lines, IMR-90, a normal human lung diploid fibroblast cell line and its T-transformed derivative IDH4 (Shay et al. 1992), as well as a pair of monkey cell lines, CV-1, a pseudodiploid kidney cell line, and its SV40-transformed derivative COS-1 (Gluzman 1981; data not shown).

To further confirm the observed dissociation of cyclin D complexes in transformed cells, immunoprecipitation coupled with immunoblotting experiments, was performed to avoid potential artifacts that can arise in metabolic labeling experiments. Total lysates prepared from either untransformed WI-38 or SV40-transformed VA13 cells were immunoprecipitated with a battery of antibodies, separated electrophoretically, and blotted with various secondary antibodies. CDK4 (Fig. 3A, lanes 1,2), CDK2 (data not shown), and PCNA (Fig. 3C, lanes 13,14) were either not detected (CDK4) or were present at dramatically decreased levels (CDK2 and PCNA) in anti-cyclin D1 precipitates derived from SV40-transformed cells. In each case, direct immunoblotting confirmed that the absolute level of CDK4 (Fig. 3A, lanes 3,4), CDK2 (data not shown), and PCNA (Fig. 3C, lanes 17,18) is similar in normal and transformed cells. Reciprocally, cyclin D1 was not detected in either anti-CDK2 (Fig. 3B,

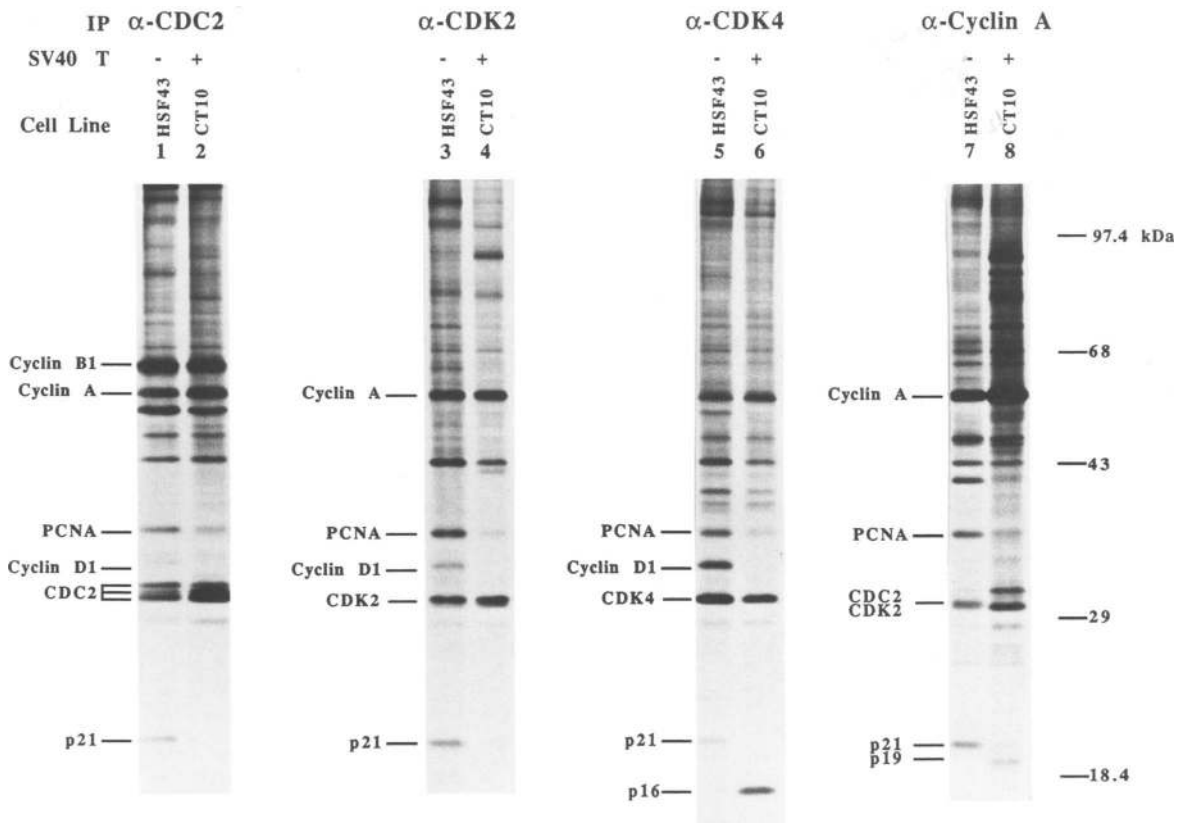

Figure 2. [^{35}]Methionine-labeled immunoprecipitates of normal HSF43 and SV40 large tumor antigen-transformed CT10 cells. Conditions for immunoprecipitations and electrophoresis, and markers for protein identities and molecular mass are the same as described in the legend to Fig. 1, except a different pair of cell lines was used. HSF43 is a diploid fibroblast cell line derived from neonatal foreskin, and CT10-2C-T1 (CT10) cell line was derived from nontransformed HSF43 by the introduction of a plasmid containing the gene for SV40 large tumor antigen (T) linked to a RSV promoter (Ray et al. 1990).

lane 2) or anti-CDK4 precipitates derived from transformed VA13 cells (Fig. 3B, lane 5). The same results were also obtained from the other pair of cell lines, HSF43 and CT10 (Fig. 3, A–C).

Subunit rearrangement of cyclin A and B complexes in transformed cells

As reported recently, PCNA and p21 not only associate with cyclin D–CDK to form a quaternary complex as described previously (Xiong et al. 1992b), but also are present as universal components of cyclin-dependent kinases in all normal human diploid fibroblast cells that we have examined (H. Zhang, Y. Xiong, and D. Beach, in prep.). Thus, we went further to examine the subunit composition of cyclin A complexes in untransformed WI-38 cells and SV40-transformed VA13 cells. Cyclin A associates with $p36^{PCNA}$, $p33^{CDC2}$, $p33^{CDK2}$, and p21 in WI-38 cells (H. Zhang, Y. Xiong, and D. Beach, in prep.; Fig. 1, lane 12). In SV40-transformed VA13 cells, the level of both cyclin A itself and cyclin A-associated CDC2/CDK2 increased approximately threefold (Fig. 1, lane 13). Reciprocally, the level of CDC2- and CDK2-associated cyclin A is also increased two- to threefold (Fig. 1, lanes 1,2 and lanes 3,5, respectively). The increased abundance and association of cyclin A and CDC2/CDK2 in transformed cells was also confirmed in immunoblotting coupled with immunoprecipitation experiments (data not shown).

The level of cyclin A-associated PCNA is reduced in immunoprecipitates of [^{35}] methionine-labeled VA13 cell lysates, compared with WI-38 cell lysates (Fig. 1, lanes 12,13). As determined by immunoblotting, however, the level of cyclin A-associated PCNA in transformed VA13 cells is higher than that in untransformed WI-38 cells (Fig. 3C, lanes 5,6). Also, the level of CDK2-associated PCNA is higher in VA13 cells compared with WI-38 cells (Fig. 3C, lanes 1,2). Using a different pair of fibroblast cell lines, essentially the same results were obtained. The level of CDC2-, CDK2-, and cyclin A-associated PCNA is lower in SV40 large tumor antigen-transformed CT10 cells than that in untransformed diploid HSF43 cells, as measured in immunoprecipitates of [^{35}] methionine-labeled cell lysates (Fig. 2, lanes 1,2,3,4,7,8). As determined by immunoblotting coupled with immunoprecipitation, however, the opposite result is obtained (Fig. 3C, lanes 3,4,7,8). A possible explanation for this experimental discrepancy is that more pre-

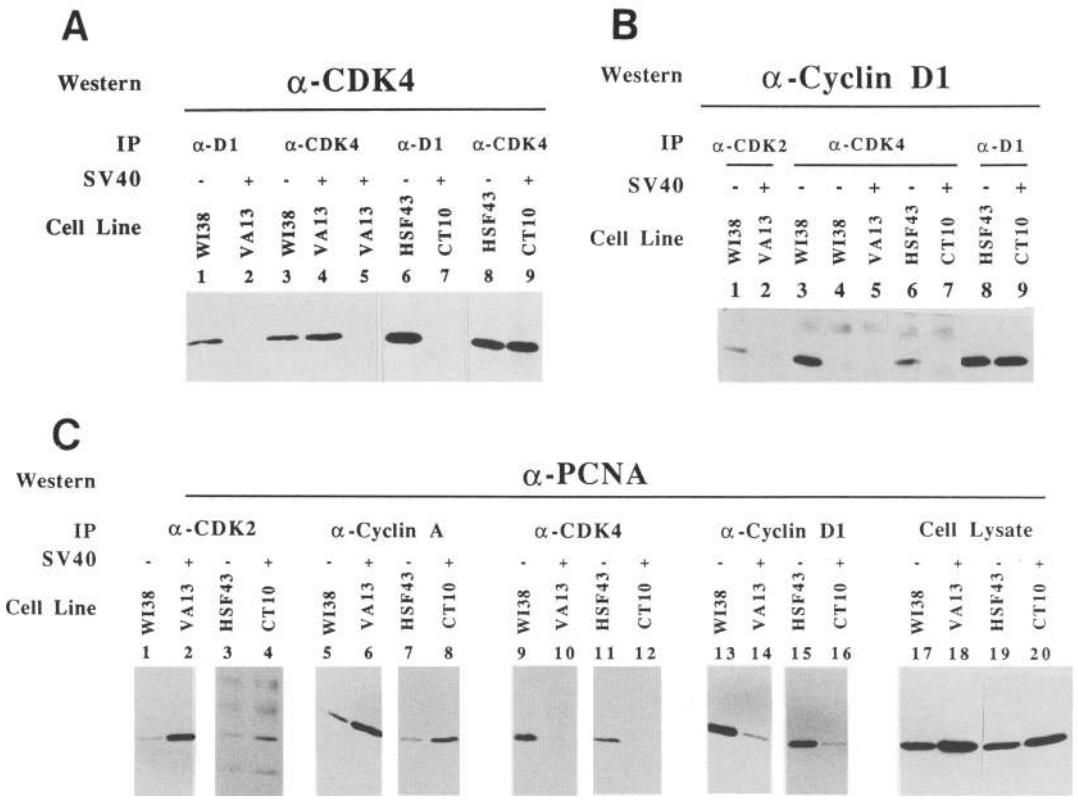

Figure 3. Immunoblotting analysis of cyclin–CDK complexes in normal and transformed cells. (*A*) Cell lysates prepared from normal WI-38 and HSF43 cells (indicated by SV40 −) or transformed VA13 and CT10 cells (+) were immunoprecipitated by a variety of antisera as indicated at the *top* of each lane. Following SDS-PAGE, the blot was probed with anti-CDK4 serum. Lane *5* is the same as lane *4*, except a competing CDK4 peptide was included in the immunoprecipitation. (*B*) Cell lysates prepared from normal WI-38 and HSF43 cells (indicated by SV40 −) or transformed VA13 and CT10 cells (+) were immunoprecipitated by a variety of antisera as indicated at the *top* of each lane. Following SDS-PAGE, the blot was probed with anti-cyclin D1 serum. Lane *4* is the same as lane *3*, except a competing CDK4 peptide was included in the immunoprecipitation. (*C*) Cell lysates prepared from normal WI-38 and HSF43 cells (indicated by SV40 −) or transformed VA13 and CT10 cells (+) were immunoprecipitated by a variety of antisera as indicated at the *top* of each lane, except lanes *17–20*, where total cell lysates from different cells were loaded directly onto the SDS gel without prior immunoprecipitation. Following SDS-PAGE, the blot was probed with anti-PCNA serum.

existing PCNA, rather than newly synthesized PCNA, associates with cyclin–CDK in transformed cells rather than normal cells. This observation suggests that both metabolic labeling and immunoprecipitation coupled with immunoblotting should be used to assess the status of any given molecular interaction. An approximate estimation of the stoichiometry of the cyclin/CDK/PCNA/p21 interaction is given in H. Zhang, Y. Xiong, and D. Beach (in prep.).

With regard to the association of p21 with members of the cyclin A/CDK family only metabolic labeling can presently be used. The level of cyclin A-associated p21, as determined by immunoprecipitation of [^{35}]methionine-labeled cell lysates, is reduced in VA13 cells as compared with WI-38 cells (Fig. 1, lanes 12,13). A low level of p21 is also present in the immunoprecipitates prepared from VA13 cells using antisera against CDK2, the major catalytic partner of cyclin A (Fig. 1, lane 5). Consistently, the same results were obtained using HSF43 and CT10 cells (Fig. 2, lanes 7,8), in addition to normal IMR-90 cells versus their T-transformed IDH4 cells, and monkey CV-1/COS-1 pair cell lines (data not shown). The lack of immunological reagents for p21 prevented us from determining whether the decrease of cyclin A-, and CDK2-associated p21 is attributable to lower abundance or slower synthesis in transformed cells.

A number of other polypeptides were noticed in cyclin A precipitates that are specific to untransformed cells or to transformed cells. For example, a protein with a molecular mass of 42 kD is seen as a predominant band in untransformed WI-38 or HSF43 cells but not in transformed VA13 or CT10 cells (Fig. 1, lanes 12,13; Fig. 2, lanes 7,8). Conversely, a polypeptide of 95 kD, which might correspond to the T antigen, is seen only in transformed cells.

We have also examined the subunit composition of cyclin B1 complexes in normal and transformed cells. In common with cyclin D1, the quaternary complex of cyclin B1/CDK/p21/PCNA is altered in both SV40-transformed VA13 cells and T-transformed CT10 cells as

compared with nontransformed WI-38 and HSF43 cells (Fig. 4, lanes 1–4). Thus, neither PCNA nor p21 associates with cyclin B/CDK in the transformed cells. As reported previously (Draetta and Beach 1988), however, CDC2 is associated with cyclin B1 (p62) in VA13, CT10, 293, and HeLa cells (Fig. 4).

Cyclin and CDK complexes in other DNA tumor virus-transformed cells

To investigate whether cyclin–CDK complexes are also altered in other human tumor cells, papilloma virus-containing cervix carcinoma HeLa cells and adenovirus-transformed primary kidney 293 cells were examined for the subunit composition of various cyclin and CDK complexes. Both of these cell lines have been used widely in biochemical studies of the mammalian cell cycle (see Draetta and Beach 1988). Cyclin D1 is expressed at a low level in HeLa cells and is barely detectable in 293 cells (Fig. 5, lanes 11,12). The subunit composition of other cyclin/CDK complexes, however, can be investigated.

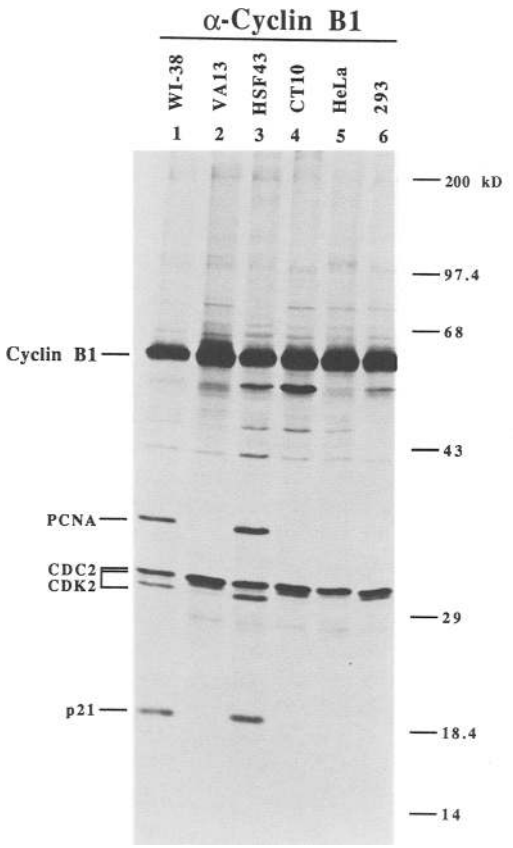

Figure 4. Analysis of [^{35}]methionine-labeled α-cyclin B1 immunoprecipitates. Subunit composition of cyclin B1 complexes was examined in normal WI38 (lane *1*) and HSF43 (lane *3*) fibroblasts, SV40-transformed VA13 (lane *2*) and CT10 (lane *4*) cells, and two other DNA tumor virus-transformed cells, HeLa (papilloma virus, lane *5*) and 293 (adenovirus, lane *6*). [^{35}]Methionine-labeled lysates prepared from each cell line were immunoprecipitated by an α-cyclin B1 serum and electrophoretically separated on a 15% SDS–polyacrylamide gel as described in Materials and methods. Proteins whose identities have been established are marked at *left*, and the positions of molecular size markers are indicated at *right*.

The well-established association of CDC2–cyclin B1, CDC2–cyclin A, and CDK2–cyclin A were all readily detected in both HeLa and 293 cells (Fig. 4, lanes 5,6; Fig. 5, lanes 1,2,3,4,9,10). No p21 was seen, however, in anti-CDC2 (Fig. 5, lanes 1,2), anti-CDK2 (Fig. 5, lanes 3,4), anti-cyclin A (Fig. 5, lanes 9,10), or anti-cyclin B1 (Fig. 4, lanes 5,6) immunoprecipitates from either HeLa or 293 cells. Thus, the quaternary complexes of cyclin A/CDK/p21/PCNA and cyclin B1/CDK/p21/PCNA are either not assembled or are present at extremely low levels in these DNA tumor virus-transformed cells. Interestingly, a new cyclin A complex appeared, in which p21 seems to be replaced by a polypeptide of 19 kD (p19, Fig. 5, lanes 9,10). The specificity of the cyclin A–p19 association and the potential significance of this new complex is unclear, but a similar 19 kD polypeptide is present in anti-cyclin A precipitates from SV40-transformed human VA13 (Fig. 1, lane 13), CT10 (Fig. 2, lane 8), IDH4 (data not shown), and even monkey COS-1 cells (data not shown). Furthermore, in 293 and HeLa cells, the subunit composition of CDK4 is identical to that of the T-antigen-transformed cells. Cyclin D1 is not associated with CDK4 (trivially attributable to absence of cyclin D from the cell), but PCNA and p21 are also absent. p21 has been replaced by p16 in HeLa and 293 cells. In a much longer exposure of Figures 1 and 2, we have also seen CDK4-associated p16 in untransformed WI-38 or HSF43 cells. Proteolytic cleavage mapping in each case reveals that the CDK4-associated p16 from HeLa, 293, VA13 and WI-38 cells is identical, but different from p21 (Fig. 6). Presence of p19 and p16 in cells transformed by three different viruses and p16 in untransformed cells indicates that they are not virally encoded proteins. The molecular identities of p21, p19, and p16 are currently unknown.

Alteration of cyclin and CDK complexes in Li–Fraumeni cells

Alteration of subunit composition of CDKs in cells transformed by three different DNA tumor viruses provoked us to ask further whether cyclin–CDK complexes are also altered in nonvirally transformed cells. Unlike mouse cells, which can be readily transformed by a various oncogenes, it is exceedingly difficult to experimentally transform or induce the immortalization of normal human cells by chemicals, physical methods, or oncogenes other than DNA tumor viruses (for review, see McCormick et al. 1988; Shay et al. 1991). Fibroblasts from familial Li–Fraumeni patients, however, exhibit an elevated rate of spontaneous abnormalities, including aneuploidy and immortalization (Li and Fraumeni 1969; Bischoff et al. 1990). These cells do not contain any known viruses; instead, single-base heterozygous mutations of the tumor suppressor gene p53 are the only reported germ-line alteration (Malkin et al. 1990; Srivas-

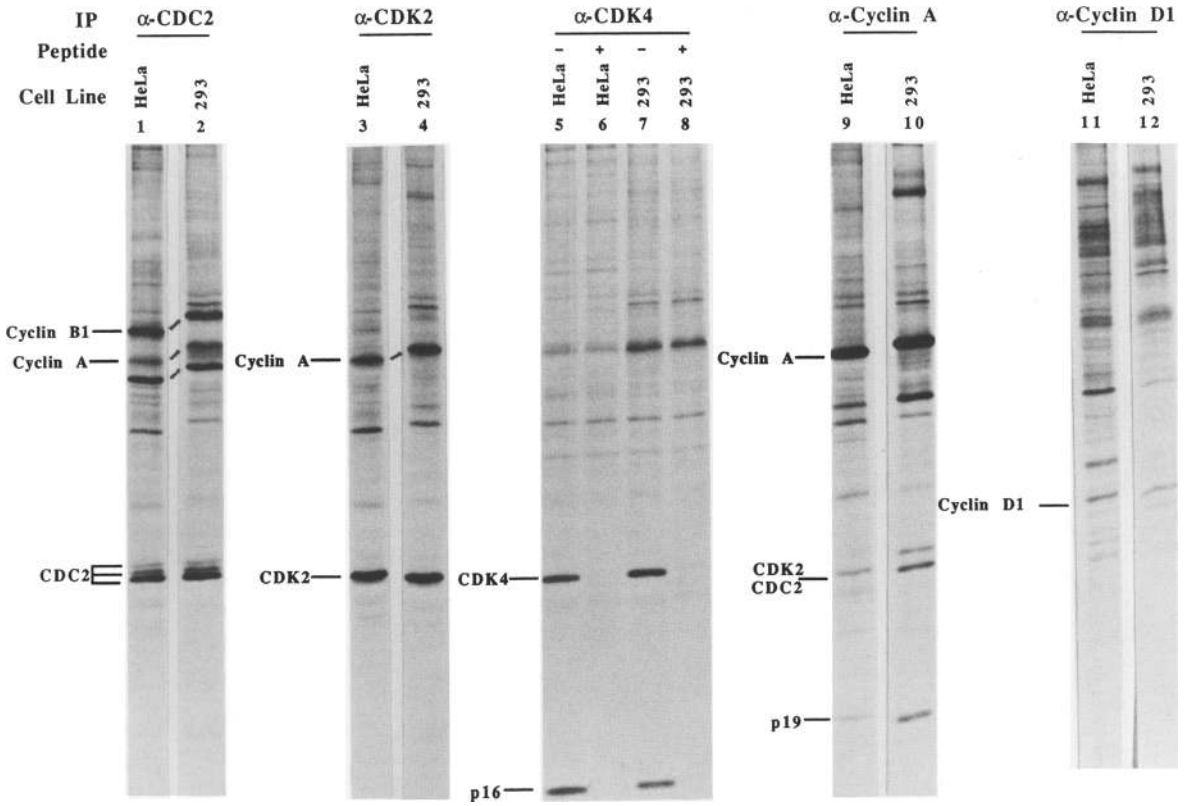

Figure 5. Analysis of [^{35}]methionine-labeled immunoprecipitates of HeLa and 293 cells. Subunit composition of a variety of cell cycle complexes was examined further in two different DNA tumor virus-transformed cells, HeLa (papilloma virus) and 293 (adenovirus). [^{35}] Methionine-labeled lysates prepared from either cell line were immunoprecipitated by a variety of antisera as indicated at the *top* of each lane. Conditions for immunoprecipitations and electrophoresis are described in Materials and methods. Proteins in each immunoprecipitate whose identities have been established are marked at *left* of each panel.

tava et al. 1990). Escape from senescence and spontaneous immortalization of Li–Fraumeni fibroblasts during continued passage in vitro is associated with secondary loss of the wild-type p53 allele (Yin et al. 1992). [^{35}]Methionine-labeled cell lysates from a spontaneously immortalized Li–Fraumeni cell line, LCS041 (previously MDAH041; Little et al. 1987; Bischoff et al. 1990, passage >170) were immunoprecipitated with a variety of antibodies and analyzed by SDS-PAGE. The level of most of these proteins in LCS041 cells is similar to that in normal diploid fibroblasts, as determined by metabolic labeling and immunoblotting, except that cyclin A is expressed at a much lower level compared with normal fibroblasts (data not shown). The subunit composition of cyclin and CDK complexes is grossly abnormal in LCS041 cells (Fig. 7A). Cyclin B1–CDC2 complexes exist as in HeLa and 293 cells and are not associated with p21 or PCNA (Fig. 7A, lanes 1,7). The cyclin D1–CDK4 association is reduced to a barely detectable level in LCS041 cells, and both PCNA and p21 are absent (Fig.

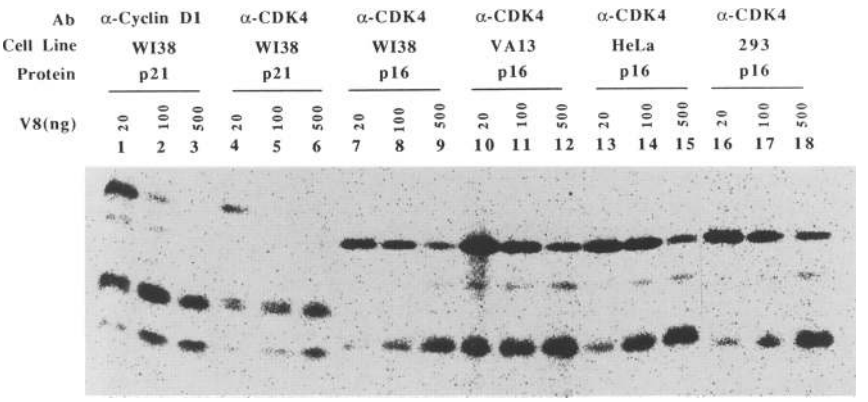

Figure 6. Partial V8 proteolytic mapping of CDK4-associated p16. p16 polypeptides coprecipitated with CDK4 were purified from WI-38 (lanes *7–9*), VA13 (lanes *10–12*), HeLa (lanes *13–15*), and 293 cells (lanes *16–18*). They were analyzed by a partial V8 protease digestion, as described in Materials and methods, and compared with p21 purified from anti-cyclin D1 (lanes *1–3*) or anti-CDK4 immunoprecipitates (lanes *4–6*) derived from WI-38 cells.

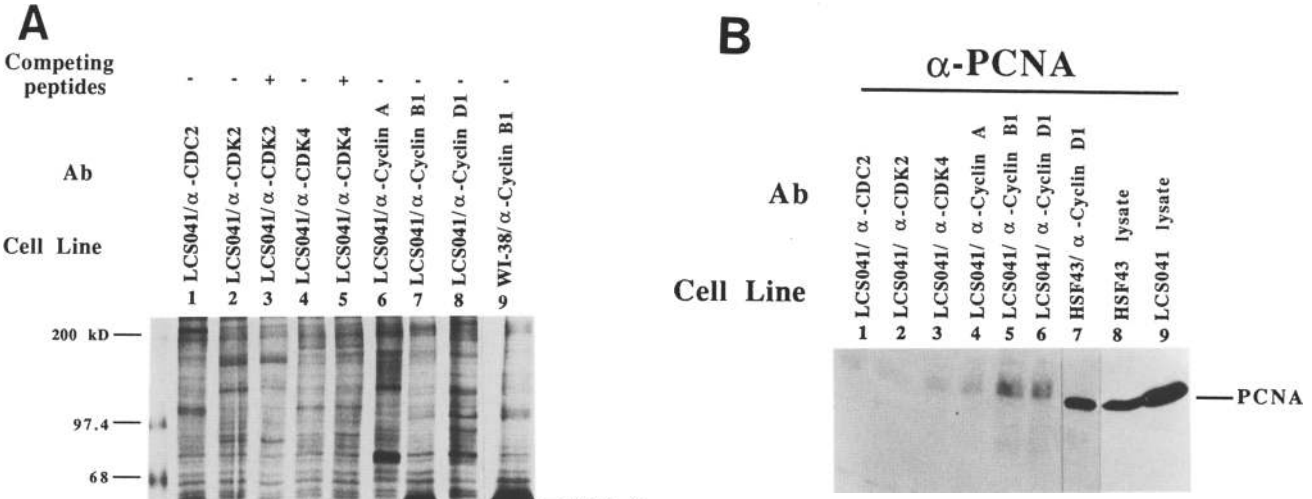

Figure 7. Analysis of cyclin and CDK complexes in Li–Fraumeni cells. (*A*) [^{35}] Methionine-labeled cell lysates prepared from LCS041 cells of a Li–Fraumeni patient (lanes *1–8*) and normal human diploid fibroblast WI-38 cells (lane *9*) were immunoprecipitated with a variety of antisera as indicated at the *top* of each lane. Conditions for immunoprecipitations, peptide competition, and electrophoresis are described in Materials and methods. Proteins in the α-cyclin B1 immunoprecipitate of normal WI-38 cell lysate whose identities have been established are marked at *right*; the positions of molecular size markers are indicated at *left*. Presence and absence of competing peptide in anti-CDK2 and anti-CDK4 immunoprecipitations were indicated by + and −, respectively, at the top of each lane. (*B*) Equal amounts of cell lysates prepared from LCS041 cells of a Li–Fraumeni patient were immunoprecipitated by a variety of antisera as indicated at the *top* of each lane. Lane 7 is an α-cyclin D1 immunoprecipitate prepared from normal diploid HSF43 fibroblasts. (Lanes *8,9*) Total cell lysates from either LCS041 or HSF43 cells were loaded directly on to the SDS gel without prior immunoprecipitation. Following SDS-PAGE, the blot was probed with anti-PCNA serum. The bands seen in lanes *1–6* correspond to the IgG heavy chain, and all lanes were from the same gel.

7A, lanes 4,8). p21 was not detected in any of the cyclin–CDK complexes investigated in LCS041 cells and was not replaced by p16 or p19 proteins as occurs in T-antigen-transformed fibroblasts (Fig. 7A). The level of PCNA associated with cyclin–CDK complexes is also dramatically reduced in every case. Direct immunoblotting demonstrated that the abundance of PCNA is higher in LCS041 cells, compared with normal fibroblasts (Fig. 7B, lanes 8,9), but PCNA is dramatically reduced or entirely absent from immunocomplexes isolated with anti-CDC2, CDK2, CDK4, cyclin A, cyclin B1, and cyclin D1 (Fig. 7B, lanes 1–6). Essentially identical results were also obtained by use of another Li–Fraumeni cell line, LCS087 (previously MADH087), obtained from a different patient (data not shown).

Discussion

The observations in this paper provide striking evidence that the cyclin/CDK family of enzymes that act at multiple key steps in the cell division cycle are grossly altered in a variety of oncogenically transformed cells. To date, we have only used so-called normal diploid fibroblasts as a model for untransformed cells. In a wide variety of fibroblasts, from different sources, each member of the cyclin/CDK family that we have investigated (cyclin E was not studied because of its relatively low abundance in fibroblasts) associated, to some extent, with PCNA and p21. Cell transformation by T antigen is accompanied by apparent total dissolution of p21, PCNA, and CDK4 and substantial dissociation of CDK2 from cyclin D. This pattern is partially shared by the cyclin B–CDC2, CDK2 enzyme. Both p21 and PCNA dissociated, but as described originally (Draetta and Beach 1989), the core enzyme is present in transformed cells. The pattern of behavior of the cyclin A–CDC2, CDK2 enzyme is quite different. The abundance of the cyclin–CDK complex increases, as does the association with PCNA. In many of the cell cycle kinase complexes p21 is not simply altered in its relative stoichiometry in transformed cells, but it is replaced by a different polypeptide

of a slightly different apparent molecular mass (p16 and p19). The possibility of any of these proteins bearing any relationship to the cdc2-associated $p13^{suc1}$/CKS (Brizuela et al. 1987; Draetta et al. 1987; Richardson et al. 1990) awaits molecular cloning of p21, p19, and p16.

The pattern of subunit alteration in fibroblasts transformed by T antigen is not restricted to this particular oncoprotein. Human cervix carcinoma HeLa cells (papilloma transformed) and 293 cells (adenovirus-transformed) carry different DNA tumor viral oncoproteins, in addition to a mass of other oncogenic lesions. Both, however, broadly share T-antigen-associated patterns of subunit composition of the cell cycle kinases, with the exception of cyclin D, which is present at a very low level in these cell types. Furthermore, subunit alteration of CDK complexes was also found in several other human tumor cell lines that we have examined, including epidermoid carcinoma A-431, pharynx squamous cell carcinoma FaDu, and breast adenocarcinoma MCF7. No quaternary CDK complex was detected for any cyclins or CDKs in these lines (Y. Xiong and D. Beach, unpubl.). From these experiments and the studies on Li–Fraumeni cells, as discussed below, we suggest that subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation in general.

It is well established that DNA tumor antigens can interact with components of the cell cycle regulatory apparatus (Giordano et al. 1989, 1991; Tsai et al. 1991; Ewen et al. 1992; Faha et al. 1992). Thus, we were particularly interested in the study of fibroblasts that are transformed by means other than DNA tumor viruses. Fibroblasts derived from Li–Fraumeni patients present an ideal opportunity. These fibroblasts are initially heterozygous with regard to mutations in the p53 tumor suppressor gene. During passage in culture, the wild-type allele of p53 becomes mutant and the cells display certain characterization of morphological transformation (Bischoff et al. 1990; Yin et al. 1992). We found that the pattern of subunit composition of the cyclin kinase family was grossly abnormal in Li–Fraumeni fibroblasts. The pattern partially resembled that of T-antigen-transformed cells, but p21 was apparently not replaced by either p19 or p16 in any of the cell cycle kinase complexes. These results strongly suggest that the alteration of cyclin/CDK enzymes in transformed cells is not simply attributable to direct or indirect binding of a DNA tumor viral oncoprotein to one or more individual subunits of the cyclin–CDK complexes. All three DNA tumor viruses are believed to share a common mechanism in cellular immortalization and transformation. Each encodes an oncoprotein, T antigen of SV40, E6 of papilloma virus, and E1B of adenovirus, which can bind to and presumably inactivate the function of the p53 protein. From these observations and our studies on Li–Fraumeni cells, we suggest that the p53 regulates the cyclin–CDK complexes in normal cells and loss of the p53 function in tumor cells may contribute to the alteration of these complexes. We believe that the striking observations in this paper were not noticed previously because, since the original biochemical analysis of cdc2 in human HeLa cells (Draetta and Beach 1988), investigators have overwhelmingly used transformed cells for cell cycle studies.

The findings in this study raise a large number of questions. Alone among the known elements of the cell cycle kinases, cyclin D1 has been directly implicated in oncogenesis as *PRAD1* and *bcl-1* oncogene (Motokura et al. 1991; Withers et al. 1991) and as a candidate oncogene at the 11q13 locus that is amplified in a variety of clinical conditions. Since the discovery that cyclins A and B directly interact with cdc2 (Draetta et al. 1989), it has become increasingly apparent that cyclins act by virtue of their association with a CDC or CDK catalytic subunit. Cyclin D1 associates with CDC2, CDK2, CDK4, and CDK5 (Matsushime et al. 1992; Xiong et al. 1992b; Zhang et al. 1993). Thus, the original hypothesis regarding the molecular function of cyclins (Draetta et al. 1989) has not been violated. In T-antigen-transformed fibroblasts, however, association of cyclin D1 with CDK4 and CDC2 is totally disrupted and cyclin D1 and CDK2 association is substantially reduced (this study). It has been shown recently (Baldin et al. 1993) that cyclin D1 is essential for the G_1/S-phase transition in normal fibroblasts, and it will be of interest to know whether this is also the case in transformed fibroblasts.

In this context, it has been demonstrated that cyclin D can bind directly to the retinoblastoma (Rb) anti-oncoprotein in vitro (Matsushime et al. 1992; Hall et al. 1993; Kato et al. 1993). Addition of a CDK4 catalytic subunit results in phosphorylation of Rb and dissociation of the Rb–cyclin D complex (Kato et al. 1993). Phosphorylation of Rb is thought to negate its growth- suppressive effect (Buchkovich et al. 1989; DeCaprio et al. 1989; Ludlow et al. 1990). It is quite possible that cyclin D might inhibit Rb function (and/or that of related proteins) not only by phosphorylation but also by direct Rb–cyclin D association, in the absence of catalytic function, thus creating a substrate trap. We would emphasize that although cyclin D1 apparently dissociates totally from CDKs in T-antigen-transformed cells, this particular effect is less apparent in certain other tumor cell lines such as A431 or MCF7, which express high levels of cyclin D1.

A further question raised by other studies concerns the role of PCNA. The function of PCNA in DNA replication and repair is well established by biochemical experiments (Prelich et al. 1987a, 1987b; Prelich and Stillman 1988; Toschi and Bravo 1988; Shiviji et al. 1992). We now find PCNA in the mitotic cyclin B–CDC2 complexes of normal cells, however, but not in any transformed cells that we have investigated. The function of PCNA in the cell cycle kinase complexes is unknown at present, but we suggest that this protein has a far broader function than has been suspected previously. For example, the PCNA (and p21) that is associated with cyclin–CDK complexes may function as a sensor for detecting DNA damage, growth conditions, or other signals.

A variety of lines of evidence suggest that the association between cyclins and their catalytic partners is not entirely passive. In particular, phosphorylation of the threonine residue 161 of cdc2 (or its equivalent in CDK2) by a protein kinase termed cdc2-activating kinase (CAK)

is required for full binding of cyclin/CDK (Ducommun et al. 1991). Cyclin A, and recently cyclin D1 as well, were found to be phosphorylated on tyrosine residues in vivo (Hall et al. 1991; 1993). We do not know whether the cyclin kinase subunit rearrangements that we have observed in this study reflect altered activity of CAK or phosphatases that might act on an individual subunit of cyclin–CDK complexes; however, these are attractive possibilities that can be investigated.

A final key question concerns the biochemical and physiological consequences of the observations described here. For many years, there has been debate about the relative nature of the cell division cycle of the normal and cancer cell. In very general terms, it has been concluded that both normal and cancer cells of any given type have roughly similar G_1, S, G_2, and M phases, and that the difference between normal and tumor tissue lies instead in the growth fraction, or the percentage of cells that are proliferatively active in any given situation. This finding has tended to focus attention on growth factors, their receptors, and signal transduction pathways that regulate the growth fraction. We would point out, however, that an altered response to a given physiological milieu might be obtained just as readily by alteration of the underlying cell cycle regulatory machinery (cyclins, CDK, etc.), as by alteration of the signal transduction pathways themselves. This study suggests that abnormality of signal transduction and cell cycle pathways are likely to be equally involved in oncogenesis.

Materials and methods

Cell culture

Human diploid lung fibroblast WI-38 cells were obtained from the American Type Culture Collection (ATCC, CCL75) at passage 13 and used between passages 16 and 25. VA13, subline 2RA (ATCC CCL 75.1), is an SV40 virus-transformed derivative of the WI-38 cell line and was obtained from ATCC. HSF43 is a human diploid fibroblast strain derived from neonatal foreskin, and CT10-2C-T1 (CT10) was derived from nontransformed HSF43 by introduction of a plasmid containing the SV40 virus large tumor antigen (T) gene linked to a RSV promoter (Ray et al. 1990). Both HSF43 and CT10 cell lines were kindly provided by Dr. F. Andrew Ray (Los Alamos National Laboratory, NM). CV-1 is an African green monkey kidney cell line, and COS-1 is a SV40 virus-transformed derivative of CV-1 cell line. HeLa is a human cervix epitheloid carcinoma cell line, and 293 is an adenovirus-transformed human embryonal kidney cell line. LCS041 (previously MDAH041) and LCS087 (previously MADH087) fibroblasts were isolated from skin biopsies of two patients with Li–Fraumeni syndrome as described previously [Little et al. 1987; Bischoff et al. 1990; kindly provided by Dr. S. Friend (Massachusetts General Hospital, Boston) and M. Tainsky (M.D. Anderson Cancer Center of The University of Texas)] and were used between passages 170 and 175 and 70 and 75, respectively. All cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 37°C incubator with 5% CO_2, except HeLa and 293, which were cultured in DMEM supplemented with 5% calf serum.

Antibodies and immunological methods

Antisera against human cyclin D1 (Xiong et al. 1992b), human cyclin A (Pagano et al. 1992b), and human CDC2 (G6, Draetta and Beach 1988) have been described previously. Anti-human CDK2 peptide antibody was raised against a synthetic peptide corresponding to the carboxy-terminal region of human CDK2 (K. Galaktionov, unpubl.). Anti-human CDK4 peptide antibody was raised against a synthetic peptide CHSYLHKDEGNPE, with the underlined region corresponding to the carboxy-terminal region of human CDK4 (H. Zhang, Y. Xiong, and D. Beach, in prep.). Affinity-purified anti-PCNA monoclonal antibody was purchased from Oncogene Science, Inc. (Uniondale, NY). Immunoprecipitation and Western blotting procedures were essentially the same as described previously with a modification only in the NP-40 lysis buffer (50 mM Tris-HCl at pH7.4, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 25 μg/ml of leupeptin, 25 μg/ml of aprotitin, 1 mM benzamidine, 10 μg/ml of trypsin inhibitor). For the peptide competition experiments, 1 μg of synthetic peptides was preincubated with 1 μl of specific antisera before the immunoprecipitation.

Partial proteolytic peptide mapping

Immunoprecipitation of [^{35}]methionine-labeled lysates and SDS-PAGE were the same as described above. Polyacrylamide gels were dried without prior fixation and enhancer treatment, exposed to Fuji image plates, and visualized on Fuji Bio-imaging Analyzer BAS2000. Appropriate protein bands were excised from the gels, with the image printout as template, partially digested in the gel with various amount of *Staphylococcus aureus* V8 protease according to Cleveland et al. (1977) and Harlow and Lane (1988), separated on a 17.5% SDS-PAGE. Gels were analyzed on Fuji image analyzer BAS2000.

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