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Senescent cells fail to express *cdc2*, *cycA*, and *cycB* in response to mitogen stimulation

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ABSTRACT **Senescent human diploid fibroblasts (HDF) contain no detectable *cdc2* mRNA or p34^{cdc2} protein. Similarly, young quiescent HDF have only low levels of *cdc2* mRNA and protein. After serum stimulation, quiescent HDF accumulate increasing amounts of *cdc2* mRNA and protein and go through DNA synthesis and mitosis. In contrast, serum-stimulated senescent HDF fail to accumulate detectable amounts of *cdc2* mRNA and protein and fail to enter S phase. Mitosis is likewise deficient in senescent cells even when they have been induced to synthesize DNA by simian virus 40 large tumor antigen. Since p34^{cdc2} or its homologues appear to be required for DNA synthesis and mitosis in eukaryotes, a lack of these molecules in serum-stimulated senescent HDF could be an important reason for their inability to enter S phase or mitosis. Nuclear microinjection of *cdc2* DNA into senescent HDF causes rounding up of the cells but no induction of DNA synthesis. Since cyclins A and B are important cofactors of the protein kinase activity of p34^{cdc2} or its homologues, we analyzed expression of these genes in serum-stimulated senescent HDF and determined that they contain little or no *cycA* or *cycB* mRNA. These deficiencies may be relevant to the lack of DNA synthesis and mitosis in senescent HDF.**

The regulation of cell proliferation in eukaryotes involves two control points: a point in G_1 phase that commits the cell to entry into S phase and a point in G_2 phase that regulates entry into mitosis. The *cdc2* gene, which was identified and characterized in the fission yeast *Schizosaccharomyces pombe*, is required for passage through both control points (1). Homologues to *cdc2* have been found in a wide variety of organisms, including man (2, 3). In all cases studied, the *cdc2* kinase acts as the catalytic subunit of M phase promoting factor (4). The hypothesis that p34^{cdc2}, or a closely related homologue, also plays a role in the transition from G_1 to S phase in higher eukaryotes is not as well established, but recent results support this contention. D'Urso *et al.* (5) have partially purified a human cell replication factor (RF-S) that is able to activate replication of simian virus 40 (SV40) DNA in cell-free extracts of G_1 phase cells. RF-S contains p34^{cdc2} and RF-S activity is dependent on this kinase. Blow and Nurse (6) have likewise shown that *Xenopus* p34^{cdc2}, or a closely related protein, is involved in the initiation of DNA replication in *Xenopus* egg extracts. Furukawa *et al.* (7) have found that *cdc2* antisense oligonucleotides reduce *cdc2* expression and DNA synthesis in phytohemagglutinin-stimulated human T lymphocytes without affecting several other early and mid-G_1 events. Furthermore, the original experiments of Lee and Nurse (2), which identified the human homologue of *cdc2*, showed that the human *cdc2* gene can supply the G_1/S and G_2/M functions of *S. pombe cdc2* in mutants of that organism. Finally, Lee *et al.* (8) have shown that the increase in *cdc2* RNA and protein narrowly precedes the increase in DNA synthesis in serum-stimulated quiescent human diploid fibroblasts (HDF). On the other hand, there are reports that injection of rat fibroblasts with antisera to p34^{cdc2} did not block entry into S phase (9) and that mouse cells with a temperature defect in p34^{cdc2} arrested in G_2 but not in G_1 at the nonpermissive temperature (10); however, both studies discuss the possibility that a G_1/S function of p34^{cdc2} was not affected in these experiments.

Cell proliferation in HDF is regulated at three levels: passage through the mitotic cycle, entry into and exit from quiescence, and cessation of proliferation owing to cellular senescence. In senescence and quiescence, the cells are arrested with G_1 phase DNA contents and can be maintained in the nonreplicative state for many months (11, 12). When serum stimulated, senescent HDF express many of the same cell-cycle-regulated genes as do serum-stimulated quiescent HDF—e.g., expression of c-*myc*, c-*jun*, and c-Ha-*ras* (13–15). However, in contrast to serum-stimulated quiescent HDF, serum-stimulated senescent HDF fail to express c-*fos* (15) and fail to phosphorylate the protein product of the retinoblastoma susceptibility gene (RB protein) (16). These data indicate that only a subset of mitogen-induced prereplicative events is deficient in senescent cells. Thus, by identifying this subset of events, we may be able to pinpoint the specific pathway or pathways that are responsible for the cessation of proliferation in senescent HDF. Because of the key role of p34^{cdc2} in commitment to entry into S phase in other systems, and because of its suggested role as the kinase responsible for phosphorylation of the RB protein (17, 18), we investigated whether altered expression of *cdc2* transcripts and protein could play a role in cellular senescence. Furthermore, since p34^{cdc2} and its homologues must associate with a cyclin to be functional, we examined the expression of cyclins A (19, 20) and B (21) in senescent HDF to determine if altered expression of these genes also contributes to the senescent phenotype.

MATERIALS AND METHODS

Cell Culture. IMR-90 human fetal lung fibroblasts (Coriell Institute for Medical Research, Camden, NJ) were cultured as described (22). Young cells (population doubling, 20–32) were made quiescent by growth to high cell density. Senescent cells (population doubling, 63–70) were cultured in growth medium containing 10% serum until they reached the point where they could no longer achieve even one population doubling in 3 weeks with weekly refeedings of sparse cultures. Serum-deprived senescent or quiescent HDF were

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Abbreviations: HDF, human diploid fibroblasts; RB, retinoblastoma susceptibility gene; SV40, simian virus 40; T antigen, large tumor antigen.

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incubated for an additional 4 days in medium containing 0.1% serum. Senescent or quiescent cultures had <5% labeled nuclei following a 24-h period of [^{3}H]thymidine incorporation (22).

CSC303 HDF, which were derived from neonatal foreskin fibroblasts, were used for the nuclear microinjection experiments and to confirm the Northern blot results obtained with IMR-90 cells. These cells were cultured as described (23).

Analysis of Steady-State Levels of RNA (Northern Blots). Poly(A)$^+$ RNA (24) from IMR-90 cells in different growth states was fractionated on formaldehyde/agarose gels and blotted onto nylon-reinforced nitrocellulose. [^{32}P]DNA probes were prepared by primer extension. The Northern blots were probed with either pOB231 (2), pCycA (20), or pCycB (21), which contain human cDNAs for *cdc2*, cyclin A (*cycA*), and cyclin B (*cycB*), respectively. These plasmids were gifts from Paul Nurse, University of Oxford (pOB231), and Jonathan Pines, The Salk Institute for Biological Studies (pCycA and pCycB). Then the blots were reprobed with a plasmid that contains 819 base pairs of the cDNA for human cytoplasmic β-actin (ref. 25; a gift from Donald Cleveland, The Johns Hopkins University). The data were quantitated by either scanning the blot with an Ambis radioanalytic scanner or scanning an autoradiograph of the blot with a densitometer.

Immunoblotting. Whole cell lysates were prepared from IMR-90 cells in different growth states (16) and aliquots containing 70 μg of protein were fractionated on 12% Laemmli SDS/polyacrylamide minigels and transferred to nitrocellulose. Immunoblotting was carried out with rabbit antisera raised against either the PSTAIR peptide (EGVPSTAIREISLLKE) or a C-terminal peptide (LDNQIKKM) of p34^{cdc2}. These antisera were gifts from Paul Nurse and Christopher Norbury, University of Oxford. Peptide-treated antisera were prepared by preincubating the antisera with an excess of the appropriate peptide for 1 h before immunoblotting. Color development was accomplished with alkaline phosphatase-conjugated secondary antibodies and the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (16).

Microinjection. Nuclear microinjection of senescent CSC303 cells was performed as described (23). Briefly, 1 × 10^5 senescent cells were plated onto etched grid coverslips (Bellco Glass) in 35-mm tissue culture dishes. Three to 4 days later, nuclear microinjections were performed on a minimum of 150 uncrowded cells, using either plasmid pOB231 (5) or plasmid pCMVcdc2 containing the *cdc2* cDNA insert from pOB231 in an expression vector driven by the cytomegalovirus promoter. Plasmid DNA was microinjected at a concentration of 1 mg/ml. In some experiments, the cells were refed with fresh serum-containing medium to provide a fresh mitogenic stimulus. The cells were labeled with [^{3}H]thymidine for 26 or 48 h after microinjection and then fixed and processed for autoradiography. The fraction of labeled nuclei in the injected cells was compared to the fraction of labeled nuclei in at least 250 uninjected nuclei on adjacent sections of the same coverslip.

Immunofluorescence Staining. Twenty-four hours after microinjection, the cells were fixed according to Riabowol *et al.* (9). The cells were exposed to primary antiserum G15, which recognizes the C-terminal region of p34^{cdc2}, for 45 min at 37°C in a moist chamber, washed with phosphate-buffered saline (PBS), and treated with biotinylated donkey anti-rabbit antiserum for 1 h at 37°C. After another PBS wash, the cells were exposed to fluorescein isothiocyanate-labeled avidin for 45 min at 37°C, washed again, and mounted for observation under a fluorescent microscope. Antiserum G15 was a gift from David Beach and Jim Bischoff, Cold Spring Harbor Laboratory.

RESULTS

The *cdc2* gene is expressed in asynchronous replicating cultures of young HDF. In Northern blots of poly(A)$^+$ RNA prepared from IMR-90 fetal lung fibroblasts, two transcripts of 2.0 kilobases (kb) and 1.4 kb hybridize to DNA probes prepared either from pOB231 (Fig. 1), which contains a human *cdc2* cDNA insert (2), or from the gel-purified insert alone (data not shown). The larger transcript represents 60–65% of the total *cdc2* mRNA in IMR-90 cells. This proportion is the same in IMR-90 fetal HDF and A41 adult HDF (data not shown), but it can vary in different human cell lines—e.g., in HeLa cells the 1.4-kb band is dominant (26, 27). The relationship between the two transcripts is not known.

***cdc2* Transcripts in Quiescent and Senescent HDF.** IMR-90 cells made quiescent by either growth to high cell density (Fig. 1) or serum deprivation (data not shown) contain low levels of *cdc2* mRNA (4- to 5-fold reduction compared to replicating cells). By 24 h after serum stimulation, the *cdc2* mRNA content of these cells is increased 3- to 6-fold and 34–52% of the cells have entered S phase (Fig. 1 and data not shown). The 2.0-kb and 1.4-kb transcripts are induced in their normal proportions in serum-stimulated quiescent IMR-90 cells. In contrast, senescent HDF that are at the end of their proliferative life-span in 10% serum-containing medium contain no apparent *cdc2* mRNA either before or 24 h after serum stimulation (Fig. 1). Furthermore, there is no significant induction of DNA synthesis in these cells (1–5% labeled

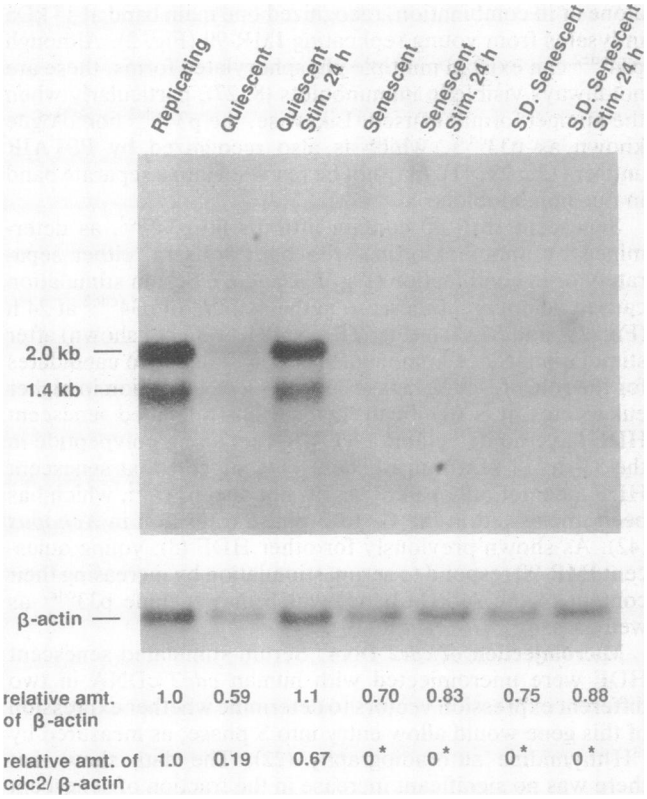

FIG. 1. Steady-state levels of *cdc2* transcripts in senescent, quiescent, and replicating IMR-90 cells. Poly(A)$^+$ RNA was prepared from IMR-90 cells in different growth states and analyzed for the presence of *cdc2* transcripts by Northern blot assay. The headings over each lane summarize the growth state of the cells at the time of harvest. The amount of *cdc2* mRNA in each sample was normalized to the amount of cytoplasmic β-actin in that sample and the relative amount of *cdc2*/β-actin is given at the bottom of the figure. S.D., serum deprived. Asterisks indicate no band was detectable by densitometry.

nuclei during the first 24 h after stimulation). Other senescent IMR-90 cultures were serum-deprived for 4 days prior to serum stimulation to determine whether a change from low to high serum content would induce *cdc2* expression. There is no detectable *cdc2* mRNA in senescent IMR-90 cells regardless of how they are stimulated.

An alternative explanation for the preceding data is that an accumulation of *cdc2* mRNA was not seen in serum-stimulated senescent HDF because it occurred at a different time in these cells than in serum-stimulated quiescent HDF. By the parameter of degree of chromosome condensation, senescent HDF resemble cells at the G_1/S boundary, whereas quiescent HDF are similar to early G_1 cells (28). If senescent HDF were arrested at the G_1/S boundary, then one might expect that *cdc2* expression would occur early after serum stimulation of senescent HDF. To test this hypothesis, we examined the expression of *cdc2* at 2 and 6 h after serum stimulation. No transcripts were detected at these times (data not shown).

$p34^{cdc2}$ in Replicating, Quiescent, and Senescent HDF. Recent studies by McGowan *et al.* (27) indicate that $p34^{cdc2}$ levels remain constant during the HeLa cell cycle even though *cdc2* transcripts are low in G_1 phase and high in S and G_2 phases. Furthermore, Lee *et al.* (8) found that although the level of $p34^{cdc2}$ was decreased in quiescent HDF, it was still readily detectable even though the mRNA transcripts were not. Consequently, we investigated whether senescent HDF contain detectable amounts of $p34^{cdc2}$ by immunoblotting with antisera raised against either the PSTAIR peptide or a C-terminal peptide of $p34^{cdc2}$. These antisera, when used alone or in combination, recognized one main band at 34 kDa in lysates from young replicating IMR-90 (Fig. 2). Although $p34^{cdc2}$ can exist in multiple phosphorylated forms, these are not always visible in immunoblots (8, 27), particularly when the minigel format is used. Likewise, the $p34^{cdc2}$ homologue known as $p33^{cdk2}$, which is also recognized by PSTAIR antisera (20, 29, 41), may not be resolved into a separate band in our immunoblots.

Senescent IMR-90 contain little or no $p34^{cdc2}$, as determined by immunoblotting with both antisera, either separately or in combination (Fig. 2 *A* and *B*). Serum stimulation caused no apparent increase in the amount of $p34^{cdc2}$ at 24 h (Fig. 2 *A* and *B*), 30 h (Fig. 2*B*), or 40 h (data not shown) after stimulation. Since homologues of $p34^{cdc2}$ are also candidates for the role of "cdc2 kinase" in the G_1/S transition in higher eukaryotes, it is significant that serum-stimulated senescent HDF have no detectable PSTAIR-containing polypeptide in the 33- to 34-kDa range. These data suggest that senescent HDF lack not only $p34^{cdc2}$ itself, but also $p33^{cdk2}$, which has been implicated in the G_1 to S phase transition in *Xenopus* (42). As shown previously for other HDF (8), young quiescent IMR-90 respond to serum stimulation by increasing their content of the 34-kDa band, which may include $p33^{cdk2}$ as well as $p34^{cdc2}$.

Microinjection of *cdc2* DNA. Serum-stimulated senescent HDF were microinjected with human *cdc2* cDNA in two different expression vectors to determine whether expression of this gene would allow entry into S phase, as measured by [^{3}H]thymidine autoradiography (22). The data show that there was no significant increase in the fraction of senescent HDF synthesizing DNA following microinjection of *cdc2* DNA (Table 1). In control experiments in which SV40 DNA was microinjected into senescent HDF, 32% of the cells were large tumor antigen (T-antigen) positive and 69% of those T-antigen-positive cells incorporated [^{3}H]thymidine. A comparable fraction (33%) of senescent HDF microinjected with pCMVcdc2 expressed $p34^{cdc2}$ as detected by immunofluorescence (Fig. 3), yet no induction of DNA synthesis occurred. In addition, 20–30 h after microinjection, the senescent HDF exhibited changes in morphology similar to those

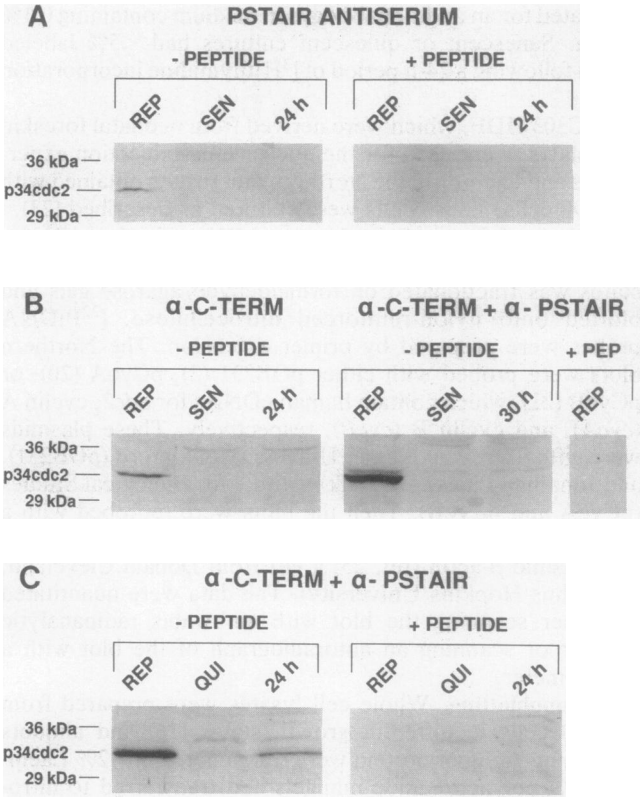

FIG. 2. Steady-state levels of $p34^{cdc2}$ in senescent, quiescent, and replicating IMR-90 cells. Whole cell lysates were prepared from IMR-90 cells in different growth states including serum stimulation of senescent and quiescent cultures. The state of the cells at the time of harvest is indicated by the headings over each lane: REP, replicating cells; SEN, senescent cells; QUI, quiescent cells; 24 h or 30 h, duration of serum stimulation of SEN or QUI cells. The α-PSTAIR and α-C-TERM antisera were used separately and in combination. The combination of both antisera gave the strongest recognition of $p34^{cdc2}$ but also increased the background somewhat. These antisera were also used with and without pretreatment with the corresponding peptide or peptides to confirm the identity of the peptide-specific bands. The molecular mass markers shown are bovine carbonic anhydrase (29 kDa) and rabbit lactate dehydrogenase (36 kDa).

observed by Lamb *et al.* (30) following microinjection of the $p34^{cdc2}$ kinase into rat embryo fibroblasts. The cells rounded up as if preparing for mitosis but did not actually complete mitosis. This morphological change was not observed in uninjected cells or cells injected with SV40 DNA, indicating that it was caused by expression of the *cdc2* DNA in the injected cells. It is not surprising that reexpression of *cdc2* alone is not sufficient to induce either DNA synthesis or mitosis in senescent HDF because the activity of $p34^{cdc2}$ is known to be modulated by its post-translational modifications and its interactions with other proteins, such as cyclins.

Table 1. Microinjection of *cdc2* DNA into senescent HDF

Exp.	Fraction of nuclei labeled		Labeling period, h	Serum stimulation
	Injected	Uninjected		
1	0/151	1/300	26	No
2	2/157	2/250	26	Yes
3	2/204	1/250	48	Yes

Plasmid pOB231, which contains *cdc2* cDNA in an expression vector driven by the SV40 promoter, was used for experiments 1 and 2. Plasmid pCMVcdc2, which contains *cdc2* cDNA in an expression vector driven by the cytomegalovirus promoter, was used in experiment 3.

FIG. 3. Immunofluorescence of senescent HDF microinjected with pCMVcdc2 DNA. Senescent HDF were microinjected with pCMVcdc2 DNA as in Table 1, fixed, and processed for immunofluorescence using primary antibody to the C-terminal region of $p34^{cdc2}$. In the field shown, five cells were positive for $p34^{cdc2}$ expression and two cells (indicated by arrowheads) were negative. (×115.)

Cyclins A and B in Senescent and Quiescent HDF. Since the cyclins are proteins that interact with $p34^{cdc2}$ and/or closely related molecules, we investigated whether they too are down-regulated in senescent HDF. Senescent HDF contain no detectable mRNA for either *cycA* or *cycB*, whereas replicating HDF express two *cycA* transcripts (2.7 kb and 1.8 kb) and one *cycB* transcript (1.7 kb) (Fig. 4), as previously reported for human cells (19, 21). *cycB* mRNA levels reach their maximum in the G_2/M phase of HeLa cells and *cycA* mRNA levels reach their maximum ≈2 h earlier (20). Since serum-stimulated quiescent HDF reach their peak of mitosis at ≈27 h after stimulation (G.H.S., unpublished results), we determined *cycA* and *cycB* transcript levels 24 h after serum stimulation. *cycA* and *cycB* transcript levels are low in unstimulated quiescent HDF and increase 2.5- to 7-fold after stimulation (Fig. 4). In contrast, there are no significant amounts of either *cycA* or *cycB* transcripts in serum-stimulated senescent HDF. These data suggest that a lack of cyclins also contributes to the inability of senescent HDF to traverse the cell cycle.

DISCUSSION

Senescent HDF cannot be induced to enter S phase by stimulation with serum or other mitogens. Furthermore, senescent HDF that have been stimulated to synthesize DNA by either SV40 infection or microinjection of SV40 DNA are unable to go through mitosis (31, 32). The data presented in this paper show that senescent HDF and serum-stimulated senescent HDF lack significant amounts of *cdc2* mRNA and $p34^{cdc2}$ protein. Furthermore, the data suggest, but do not prove, that the $p34^{cdc2}$ homologue $p33^{cdk2}$ is also deficient in senescent cells. Since current evidence supports the hypothesis that $p34^{cdc2}$ is necessary for mitosis and that either $p34^{cdc2}$ or a closely related homologue is necessary for entry into S phase (42), these data suggest that the lack of *cdc2* or *cdc2*-like molecules in senescent HDF could be an important reason for their inability to traverse the cell cycle.

Senescent HDF and serum-stimulated senescent HDF also lack transcripts of the *cycA* and *cycB* genes. Cyclin B is a cofactor for the kinase activity of $p34^{cdc2}$ at mitosis, but the role of cyclin A as either an interphase or mitotic cyclin is less well understood (4, 19–21, 33). Very recent results suggest

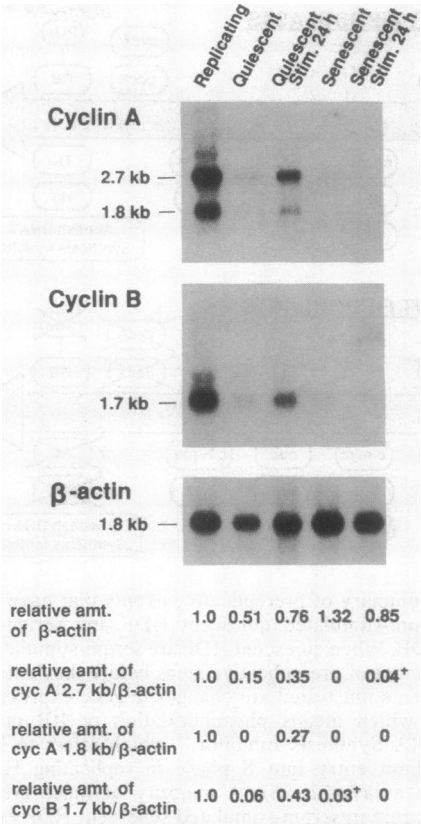

FIG. 4. Steady-state levels of cyclin A and cyclin B transcripts in senescent, quiescent, and replicating IMR-90 cells. A Northern blot of poly(A)$^+$ RNA from IMR-90 cells in different growth states was prepared and analyzed as in Fig. 1. The same blot was probed sequentially for cyclin A, cyclin B, and β-actin transcripts. Daggers indicate values are not significantly different from zero.

that cyclin A may act at the initiation of S phase by stimulating $p34^{cdc2}$ and possibly other kinases to phosphorylate one of the proteins that helps get DNA replication under way (34). If so, then a lack of cyclin A in serum-stimulated senescent HDF may be an important reason why these cells cannot initiate DNA synthesis. On the other hand, a lack of cyclin B in serum-stimulated senescent HDF may help explain why SV40-induced DNA synthesis in senescent HDF does not lead to mitosis, even though it is possible that SV40 T antigen induces expression of *cdc2* in these cells (see ref. 35). The lack of *cycA* and *cycB* transcripts in serum-stimulated senescent HDF that also lack *cdc2* transcripts suggests further that there may be coordinate control of expression of these genes in human cells.

Since serum stimulation induces many of the same prereplicative events in senescent HDF as in quiescent HDF (Fig. 5), it is possible that the altered events in senescent HDF are clustered in just one or a few pathways that are deficient in senescent HDF. Prereplicative events that are altered in serum-stimulated senescent HDF include failure to show increased levels of c-*fos* (15) and *cdc2*, failure to phosphorylate RB protein (16), failure to abolish the inhibitor of entry into S phase as measured by the heterokaryon assay (22, 36), and failure to enter S phase (Fig. 5). There are already some connections that can be made between these events. (*i*) Unphosphorylated RB protein, a putative inhibitor of cell proliferation (39), is likely to be at least one component of the inhibitor of entry into S phase measured by the heterokaryon assay because only cells that contain RB-binding oncoproteins—i.e., SV40 T-antigen, adenovirus E1A, and HPV E7—are able to overcome the inhibitory effect of senescent HDF in heterokaryons (22). (*ii*) Several studies suggest that the

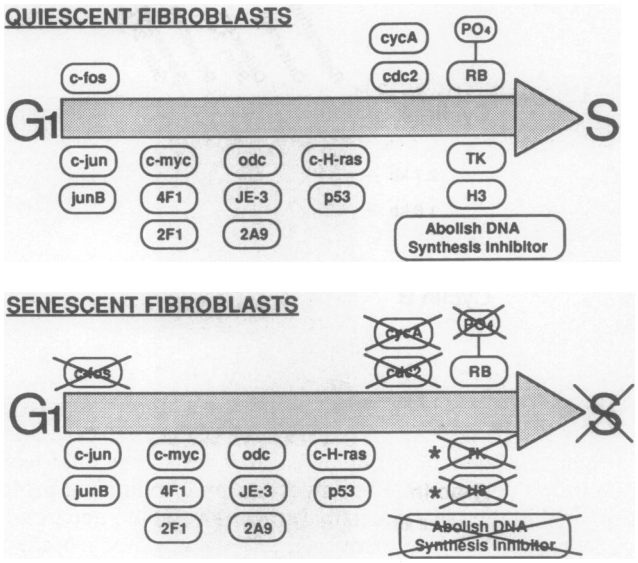

FIG. 5. Summary of prereplicative events that have been compared in serum-stimulated quiescent HDF and serum-stimulated senescent HDF. When quiescent HDF are serum-stimulated, they go through a number of prereplicative steps before they enter S phase. All of the events illustrated are changes in gene expression except "PO_4–RB," which means phosphorylation of RB protein, and "Abolish DNA Synthesis Inhibitor," which refers to loss of the ability to inhibit entry into S phase in replicating HDF in the heterokaryon assay (22, 36). A majority of these prereplicative events still occurs in serum-stimulated senescent HDF even though these cells are unable to enter S phase (13–15). The key differences are the events that are crossed out in the senescent HDF diagram. The status of the events marked with asterisks is uncertain because there are contradictory reports in the literature concerning whether they occur in serum-stimulated senescent HDF (13, 15, 37, 38). *cycB* is not included in this diagram because its function is postreplicative (21), whereas *cycA* may have a prereplicative role (33, 34). odc, Ornithine decarboxylase; TK, thymidine kinase; H3, histone H3.

$p34^{cdc2}$ kinase may be responsible for phosphorylation of RB protein *in vivo* (17, 18, 43). Thus, at least one reason that $p34^{cdc2}$ might be necessary for entry into S phase could be its role in phosphorylating RB protein. (*iii*) RB itself may play a role in regulating the transcription of c-*fos* because recent studies show that transient expression of RB in cycling and serum-stimulated mouse 3T3 cells lowers the level of expression of c-*fos* (40). (*iv*) Taken together, the data from senescent HDF suggest the possibility that c-*fos* may play an upstream role in a cascade of events that ultimately regulates expression of *cdc2*, *cycA*, and *cycB*.

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