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ARTICLES

SV40 T Antigen Increases the Expression and Activities of $p34^{cdc2}$, Cyclin A, and Cyclin B Prior to Immortalization of Human Diploid Fibroblasts

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Abstract SV40 T antigen induces karyotype instability soon after it is expressed in human diploid fibroblasts and ultimately promotes cell immortalization and tumorigenesis. Protein levels and activities of mitotic cell cycle proteins have been shown to be elevated in several immortal cell lines relative to their normal parental cells, suggesting a possible role for the aberrant regulation of mitosis in karyotype instability. We show here that IMR-90 human diploid lung fibroblasts expressing the SV40 tumor antigens display increased protein levels and associated enzymatic activities of cyclin A, cyclin B, and $p34^{cdc2}$ long before crisis and immortalization. These elevations cannot be explained by faster cell growth or altered cell cycle distributions. Increased protein levels were not totally accounted for by elevated levels of the corresponding mRNA, indicating that T antigen modulates expression at least partially by posttranscriptional mechanisms. These results indicate that perturbation of mitotic regulatory proteins precedes crisis, and imply that altered mitotic control is a direct consequence of T antigen expression rather than an outcome of secondary events associated with immortalization.

Key words: mitosis, cell cycle, cyclin A, cyclin B, $p34^{cdc2}$, immortalization, SV40, T antigen, DNA tumor virus

One of the hallmarks of cancer is the loss of critical checkpoints within the cell cycle, resulting in dysregulated proliferation. Cyclins and cyclin-dependent kinases are believed to drive cell cycle progression by transiently dimerizing and triggering the phosphorylation of key structural and growth-regulatory targets [reviewed in Norbury and Nurse, 1992]. Altered expression or activity of these cyclins and cyclin-dependent kinases may contribute to uncontrolled cell proliferation [reviewed in Marx, 1994; Hunter and Pines, 1991]. For example, the cyclin A gene was the hepatitis B viral integration site in a hepatocellular carcinoma, resulting in elevated expression of a cyclin A fusion protein [Wang et al., 1990, 1992]. Cyclin A, cyclin B, and $p34^{cdc2}$ are components of the cell cycle machinery known to regulate the onset of mitosis in eukaryotic cells [reviewed in Norbury and Nurse, 1992]. At the G_2/M boundary, the $p34^{cdc2}$/cyclin B kinase heterodimer is dephosphorylated and activated by cdc25. This activated kinase, known as maturation promoting factor (MPF), is necessary for the initiation of mitosis in eukaryotic cells. In mitosis, MPF phosphorylates key structural targets, such as histone H1, vimentins, and nuclear lamins, presumably precipitating events such as chromatin condensation and nuclear envelope breakdown [reviewed in Nigg, 1993a,b]. The $p34^{cdc2}$/cyclin A complex, also necessary for the G_2-to-M transition, is activated somewhat earlier than MPF, but appears to share many of the same phosphorylation substrates [Peeper et al., 1993; reviewed in Nigg, 1993a].

Primary human cells can be immortalized by the oncoproteins of certain DNA tumor viruses, such as the simian virus 40 (SV40) tumor antigens. SV40 large T antigen is a multifunctional protein that, when expressed continuously, extends the limited proliferative lifespan of primary cells past the point at which they normally would have become senescent [reviewed in Fanning and Knippers, 1992]. Subsequently, such cells enter a crisis stage, from which immortal clones emerge at a low frequency [Shay and

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Wright, 1989; Ray and Kraemer, 1993]. Upon extended serial passaging, these immortal cells can become tumorigenic in nude mice [Ray and Kraemer, 1993]. SV40 large T antigen alone induces cytogenetic damage in primary human cells long before crisis [Ray et al., 1990; Stewart and Bacchetti, 1991], and the resulting genomic instability is believed to play an important role in cell immortalization and tumor formation.

The mechanism by which T antigen causes chromosomal damage is unknown, but aberrant regulation of mitosis is a likely contributor. Improperly regulated enzymatic activation of the mitotic cell cycle proteins can result in mitotic catastrophe, where mitotic events are uncoupled from other cell cycle processes. Chemicals such as caffeine, 6-dimethylaminopurine, 2-aminopurine, staurosporine, and okadaic acid, when administered to hamster cells in S phase, can induce a premature mitotic state characterized by MPF activation and pulverized, condensed chromatin [Schlegel and Pardee, 1986; Schlegel et al., 1990; Yamashita et al., 1990; Steinmann et al., 1991; Tam and Schlegel, 1992]. In BHK-21 hamster cells, premature mitosis also results from inactivation of the RCC1 gene product [Nishimoto et al., 1978; Uchida et al., 1990] and from the simultaneous, ectopic overexpression of cdc25C and cyclin B [Heald et al., 1993]. In human cells, caffeine, 6-dimethylaminopurine, staurosporine, and okadaic acid can induce apoptosis, characterized by chromatin condensation and activation of cyclin A-associated kinases [Meikrantz et al., 1994]. The lethal mitosis phenotype is seen in fission yeast mutants that either do not express wee1 and mik1 (both inhibitors of cdc2) [Lundgren et al., 1991], do not express wee1 and overexpress cdc25 [Russell and Nurse, 1986], express a semidominant activating allele of cdc2 (cdc2-3w) and do not express wee1 [Russell and Nurse, 1987], or express cdc2-3w and are subjected to hydroxyurea-induced S-phase arrest [Enoch and Nurse, 1990]. Inappropriate activation of mitotic cell cycle regulators also occurs in *rum1* mutants of fission yeast, in conjunction with severe chromosomal damage and cell death [Moreno and Nurse, 1994]. Subtler dysregulation of mitotic control might result in the accumulation of less severe chromosomal aberrations that are not acutely lethal, such as those seen in cells expressing T antigen.

A positive correlation between the immortal state and elevated expression and activities of mitotic cell cycle proteins has been shown in a number of cell systems. Human foreskin keratinocytes immortalized by the oncogenic human papillomavirus (HPV) types 16 and 18, and by the E6 and E7 oncoproteins of HPV type 16, all displayed substantially higher levels and associated kinase activities of cyclin A, cyclin B, and $p34^{cdc2}$ than their normal matched parental keratinocytes [Steinmann et al., 1994]. During the spontaneous immortalization of normal keratinocytes, a sudden increase in cyclin A, cyclin B, and $p34^{cdc2}$ protein levels was noted at a passage coinciding with the emergence of immortal cells, with the appearance of the first chromosomal defect, and with the onset of increasing colony-forming efficiency [Rice et al., 1993]. In a rat epithelial cell line expressing a thermolabile SV40 T antigen, elevated levels of $p34^{cdc2}$ and cyclin A persisted only at the permissive temperature [Oshima et al., 1993]. In addition, increased mRNA and protein levels of cyclins A and B have been seen in human breast cancers relative to normal breast tissue [Keyomarsi and Pardee, 1993; Buckley et al., 1993].

Since chromosomal damage is evident shortly after expression of SV40 tumor antigens and long before crisis and the emergence of immortal clones, we examined the expression and activities of mitotic regulatory proteins in immortal and precrisis human diploid fibroblasts. We report here that SV40-immortalized human diploid fibroblasts display increased protein levels and associated histone H1 kinase activities of cyclin A, cyclin B, and $p34^{cdc2}$ relative to the parental cells, suggesting that such elevations may be a typical event during the immortalization of human cells by DNA tumor viruses. Furthermore, these elevations are induced by the expression of SV40 tumor antigens long before crisis and the appearance of immortal clones, indicating that these changes are consistent with a role in the multistep process of tumorigenesis and are not simply a secondary consequence of immortalization.

MATERIALS AND METHODS

Cell Culture

IMR-90 human diploid lung fibroblasts (catalog #I90 P04) [Nichols et al., 1976] and an SV40-immortalized IMR-90 cell line (IMR-90 SV40, catalog #AGO2804C) [Stein, 1985] were obtained from the National Institute on Aging Cell Repository (Camden, NJ). Cells were grown

in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) containing 10% iron-supplemented calf serum (Hyclone, Logan, UT) and 3.5 mM glutamine in a humidified 10% CO_2 atmosphere at 37°C.

The plasmids used were pSV3neo, which expresses both the large T and small t antigens of SV40, and pSV2neo, which is identical to pSV3neo minus the SV40 viral coding sequences [Southern and Berg, 1982]. Plasmids were transfected into IMR-90 cells by the calcium phosphate method described by Donahue and Stein [1988]. Briefly, 25 μg of plasmid DNA was precipitated in 0.25 M $CaPO_4$, sprinkled over 1×10^6 cells, and incubated overnight. Single cell clones were selected and expanded in 250 μg/ml G418 antibiotic (Gibco BRL, Gaithersburg, MD) and the presence of T and t antigens was confirmed by immunoblotting, using the procedure described below. Subsequent culture revealed that at the time of analysis the pSV3neo clones still had a proliferative capacity of at least 30 additional population doublings before crisis.

Growth Curves

Cells (2×10^4) were plated onto 35-mm dishes. Twenty-four h later, the medium was replaced. Cell number was determined from triplicate plates at the time of medium replacement and on the three days thereafter by trypsinizing cells and counting cell suspensions in a Coulter Electronics ZM counter.

Flow Cytometry

Cells (2×10^5) were plated in 75-cm^2 flasks; 48 h after plating, the cells were harvested by trypsinization, resuspended in 0.2 ml phosphate-buffered saline (PBS) and fixed by the dropwise addition of 0.8 ml cold 70% ethanol. Fixed cells were stored at 4°C for at least 1 week before staining and analysis. Cells were washed with PBS/0.1% bovine serum albumin and stained in a PBS solution containing propidium iodide (100 μg/ml) and ribonuclease A (40 μg/ml) for 1 h at room temperature. Five thousand cells per sample were analyzed on an Ortho Cytofluorograf system 2151, using the Cytomation Cicero program to compile fluorescence histograms.

Immunoblotting

Cellular proteins were extracted by boiling cell pellets for 5 min in Laemmli sample buffer (10 mM Tris pH 6.8, 2% SDS, 4% glycerol, 5% β-mercaptoethanol) and separated by electrophoresis through 10% denaturing polyacrylamide gels [Laemmli, 1970]. Each lane contained 100 μg of protein, as estimated by a modified Lowry protein assay [Peterson, 1977] using γ-globulin as the standard. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P from Millipore, Bedford, MA) in a tank blotting system at 30 V overnight, followed by 1 h at 60 V, in 25 mM Tris pH 8.3/192 mM glycine/20% (v/v) methanol.

Proteins were probed with antiserum raised against the C-terminal peptide of human $p34^{cdc2}$ (LDNQIKKM) [Oshima et al., 1993], with antisera raised against full-length human cyclin A and cyclin B proteins [Oshima et al., 1993], with antiserum 692 recognizing the extracellular signal-regulated kinases ERK1 and ERK2 [Boulton and Cobb, 1991], with supernatant from the PAb108 hybridoma recognizing the amino terminus of SV40 large T and small t antigens [Gurney et al., 1986], and with monoclonal antibody recognizing the C-terminus of SV40 large T antigen, partially purified by ammonium sulfate precipitation from the supernatant of the PAb 423 hybridoma, previously designated as L23 [Harlow et al., 1981]. Probing and washing procedures are as described [Oshima et al., 1993]. Briefly, for cyclin A, cyclin B, and $p34^{cdc2}$, the blots were blocked for one hour in 20 mM Tris pH 7.5/300 mM NaCl/0.3% Tween-20 (TBST) containing 5% Carnation nonfat dry milk and incubated overnight at 4°C in a 1:1,000 dilution of antiserum in the same blocking solution. For T antigen, t antigen, and ERKs, the blots were incubated overnight at 4°C in a 1:1,000 dilution of antiserum or a 1:200 dilution of hybridoma supernatant (for PAb 108) in TBST. All membranes were washed three times with TBST, incubated at room temperature with 0.5 μCi/ml ^{125}I-labeled protein A (ICN, Irvine, CA) in TBST, and washed four times in TBST before detection by indirect autoradiography using Kodak X-OMAT film and an intensifying screen. Bands were quantified by scanning the autoradiographs on a XRS 3cx 256-greyscale flatbed scanner and analyzing the band densities with the BioImage Visage release 4.6 program (Millipore, Bedford, MA).

Histone H1 kinase activity assays

This assay is a modification of the procedure used by Tam and Schlegel [1992]. Asynchronous

cells were washed twice with warm PBS, detached from the monolayer by incubation in PBS/0.5 mM EDTA, lysed by resuspension in lysis buffer (40 mM HEPES pH 7.5, 250 mM NaCl, 15 mM $MgCl_2$, 1% Triton X-100, 5 mM NaF, 5 mM β-glycerophosphate, 2 mM EGTA, 2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM Na_4VO_3, 10 μg/ml each of aprotinin, leupeptin, and pepstatin) containing 18% sucrose, passed ten times through a 25-gauge needle and kept on ice for 30 min. The extract was then clarified by centrifugation, aliquoted, stored at −80°C, and assayed for protein concentration as described above [Peterson, 1977]. Cyclin A, cyclin B and $p34^{cdc2}$ were immunoprecipitated from 50 μg of extract using a 1:100 dilution of the appropriate antiserum at 4°C for 1 h, followed by a 1-h incubation at 4°C in 20 μg/ml protein A–Sepharose beads. $p33^{cdk2}$ and $p34^{cdc2}$ were affinity precipitated from 50 μg of extract by mixing for 1 h at 4°C with 50 μl of $p13^{suc1}$–Sepharose beads, prepared as previously described [Brizuela et al., 1987] using the $p13^{suc1}$-producing bacterial strain BL21(DE3)LysS obtained from Dr. David Beach. Following antibody and $p13^{suc1}$ precipitations, the beads were washed twice in 0.5 ml lysis buffer and three times in 0.5 ml washing buffer (20 mM HEPES pH 7.5, 15 mM EGTA, 20 mM $MgCl_2$). Histone H1 kinase activity was determined by incubation of beads in 40 μl of washing buffer containing 1 mM dithiothreitol, 10 μg/ml A kinase inhibitory peptide (Sigma, St. Louis, MO), 0.5 mg/ml histone H1 (Boehringer Mannheim, Indianapolis, IN), and 50 mM ATP (5500 cpm/pmol) for 20 min at 30°C. The reaction was stopped by adding 40 μl of 2× Laemmli sample buffer (Laemmli, 1970) and boiling for 10 min. Proteins were separated on a 12% SDS-polyacrylamide minigel, fixed in 25% (w/v) trichloroacetic acid, and stained with Coomassie Brilliant Blue R-250. Relative kinase activities were determined by densitometry of the histone H1 bands on the autoradiograph, as described above for immunoblots.

Northern Blotting

Total RNA was isolated from cells lysed in guanidinium isothiocyanate, as described by Chomczinski and Sacchi [1987]; 15 μg RNA was separated in a 1% agarose/formaldehyde gel and transferred to a nylon membrane (Nytran+ from Schleicher and Schuell, Keene, NH) by capillary action. DNA probes were labeled with ^{32}P-dCTP by random priming (Boehringer-Mannheim, Indianapolis, IN), and hybridization was conducted according to manufacturer's instructions (QuikHyb from Stratagene, La Jolla, CA). Detection was by autoradiography using Kodak X-OMAT film, and relative mRNA abundance was determined by densitometry, as described above for immunoblotting. The cdc2 probe used was the *Bam*HI fragment of human cdc2 cDNA (pOB231) described previously [Lee and Nurse, 1987]. The cyclin A and B probes used were the *Eco*RI fragment of pGEM-cyclinA and the *Bam*HI fragment of pGEM-cyclin B, respectively [Pines and Hunter, 1989, 1990]. The PO probe used was the *Pst*I fragment of 36B4 cDNA, described previously [Laborda, 1991].

RESULTS

Expression of SV40 Tumor Antigens Increases the Abundance of Mitotic Regulatory Proteins Prior to Cell Immortalization

In several different cell systems, the expression and associated histone H1 kinase activities of the mitotic regulatory proteins $p34^{cdc2}$, cyclin A, and cyclin B have been shown to be elevated following immortalization by DNA tumor virus oncoproteins [Steinmann et al., 1994; Oshima et al., 1993]. However, such analyses did not establish whether these changes occurred shortly after expression of the viral oncoproteins or whether they were secondary events associated with immortalization. To address this question, we examined normal human fibroblasts expressing the SV40 tumor antigens prior to crisis and cell immortalization.

Plasmids pSV3neo and pSV2neo were transfected into IMR-90 human diploid fibroblasts. pSV3neo contains the entire SV40 early region, expressing both large T and small t antigens [Southern and Berg, 1982]. pSV2neo is identical to pSV3neo, except that it does not contain coding sequences for SV40 tumor antigens [Southern and Berg, 1982]. The neo resistance marker is present on both constructs, permitting direct selection of transfected clones. Since IMR-90 fibroblasts have a long replicative lifetime before senescence (approximately 60 population doublings), cells expressing either SV40 T/t antigens or neo alone can be isolated long before the cells approach crisis or senescence.

The cell types analyzed were: early passage (approximately population doubling 26; d26) and

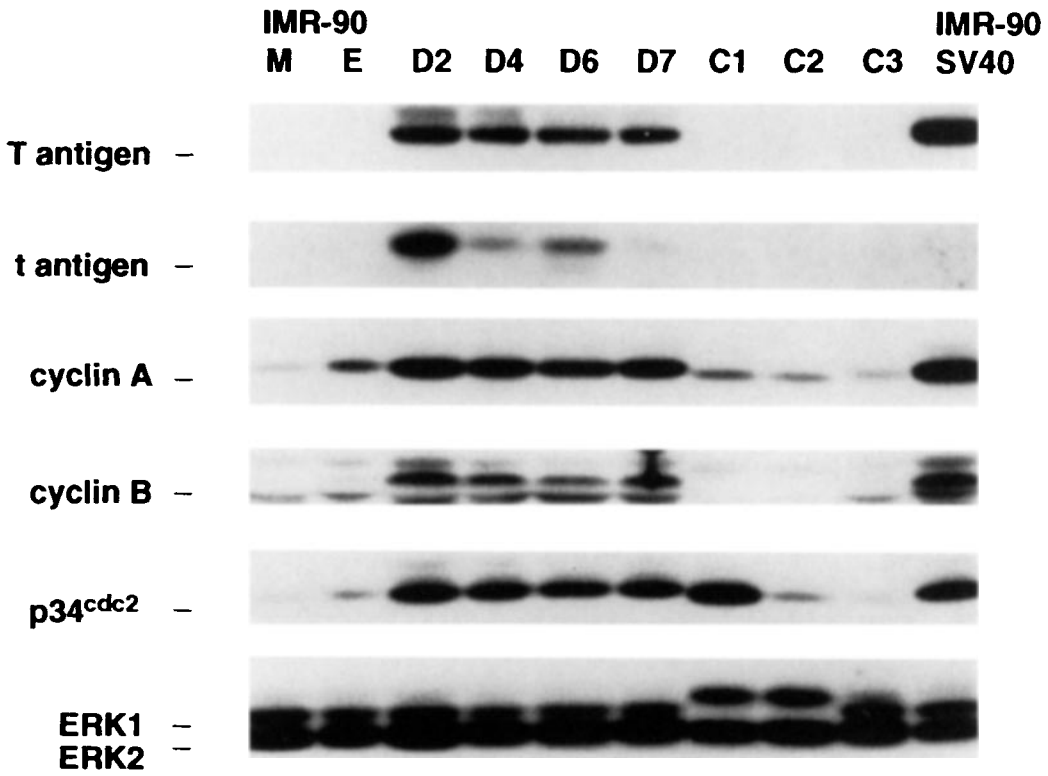

Fig. 1. Immunoblots of large T and small t antigens, cyclin A, cyclin B, $p34^{cdc2}$, and ERK1/ERK2. Laemmli protein extracts were prepared from exponentially growing early (d25, marked E) and middle passage (d40, marked M) IMR-90 fibroblasts, IMR-90 pSV2neo clones (C1, C2, C3), IMR-90 pSV3neo clones (D2, D4, D6, D7), and SV40 transformed IMR-90 cells (IMR-90 SV40); 100 μg of protein from each extract was immunoblotted with the appropriate antibodies, as described in Materials and Methods.

middle passage (d40) parental IMR-90 cells, an IMR-90 cell line transformed by SV40 virus (IMR-90 SV40), three IMR-90 pSV2neo clones (C1, C2, C3; approximate population doubling 55), and four IMR-90 pSV3neo clones (D2, D4, D6, D7; approximate population doubling 55). Equal protein amounts from asynchronously growing cultures were immunoblotted for T antigen, t antigen, cyclin A, cyclin B, $p34^{cdc2}$, and the extracellular signal-regulated kinases ERK1/ERK2 (Fig. 1). As expected, the pSV3neo clones expressed both T and t antigens. Approximately equivalent amounts of T antigen were seen in all four pSV3neo clones, while there was significant variability in the level of t antigen expressed in these clones. The SV40 transformed IMR-90 line (IMR-90 SV40) displayed roughly twice as much T antigen as the pSV3neo clones, although no t antigen was detected. Levels of cyclin A, cyclin B, and $p34^{cdc2}$ were elevated substantially (23-, >40-, and 27-fold, respectively) in the IMR-90 SV40 cell line, compared with the parental IMR-90 cells. Similar increases in cyclin A, cyclin B, and $p34^{cdc2}$ levels were also seen in the precrisis pSV3neo clones D2, D4, D6, and D7 when compared with either the parental cells or with the pSV2neo control clones C1, C2, and C3 (see Table 1 for quantification). The only exception was the pSV2neo clone C1, which expressed as much $p34^{cdc2}$ as the pSV3neo clones. We attribute this anomaly to clonal variation because there is no concomitant increase in cyclin A or cyclin B levels. Independent extracts of parental and precrisis pSV3neo clones were used in a second immunoblot analysis, yielding essentially identical results (Table I). Roughly equivalent protein levels of ERK1 and ERK2, which are required for entry into the cell cycle from quiescence [reviewed in Pelech and Sanghera, 1992; Ruderman, 1993], were seen in all cell types, demonstrating that not all cell cycle-regulated kinases are elevated by T antigen. Interestingly, control clones C1 and C2, but not C3, displayed a slower migrating form of

TABLE I. Relative Protein Levels of Cyclin A, Cyclin B, and p34^{cdc2}*

Cell type	Cyclin A	Cyclin B	p34^{cdc2}
IMR-90 d40	1.0 (1.0)	< 1.0 (1.0)	1.0 (1.0)
IMR-90 d25	4.7	1.6	3.0
C1	3.7	< 1.0	38
C2	2.5	< 1.0	4.2
C3	1.2	< 1.0	1.6
D2	20 (20)	> 32 (24)	28 (36)
D4	15 (14)	> 23 (20)	22 (26)
D6	14 (16)	> 20 (20)	20 (21)
D7	16 (18)	> 36 (18)	21 (19)
IMR-90 SV40	23	> 40	27

*Values are shown relative to IMR-90 d40. Since the cyclin B band for IMR-90 d40 was below the optical density that could be detected by densitometry, values for cyclin B are shown relative to the minimum detectable density. Values in parentheses are from a second assay conducted with independent extracts.

ERK1, suggesting increased phosphorylation of this kinase. These results indicate that T antigen-induced increases in mitotic cell cycle regulatory proteins occur as soon as stably transfected cells can be harvested for analysis and long before crisis and immortalization.

Elevated Protein Levels Are Accompanied by More Modest Increases in mRNA

To determine whether the observed increases in mitotic cell cycle proteins can be explained by increased abundance of the respective mRNA species, whole cell RNA extracts were prepared from normal IMR-90 fibroblasts, IMR-90 fibroblasts transformed by SV40 and clones D2, D4, D6, D7, C1, and C3. RNA extracts were prepared from cells that were within 10 population doublings of those used for protein analysis. A single Northern blot was successively stripped

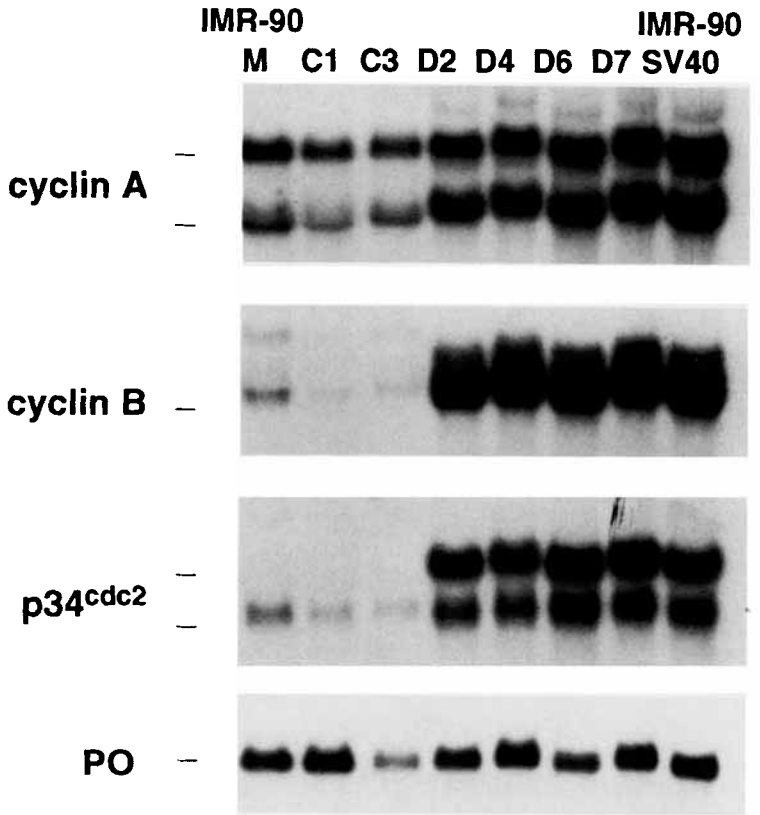

Fig. 2. Northern blots of cyclin A, cyclin B, and cdc2. Guanidinium isothiocyanate extracts were prepared from exponentially growing middle (d58, marked M) passage IMR-90 fibroblasts, IMR-90 pSV2neo clones (C1, C3), IMR-90 pSV3neo clones (D2, D4, D6, D7), and SV40 transformed IMR-90 cells (IMR-90 SV40). Northern analysis was performed as described in Materials and Methods, using successive ^{32}P-labeled DNA probes for cyclin A, cyclin B, cdc2 and PO. Clone C2 was probed on the same Northern blot as the other cell types, but insufficient RNA from this clone prevented us from making a reliable comparison of C2 with the other lanes. When normalized to PO, however, cyclin A, cyclin B and cdc2 mRNA levels for C2 were no greater than those for C1 and C3 (data not shown).

and probed for mRNA levels of cyclin A, cyclin B, cdc2 and the ribosomal phosphoprotein PO, using ^{32}P-labeled DNA probes (Fig. 2). mRNA levels of each species were quantified by densitometry of the autoradiographs using the level of PO to correct for differences in RNA loading (Table II). The steady-state mRNA levels of cyclin B and cdc2 were much higher (17- and 7-fold on average, respectively) in all pSV3neo clones and in IMR-90 SV40 cells when compared with normal parental cells and pSV2neo clones. Remarkably, IMR-90 pSV2neo clone C1, which possessed the highest level of p34^{cdc2} protein of all the examined cell types, displayed the lowest levels of cdc2 mRNA, suggesting extreme translational efficiency or protein stability of cdc2 in this clone. Cyclin A mRNA levels were elevated only 1.5-fold, on average, in precrisis and immortal T antigen-positive cells relative to the control cell populations. Since these elevations clearly cannot account for the roughly 15- to 20-fold increases in cyclin A protein, translational and/or posttranslational mechanisms must play a role in the elevation of cyclin A protein following expression of SV40 tumor antigens.

TABLE II. Relative mRNA Levels of Cyclin A, Cyclin B, and cdc2*

Cell type	Cyclin A	Cyclin B	cdc2
IMR-90 M	1.0	1.0	1.0
C1	0.4	0.2	0.4
C3	1.7	1.8	1.4
D2	1.4	16	7.2
D4	1.2	16	5.6
D6	2.3	24	11
D7	1.3	17	6.4
IMR-90 SV40	1.7	15	5.4

*Values for cyclin A, cyclin B, and cdc2 are normalized to the corresponding PO levels. Cyclin A and cdc2 values represent the sum of two mRNA species.

Elevation of Histone H1 Kinase Activities by SV40 Tumor Antigens

To ascertain whether elevated levels of mitotic regulatory proteins in precrisis pSV3neo clones led to corresponding increases in enzymatic activity, histone H1 kinase assays were performed. Histone H1 kinase activity was precipitated with p13^{suc1} beads, which bind p34^{cdc2} and p33^{cdc2} [Brizuela et al., 1987; Gabrielli et al., 1992], and with antibodies directed against cyclin A, cyclin B and p34^{cdc2}. Enzymatic activity was assayed in primary IMR-90 cells, IMR-90 SV40, and clones C1, C2, C3, D2, D4, D6 and D7 (Fig. 3). All precrisis clones were within ten population doublings of those used for immunoblot analysis. Modest elevations in p13^{suc1}-precipitable, cyclin A-, cyclin B-, and p34^{cdc2}-associated kinase activities were seen in T antigen-positive cell types relative to T antigen-negative cell types, with average increases of 3.5-, 4.5-, 8.5- and 6.2-fold, respectively, as determined by densitometry of the histone H1 band (Table III). A second kinase assay, using independent extracts, was performed on a subset of precrisis T antigen-expressing clones to verify the modest increases observed in the activities

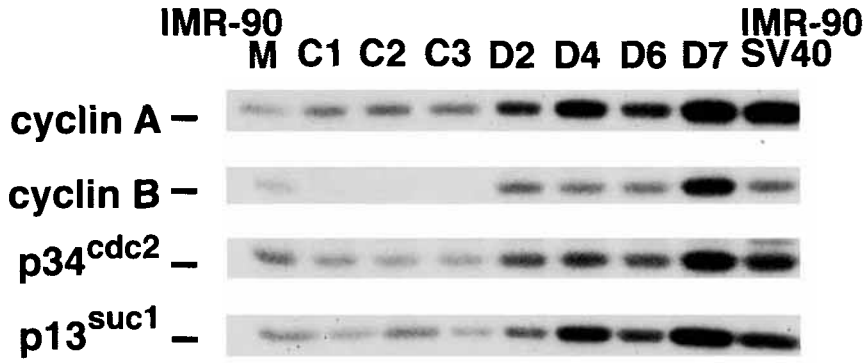

Fig. 3. Histone H1 kinase activities associated with cyclin A, cyclin B, p34^{cdc2}, and p13^{suc1}. 1% Triton X-100 extracts were prepared from exponentially growing middle passage (d37, marked M) IMR-90 fibroblasts, IMR-90 pSV3neo clones (C1, C2, C3), IMR-90 pSV3neo clones (D2, D4, D6, D7), and SV40 transformed IMR-90 cells (IMR-90 SV40); 50 μg of protein was affinity-precipitated with p13^{suc1}–Sepharose beads or immunoprecipitated with antiserum directed against cyclin A, cyclin B, or p34^{cdc2}, followed by incubation with protein-A–Sepharose beads. Kinase assays were performed on the beads, as described in Materials and Methods.

TABLE III. Histone H1 Kinase Activities*

Cell type	$p13^{suc1}$	$p34^{cdc2}$	Cyclin B	Cyclin A
IMR-90 M	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)	1.0
C1	0.63	0.51	0.14	1.6
C2	1.2	0.41	0.10	2.1
C3	0.59	0.48	0.09	2.1
D2	1.3	2.2	2.4	3.3
D4	3.7	2.6	2.2	7.2
D6	2.0 (4.2)	2.2 (3.8)	2.2 (3.9)	4.6
D7	6.0 (4.4)	7.2 (5.2)	4.9 (4.8)	11
IMR-90 SV40	3.5	4.4	2.5	12

*Values are shown relative to the activities in IMR-90 parental cells (approximate population doubling 37). Values in parentheses are from a second assay conducted with independent extracts.

associated with $p13^{suc1}$, $p34^{cdc2}$, and cyclin B (Table III). Results of the second assay were similar to those of the first. Increases in kinase activities were not as great as the corresponding increases in protein levels, presumably due to posttranslational regulatory steps that act to partially suppress activity. It is clear, however, that expression of T antigen increases the activities of mitotic cell cycle proteins prior to immortalization and, therefore, marks an early event during T antigen-induced transformation.

Increased Expression and Activity of Mitotic Regulatory Proteins Is Not a Consequence of Altered Cell Growth Rate or Cell Cycle Distribution

Elevated levels of mitotic regulatory proteins could be explained by accelerated cell growth or by an increase in the population of cells in the G_2 or M phases, where some of these proteins are maximally expressed [Pines and Hunter, 1989, 1990]. Three day growth curves, however, showed no consistent difference between the growth rates of pSV3neo clones and of middle and early passage parental cells (Fig. 4). For instance, clones D2 and D6 had doubling times equivalent to the early passage IMR-90 parental cells, and clone D4 had a longer doubling time, comparable to that of the middle passage IMR-90 cells. In addition, flow cytometry was performed on asynchronous cultures of primary IMR-90 cells, IMR-90 SV40 cells, pSV2 neo clones, and pSV3neo clones (Fig. 5). All clones were of essentially identical age when analyzed by flow cytometry and were within five population doublings of those used in protein, mRNA and kinase assays. A somewhat larger G_2/M cell population

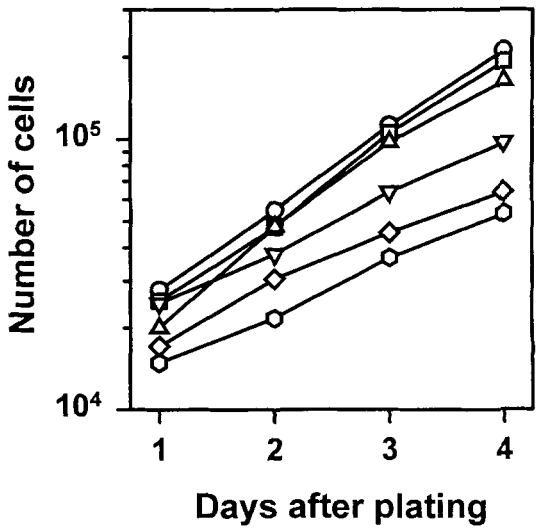

Fig. 4. Growth curves of parental and tumor antigen-expressing IMR-90 cells. IMR-90 fibroblasts, at population doublings 26 (d26; △) and 40 (d40; ◇), and IMR-90 pSV3neo clones D2 (□), D4 (○), D6 (○), and D7 (▽) were plated at 2×10^4/35 mm dish. At the indicated times, cells were trypsinized and the cell number determined with a Coulter counter. Each point is the mean cell count of three dishes, and the standard error does not exceed the symbol size.

was seen in clones D4 (19.2%) and D7 (22.4%), but not D2 (12.8%) or D6 (14.4%), when compared with IMR-90 cells (18.2%) and clones C1 (14.8%), C2 (17.6%), and C3 (12.8%). Even for clones D4 and D7, these modest differences in cell cycle distribution cannot account for the dramatic elevations in the expression of mitotic regulatory proteins. It is clear, therefore, that elevated levels and activities of cyclin A, cyclin B, and $p34^{cdc2}$ in precrisis cells expressing T antigen can occur without increases in the cell growth rate or increases in the G_2/M cell population.

DISCUSSION

We report here that the SV40 tumor antigens elevate the mRNA levels, protein levels and associated kinase activities of the mitotic cell cycle regulatory proteins cyclin A, cyclin B, and $p34^{cdc2}$. While similar elevations have been reported in immortal and tumor-derived cells [Steinmann et al., 1994; Oshima et al., 1993; Rice et al., 1993; Keyomarsi and Pardee, 1993; Buckley et al., 1993], this is the first report of preimmortal cells displaying these changes as a direct consequence of the expression of the SV40 tumor antigens. These findings are consistent with a

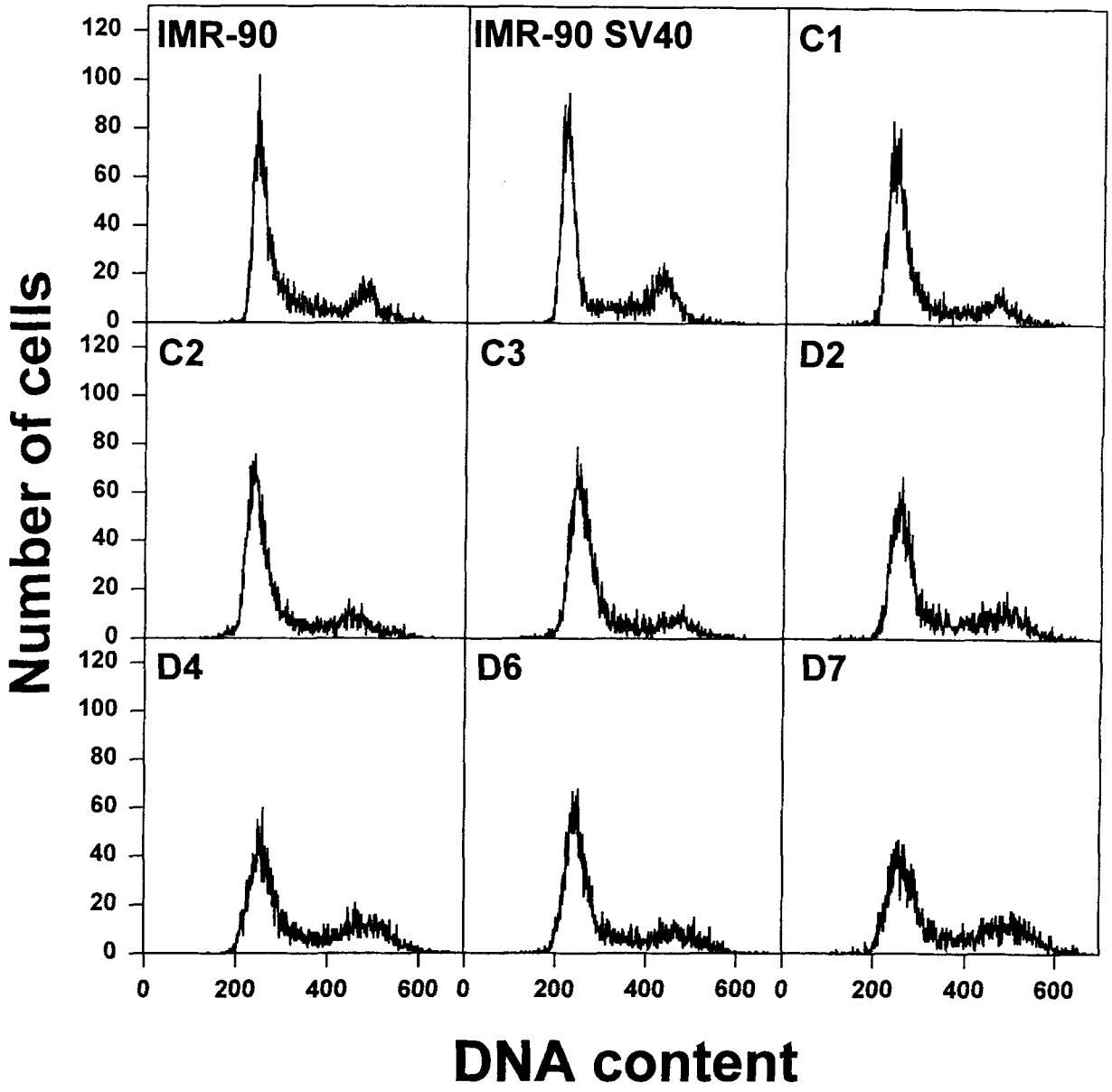

Fig. 5. Flow cytometry of parental and tumor antigen-expressing IMR-90 cells. IMR-90 fibroblasts, SV40-transformed IMR-90 cells, IMR-90 pSV2neo clones (C1, C2, C3), and IMR-90 pSV3neo clones (D2, D4, D6, D7) were prepared for flow cytometry as described in Materials and Methods; 5,000 cells from each sample were analyzed.

recent report which found that one precrisis IMR-90 cell clone transformed with SV40 virus displayed elevated cyclin B protein, $p34^{cdc2}$, and cyclin B-associated histone H1 kinase activity [Kaufmann et al., 1995]. A direct role for T antigen in regulating mitosis has been suggested by experiments using rodent and human cells immortalized with the thermolabile T antigen mutant tsA58. A shift to the restrictive temperature of T antigen causes these cells to arrest growth with an increased G_2 population [Jat and Sharp, 1989; Resnick-Silverman et al., 1991], suggesting that these cells require continuous expression of T antigen for entry into mitosis.

Improper regulation of MPF can greatly increase the frequency of chromosomal aberrations and, in some cases, can cause premature chromatin condensation and mitotic catastrophe [e.g., Nishimoto et al., 1978; Russell and

Nurse, 1986; Schlegel and Pardee, 1986; Russell and Nurse, 1987; Enoch and Nurse, 1990; Lundgren et al., 1991; Heald et al., 1993; Moreno and Nurse, 1994]. Several *S. cerevisiae* genes have been identified that are essential for the G_2 arrest following DNA damage, including *rad9, rad17, rad24, mec1, mec2,* and *mec3.* These mutants proceed into mitosis without G_2 delay and they are sensitive to radiation-induced killing [Weinert and Hartwell, 1988; Schiestl et al., 1989; Weinert et al., 1994]. Mutations in *rad9* also increase the frequency of aneuploidy in undamaged cells [Weinert and Hartwell, 1990]. In addition, moderate overexpression of cdc25, which activates cyclin B/p34^{cdc2} complexes, results in an increased frequency of aneuploidy in *S. pombe* (P. Nurse, personal communication). It is possible, therefore, that the T antigen-induced changes in expression and activities of mitotic regulatory proteins seen in the present study could contribute to the karyotype instability seen in primary human cells expressing this viral oncoprotein.

The expression of T antigen diverts normal human cells from the senescence pathway to the crisis pathway. Senescence is a nonproliferative, stable state associated with increased cell size and a reduced nucleocytoplasmic ratio [Sherwood et al., 1988]. The crisis pathway is characterized by an extended precrisis lifespan, a turbulent period of active cell division and cell death, and potential for immortalization [Stein, 1985]. Senescent human diploid fibroblasts that are stimulated with serum express little or no cdc2, cyclin A, or cyclin B mRNA, even through they can expression other genes associated with serum stimulation of proliferation-competent cells, such as c-myc, c-Ha-ras, thymidine kinase, and histone H3 [Rittling et al., 1986; Seshadri and Campisi, 1990; Stein et al., 1991]. In the crisis pathway promoted by T antigen, we found elevated expression of p34^{cdc2}, cyclin A, and cyclin B at or prior to the onset of extended lifespan. Stimulation of MPF activity may, therefore, facilitate the extended lifespan of cells expressing T antigen.

The mechanism by which T antigen modulates the expression and activities of mitotic regulators is unknown. In the simplest model, T antigen may increase the transcription of the cyclin A, cyclin B and cdc2 genes, either directly or indirectly, with increased protein levels and activities following directly from increased messenger RNA levels. T antigen itself is a transcriptional activator of cellular genes [Segawa and Yamaguchi, 1987; Taylor et al., 1989; reviewed in Fanning and Knippers, 1992] and may be responsible for direct transactivation of cyclin A, cyclin B, and cdc2. Alternatively, since cdc2 and cyclin A contain E2F transcription factor binding sites in their promoters [Henglein et al., 1994; Yamamoto et al., 1994], T antigen could release free E2F by disrupting pRb/E2F complexes [Chellapan et al., 1992]. This latter model is not supported, however, by the earlier finding that rat epithelial cells immortalized by a T antigen mutant defective for pRb binding display increased expression of p34^{cdc2} and cyclin A [Oshima et al., 1993]. T antigen may also stimulate the transcription of mitotic regulatory genes by interacting directly with cellular transcription factors. The promoters of cdc2 and cyclin A contain ATF binding sites [Henglein et al., 1994; Yamamoto et al., 1994], and T antigen may interact with the DNA binding domain of ATF transcription factors in a fashion analagous to adenovirus E1A [Liu and Green, 1994]. Although increased mRNA levels of cyclins and cyclin-dependent kinases could also arise by amplification of these genes, cyclin A and cyclin B are not amplified in several breast cancer lines that overexpress these proteins [Keyomarsi and Pardee, 1993; Buckley et al., 1993], and it is highly unlikely that gene amplification events, which occur at very low frequencies, could explain overexpression in all four SV40 tumor antigen-expressing clones examined. Because increases in cyclin A protein are greater than the increases in the corresponding mRNA, T antigen must also increase translational efficiency and/or protein stability. Similar posttranscriptional increases of cyclin A, cyclin B, and p34^{cdc2} levels have been observed in human foreskin keratinocytes immortalized by human papillomaviruses [Steinmann et al., 1994]. It appears, therefore, that DNA tumor virus oncoproteins often increase the expression and activity of mitotic regulatory proteins, and that these effects result from both transcriptional and posttranscriptional perturbations that arise long before cell immortalization. Such cell cycle alterations may contribute to the genetic instability and neoplastic transformation induced by DNA tumor viruses.

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