Transformation by v-Jun Prevents Cell Cycle Exit and Promotes Apoptosis in the Absence of Serum Growth Factors

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Abstract
To gain insight into the mechanism of action of the v-Jun oncprotein, we compared the growth and cell cycle behavior of normal and v-Jun-transformed fibroblasts. We show that v-Jun induces marked alterations in cell cycle regulation in both the presence and absence of serum growth factors. During asynchronous growth, v-Jun-transformed fibroblasts divide more rapidly than their normal counterparts, owing to a reduction in the length of the G1 phase of the cell cycle. When deprived of serum mitogens, normal fibroblasts exit the cycle and enter a reversible state of quiescence (G0). In contrast, v-Jun-transformed fibroblasts continue to cycle and maintain increased levels of retinoblastoma tumor suppressor protein phosphorylation and elevated expression of cell cycle-dependent markers such as cyclin A, cyclin-dependent protein kinase 2 (CDK2), and CDC2. v-Jun-transformed fibroblasts nevertheless remain wholly dependent on growth factors for cell multiplication, because cell cycle progression in the absence of serum is accompanied by high rates of apoptotic cell death. We conclude that v-Jun shares the capacity of the Myc, E1A, and E2F oncproteins to promote both cell cycle progression and apoptosis under conditions of mitogen depletion.

Introduction
The v-Jun oncogene of ASV17 encodes a mutated version of the product of the c-jun proto-oncogene, p39 c-Jun (1). c-Jun and its relatives JunB and JunD constitute the prototypic family of basic region-leucine zipper transcription factors that bind to and activate transcription through TREs or AP-1 sites (2). Jun proteins can bind to TRE motifs either as homodimers (3) or by forming a variety of heterodimeric complexes with basic region-leucine zipper partner proteins encoded by the fos proto-oncogene and cAMP-responsive element binding protein/activating transcription factor families (4). Many cell types express multiple members of each family, resulting in the formation of a complex array of homodimeric and heterodimeric TRE-binding complexes that collectively define the generic transcription factor AP-1 (5, 6).

Although the specific biological functions of the individual members of the jun and fos families are only poorly understood, several lines of evidence implicate c-Jun in the control of cell proliferation. c-jun mRNA and protein expression are rapidly induced when quiescent fibroblasts are exposed to mitogens (5, 7), and intervention experiments demonstrate that inhibition of c-Jun activity during mitogenic stimulation prevents subsequent entry into S-phase (8). Furthermore, fibroblasts derived from c-jun nullizygous mouse embryos display severely retarded growth in culture, presumably reflecting the failure of some rate-limiting process or event necessary for cell cycle entry or progression (9). In view of its role as a transcriptional regulator, it seems likely that c-Jun is required for the expression of genes whose products in turn influence the cell cycle machinery directly; however, no such genes have yet been identified.

Cell cycle entry and progression require the sequential activation of a series of CDKs in association with their cognate cyclin regulatory subunits (10). Several mechanisms have been identified that modulate the catalytic activity of CDKs. These include fluctuations in the availability of cyclin subunits, phosphorylation of specific residues that activate or inhibit function, and physical association of the CDK or cyclin/CDK complex with inhibitors such as p16/INK4, p21 Waf-1/CIP1, and p27 KIP1 (reviewed in Refs. 11-13).

In normal fibroblasts, the decision to embark on a new cell cycle or to enter quiescence (G0) after mitosis is made in the early part of G1 and is determined primarily by the prevailing concentration of mitogens (14). One of the key regulatory events that occurs during G1 involves phosphorylation of the pRb at multiple sites that inactivate its growth-suppressive function and permit the cell to enter S-phase (reviewed in Ref. 15). pRb phosphorylation is thought to be catalyzed predominantly by CDK4 (or CDK6) in association with D-type cyclins (notably cyclin D1) and also by CDK2 complexed with cyclin E (collectively referred to as G1 cyclin/CDK complexes; Ref. 15). pRb phosphorylation occurs concurrently with and may be required for passage through the restriction point, a discrete time in G1 beyond which further progression through the cell cycle becomes independent of mitogens.

One important downstream target of pRb action is the E2F transcription factor. Hypophosphorylated pRb binds tightly
to E2F and inhibits its transcriptional activation function (16). This interaction is dissociated as a consequence of pRb phosphorylation in mid- to late G1, thereby releasing E2F from inhibition and enabling it to activate the expression of genes required for DNA synthesis and entry into S-phase (17-22).

The importance of this process for growth control is underscored by the fact that several viral and cellular oncogenes promote cell cycle progression by undermining or circumventing the growth-suppressive activity of pRb. For example, ectopic overexpression of E2F impels quiescent cells into S-phase (23), whereas a similar end point is achieved by the adenovirus E1A oncoprotein through physical dissociation of preexisting pRb/E2F complexes (24). pRb also seems to be an indirect target of the Myc oncoprotein because induction of Myc activity in growth factor-deprived quiescent fibroblasts triggers activation of latent cyclin D1/CDK4 and cyclin E/CDK2 complexes, leading to pRb phosphorylation and entry into S-phase (25, 26). This latter example is of particular interest because other evidence suggests that Myc may form part of the normal circuitry through which mitogens promote cell cycle entry or progression (27, 28).

Curiously, in addition to their mitogenic and transforming properties, the Myc, E1A, and E2F oncoproteins are also potent triggers of programmed cell death or apoptosis under suboptimal growth conditions (29-32). Although this is strongly suggestive of a causal link between cell cycle progression and cell death, the nature of the relationship between these processes, if any, remains obscure (33).

The requirement for c-Jun for both entry into S-phase from G0 and continued cell proliferation (8, 9) is in many ways reminiscent of similar requirements for Myc (34, 35), suggesting that the cell cycle machinery may be a principal target for both of these transcription factor oncogenes. If this is true, then clearly transformation by the v-Jun oncoprotein should result in the loss or abrogation of normal cell cycle controls.

To evaluate this possibility, we have compared the cell cycle behavior of normal fibroblasts before and after transformation by v-Jun. We show that v-Jun induces profound alterations in cell cycle control, including shortening of the G1 phase, failure to exit the cell cycle in the absence of growth factors, and increased susceptibility to apoptosis. Furthermore, these alterations are associated with loss or reduction in the mitogen-dependence of a number of biochemical processes required for cell cycle progression, including pRb phosphorylation and expression of cyclin A, CDK2, and CDC2. These results suggest that v-Jun, like Myc, E1A, and E2F, promotes both cell cycle progression and apoptosis.

**Results**

**v-Jun-transformed Fibroblasts Divide More Rapidly but Remain Dependent on Serum Growth Factors for Proliferation.** Because the function of the c-jun proto-oncogene product is required for serum-induced mitogenesis in quiescent fibroblasts (8), we considered that constitutive expression of the oncogenic derivative, v-Jun, might alleviate the requirement for serum growth factors for cell proliferation. To investigate this possibility, we compared the growth of normal and v-Jun-transformed primary CEFs in normal GM and LS (Fig. 1A; see "Materials and Methods" for details). Cultures of fibroblasts uniformly transformed by ASV17, which encodes v-Jun, were regenerated as described previously (36). For control purposes, the normal cultures were infected with nontransforming helper virus (RCAS; Ref. 36).

This experiment (Fig. 1A) showed that whereas both normal (RCAS) and v-Jun-transformed (ASV17) fibroblasts grew vigorously in normal GM, neither was able to proliferate significantly in LS, although in neither case did the number of cells remaining after 4 days decline significantly below that
Fig. 2. Kinetic analysis of cell cycle progression in normal and v-Jun-transformed fibroblasts. A, schematic diagram of the experimental protocol. Cultures of normal and v-Jun-transformed fibroblasts were pulse-labeled for 30 min with BrdUrd and then chased for the indicated times in conditioned medium. At each time point, cells were trypsinized, fixed, and analyzed by two-parameter flow cytometry. The percentage of S-phase, G2/M, and G1 cells in the labeled cohort at each time point was then calculated by deconvolution of the DNA content histograms and is presented in B, C, and D, respectively.

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originally plated. It was also apparent that v-Jun-transformed fibroblasts divided more rapidly than their normal counterparts during exponential growth phase, with doubling times estimated at 20 and 27 h, respectively.

G1 is Abbreviated in v-Jun-transformed Fibroblasts. To understand why v-Jun-transformed fibroblasts grew more rapidly than their normal counterparts, we first compared the cell cycle distribution of the cultures by flow cytometry (fluorescence-activated cell sorting) after pulse-labeling with BrdUrd to identify cells actively synthesizing DNA.

This analysis revealed that the v-Jun-transformed cell population contained a striking preponderance of cells in S-phase and a corresponding depletion of cells in G1 phase compared to normal fibroblasts. This was evident from DNA content cytograms and readily quantitated by BrdUrd labeling and two-parameter flow cytometry (Fig. 1, B and C; data not shown). Although the proportion of S-phase cells in the v-Jun-transformed culture declined somewhat with increasing cell density (from 57 to 33%; Fig. 1C), it remained consistently several-fold higher than the corresponding percentage in the normal fibroblast population (from 20 to 15%; Fig. 1C).

An alteration in the relative proportions of the G1 and S-phase compartments in an asynchronously growing population could result from either an increase in the length of S-phase, a decrease in the length of G1, or a combination of both. To distinguish between these possibilities, normal and v-Jun-transformed fibroblasts were pulse-labeled during exponential growth phase with BrdUrd and then harvested either immediately or after increasing chase periods in the absence of label (Fig. 2A). The progress of the labeled cells through the subsequent G2/M, G1, and following S-phases was then followed by fluorescence-activated cell sorting analysis using computer deconvolution of DNA content histograms of the labeled cohort (see "Materials and Methods" for details).

As shown in Fig. 2B-D, although both normal and v-Jun-transformed fibroblasts exited S-phase, passed through G2/M, and began to enter the succeeding G1 rapidly and with similar kinetics, their subsequent cell cycle progression was distinct. Normal fibroblasts accumulated in G1 between 3 and 8 h, resulting in a perceptible lag phase before any significant entry into the subsequent S-phase occurred (first evident at 10 h, Fig. 2B), whereas v-Jun-transformed fibroblasts began to enter S-phase much more rapidly (Fig. 2B) and showed a corresponding overall reduction in the number of cells that accumulated in G1 at each time point (Fig. 2D).

These results demonstrate that v-Jun-transformed fibroblasts on average spend significantly less time in G1 after passing through mitosis before entering S-phase than their normal counterparts. Thus, abbreviation of the G1 phase of the cell cycle is likely to explain the more rapid division rate of v-Jun-transformed fibroblasts. Due to the nonuniform age structure of asynchronous populations, it seems likely that this also accounts for the observed increase in the proportion of S-phase cells, although additional experiments will be required to confirm that the duration of S-phase is in fact identical in both cell types.
Expression of Cell Cycle-regulatory Proteins in v-Jun-transformed Fibroblasts. To identify possible mechanisms through which v-Jun might accelerate cell cycle progression, we investigated the expression of a number of cell cycle regulators by Western blotting. We first examined cyclin D1 and the cyclin/CDK inhibitor p27 KIP1 because these are considered to be important positive and negative determinants of G1 progression, respectively (37–39). As shown in Fig. 3, cyclin D1 protein expression was modestly increased in v-Jun-transformed cells during exponential growth (GM), and this difference was accentuated when the cultures were maintained in LS for 48 h, conditions that cause normal fibroblasts to exit the cell cycle and become quiescent (see next section). In contrast, the level of p27 KIP1 was similar in both cell types, although a small increase that occurred in quiescent normal fibroblasts was not seen in serum-deprived transformed cells (Fig. 3).

The overall levels of cyclin A, CDK2, and CDC2 were also elevated in growing v-Jun-transformed fibroblasts compared to normal fibroblasts, and strikingly, in each case, expression persisted in the transformed cells in LS at either maximal (CDC2 and CDK2) or much higher levels (cyclin A) than in quiescent normal fibroblasts (Fig. 3). As discussed herein, the persistence of these cell cycle-dependent markers reflects the failure of v-Jun-transformed fibroblasts to exit the cell cycle effectively under these conditions.

Cell Cycle Progression and pRb Phosphorylation Are Dependent on Serum Growth Factors in Primary Avian Fibroblasts. Because transformation by v-Jun advanced the onset of the G1/S-phase transition in growing cells, we reasoned that it might also disturb regulation of the G1/S transition. To investigate this possibility, we first determined the effect of growth factor deprivation followed by serum refeeding on cell cycle progression in normal fibroblasts.

As shown in Fig. 4A, when normal fibroblasts in exponential growth were transferred to LS, the percentage of cells in S-phase declined steadily, reaching a stable minimum of 2% after 48 h. This decline was accompanied by a corresponding increase in the number of cells in G0/G1 (i.e., cells with 2N DNA content). That these cells had exited the cycle and were in G0, rather than G1, was evident from the fact that only a very small proportion (<5%) became labeled even when exposed to BrdUrd for prolonged periods (Fig. 5).

Western blotting analysis of extracts prepared from replicate cultures demonstrated that pRb, which was predominantly hyperphosphorylated in growing cells, became progressively dephosphorylated after transfer to LS until only the hypophosphorylated form was detectable in quiescent cells (Fig. 4B). As already noted, exit from the cycle was also accompanied by a marked decline in the levels of cyclin A, CDC2 (Fig. 4B), and CDK2 (Fig. 3; data not shown).

Refeeding these quiescent cultures with serum growth factors induced synchronous cell cycle reentry as judged by an increase in the percentage of cells in S-phase, which rose steeply after 7 h to reach a peak at 13 h, and a reciprocal decline in the proportion of G1/S cells (Fig. 4A). Synchrony was also evident from the progress of pRb phosphorylation, which persisted in its hypophosphorylated form for 3 h after serum addition but then underwent an abrupt conversion to the hyperphosphorylated forms typical of growing cells that was complete by 5 h. Thus, as in other cell types, pRb phosphorylation is triggered in mid-to late G1 during serum-induced mitogenesis in CEFs. As expected, expression of both cyclin A and CDC2 was induced during cell cycle reentry, although with somewhat different kinetics (Fig. 4B). The level of CDK2 also increased in parallel with cyclin A (data not shown).

v-Jun-transformed Fibroblasts Continue to Cycle and Sustain pRb Phosphorylation in the Absence of Serum Growth Factors. A very different outcome was observed when v-Jun-transformed fibroblasts were subjected to the same experimental protocol (Fig. 6A). In this case, although the percentage of cells in S-phase also declined after transfer to LS, it stabilized at a much higher steady-state level (12%). Furthermore, although the number of cells with 2N DNA content also increased, unlike quiescent normal fibroblasts, most of these had not entered G0 because a significant proportion (approximately 50%) became labeled when the cultures were incubated with BrdUrd for 18 h and must therefore have successfully passed through both S-phase and mitosis during that time (Fig. 5; data not shown). Because a substantial proportion of cells in the culture continue to cycle under these conditions, the increase in the proportion of G1 cells must reflect either a slowing in the rate of S-phase entry, selective depletion from the culture of cells from subsequent phases of the cycle (see next section), or a combination of both.

Continued cell cycle progression was also evident from the behavior of the biochemical markers of cell cycle progression
that we examined: (a) the hyperphosphorylated forms of pRb typical of growing normal cells persisted in v-Jun-transformed fibroblasts after 48 h in LS, although small amounts of hypophosphorylated pRb also became evident (Fig. 6B); and (b) the levels of cyclin A and CDC2, which declined dramatically in quiescent fibroblasts (Fig. 4B), persisted at much higher levels in the transformed cells (Fig. 6B), as did CDK2 (Fig. 3; data not shown).

v-Jun-transformed fibroblasts also responded very differently to mitogenic stimulation with serum (Fig. 6A). In contrast to the synchronous cell cycle reentry seen in normal fibroblasts, the percentage of S-phase cells in the v-Jun-transformed culture began to increase slowly but immediately, with no evidence of either a G1 lag or a synchronous entry to S-phase (Fig. 6B). Furthermore, we saw no evidence of any coordinated change in the phosphorylation of pRb, which persisted as a mixture of hypo- and hyperphosphorylated forms throughout the duration of the experiment (Fig. 6B). Neither was there any change in the level of CDC2 (or CDK2, data not shown), and only a slow and relatively modest increase in the expression of cyclin A, which paralleled the increase in the number of S-phase cells (Fig. 6B). Thus, v-Jun-transformed fibroblasts not only fail to exit the cell cycle and enter G1 efficiently when deprived of mitogens, but they also sustain biochemical processes that are strictly mitogen- or cell cycle-dependent in normal cells.

Clearly, these results could be explained if v-Jun-transformed cells secreted mitogens and thus stimulated their own cell cycle progression by an autocrine mechanism. To test this idea, cultures that had been maintained in LS for 48 h were transferred to either normal GM or LS that had previously been conditioned by exposure to normal or v-Jun-transformed cells for 24 h. BrdUrd was then added, and the percentage of G0/G1 cells that became labeled in 18 h was
determined by flow cytometry. As shown in Fig. 5, conditioned medium from transformed cells had little, if any, detectable mitogenic effect on normal fibroblasts, nor did medium conditioned by normal cells inhibit cycling by transformed cells. Thus, v-Jun most likely disturbs cell cycle regulation by a cell-autonomous rather than an autocrine mechanism.

**v-Jun Promotes Apoptosis in the Absence of Serum Growth Factors.** The finding that v-Jun-transformed fibroblasts continued to cycle in the absence of growth factors was unexpected because we had previously found that they required serum for proliferation (Fig. 1A). During the course of the cell cycle studies described previously, however, we noted that large numbers of v-Jun-transformed cells detached and accumulated in suspension when the cultures were maintained in LS. Detached cells were not observed when the transformed cells were maintained in normal GM, nor were significant numbers of cells detached from cultures of quiescent normal fibroblasts in LS (Fig. 7).

To investigate this phenomenon further, we harvested the cells that detached during sequential 24-h periods after transferring growing v-Jun-transformed cultures to LS. Adherent cells from normal and v-Jun-transformed cultures in normal GM or after 48 h in LS were harvested by trypsinization for comparison. A portion of each sample was analyzed by flow cytometry, whereas genomic DNA was prepared from the remainder and analyzed by gel electrophoresis.

This analysis revealed that the detached cells were apoptotic as judged by two conventional criteria: (a) they formed a population whose mean DNA content was significantly less than 2N (Fig. 7A, arrow); and (b) genomic DNA isolated from the detached cells was found to be fragmented into the characteristic pattern of oligonucleosomal fragments diagnostic of apoptotic cell death (Fig. 7B). In contrast, the attached cells in both normal and transformed cultures had conventional DNA cytograms and contained intact genomic DNA regardless of whether the culture was growing in normal GM or had been maintained in LS for 48 h (Fig. 7, A and B). Thus, although v-Jun-transformed fibroblasts continue to cycle in LS, cell multiplication is limited by high rates of apoptotic death.

**Discussion**

Although v-jun was one of the first oncogenes known to be derived from a cellular transcription factor (1), the cellular target genes through which it elicits its oncogenic effects have still not been identified. One obstacle to uncovering these genes has been uncertainty as to the precise nature of the phenotypic changes induced by v-Jun during cell transformation. Whereas some nuclear oncogenes such as myc are considered to promote cell proliferation by acting directly or indirectly on the cell cycle machinery (25, 26), others such as fos seem to have little effect on proliferation per se but instead induce alterations in cell morphology or enhanced invasive potential (40-42). This functional heterogeneity suggests that different oncogenes promote tumorigenesis in vivo by disturbing different aspects of normal cell behavior; however, the specific effects of v-Jun on cell physiology have not been documented.

Because c-Jun is required for both proliferation and serum-induced mitogenesis in untransformed fibroblasts (8, 9), we reasoned that cell transformation as a result of constitutive overexpression of v-Jun might reflect a loss of normal cell cycle controls and/or altered growth factor responses. To evaluate this possibility, we compared the growth and cell cycle behavior of primary avian fibroblasts before and after transformation by v-Jun. As we have shown, this analysis revealed that v-Jun induces marked alterations in cell cycle regulation that suggest that it, like Myc, acts in G1 to promote cell cycle progression. Several lines of evidence point to this conclusion:

(a) During asynchronous growth, v-Jun-transformed fibroblasts divide more rapidly than normal and show a striking increase in the proportion of S-phase cells and a corresponding depletion of cells in G1. Kinetic analysis has shown that both the increased division rate and altered cell cycle distribution are attributable to a reduction in the duration of the G1 phase of the cell cycle. Thus, when cells are proliferating in optimal concentrations of growth factors, one effect of v-Jun is to accelerate the onset of S-phase as a result of more rapid progression through G1.

(b) Transformation by v-Jun prevents effective cell cycle withdrawal in the absence of serum growth factors. Whereas normal fibroblasts exit the cell cycle and enter a reversible state of quiescence (G0) when cultured in LS, v-Jun-transformed fibroblasts continue to cycle and sustain increased levels of pRb phosphorylation and elevated expression of cell cycle-dependent markers such as cyclin A, CDK2, and CDC2. Because normal fibroblasts are thought to require mitogens primarily to enable them to pass the G1 restriction
Fig. 6. v-Jun-transformed fibroblasts continue to cycle and sustain pRb phosphorylation in the absence of serum growth factors. A, the percentage of S-phase and G1/G0 cells was determined by two-parameter flow cytometry after pulse-labeling with BrdUrd for 30 min. Cultures of v-Jun-transformed CEFs in exponential growth were transferred to LS for 48 h, after which 10% NBS was added for the indicated times. Note that the time scale to the left and right of the vertical bar is different. B, progress of pRb phosphorylation and cyclin A and p34 CDC2 expression. Equal portions (50 μg) of whole-cell extracts prepared from replicate dishes corresponding to the samples shown in A were resolved by SDS-PAGE and analyzed by Western blotting using the indicated antisera.

Although v-Jun-transformed fibroblasts continue to cycle in LS, viable cell multiplication is limited by high rates of apoptotic cell death. Thus, there are many similarities between the alterations in cell cycle regulation induced by v-Jun and those induced by constitutive overexpression of Myc, which also shortens G1 during exponential growth (43) and both prevents cell cycle exit and promotes apoptosis in the absence of serum growth factors (25, 30).

Clearly, an important question arising from these studies concerns the molecular mechanism(s) through which v-Jun affects the cell cycle machinery. Although it is formally possible that nontranscriptional mechanisms are involved, we think it more probable that v-Jun, as with Myc (26), must affect the expression of genes whose products in turn influence cell cycle progression directly. Unfortunately, owing to the highly interdependent nature of cell cycle processes and the difficulties of distinguishing cause from effect, identifying the primary point of action of v-Jun may not be straightforward.

For example, although cyclin A and CDC2 expression are maintained at unusually high or maximal levels in serum-deprived v-Jun-transformed fibroblasts, it seems unlikely that either of these genes is a direct transcriptional target of v-Jun. Instead, because each is known to be regulated at least in part by E2F (17, 21), we speculate that their continued expression may be an indirect consequence of the increased levels of pRb phosphorylation that persist in the transformed cells under these conditions because this will presumably release increased amounts of free transcriptionally active E2F compared to quiescent cells.

Although this remains to be verified experimentally, it is clear that many of the other cell cycle effects that we have documented could be explained if v-Jun, like Myc, promotes
pRb phosphorylation and potentially other rate-limiting processes that are required for transit through the G1 restriction point. This could account for both the more rapid onset of S-phase during exponential growth and the inability of v-Jun-transformed cells to exit the cell cycle efficiently under conditions of mitogen deprivation.

Our attention was drawn to cyclin D1 as a potential target of v-Jun for several reasons: (a) overexpression of cyclin D1 itself accelerates G1 progression (37, 38); and (b) the cyclin D1 gene promoter has been shown to be transactivated by c-Jun in transient transfection assays (44), suggesting that c-Jun may play a role in regulating cyclin D1 expression in normal cells. Consistent with this latter possibility, cyclin D1 protein expression is modestly elevated in v-Jun-transformed cells and therefore could potentially contribute to the abbreviation of G1. Additional experiments will be required to test this idea; however, even if confirmed, it seems unlikely that this alone could account for all of the phenotypic effects of v-Jun because overexpression of cyclin D1 is insufficient to prevent cell cycle withdrawal in the absence of serum growth factors in normal fibroblasts (37, 38).

We also investigated the expression of p27 KIP1 because recent evidence suggests that the intracellular concentration of this cyclin/CDK inhibitor is a major determinant of G1 progression and restriction point control (39). Although we found little evidence that transformation by v-Jun reduced the absolute amount of p27 KIP1, we speculate that its activity may nevertheless be compromised on stoichiometric grounds because the levels of cyclin/CDK complexes (for example cyclin A/CDK2) seem likely to be higher than in normal cells, particularly under conditions of mitogen deprivation. Biochemical analysis of cyclin/CDK kinase activity and p27 KIP1 association in normal and v-Jun-transformed fibroblasts will be required to test this prediction. It will also be important to determine whether v-Jun disturbs the expression or activity of other G1 regulators that we have not yet investigated, such as cyclin D2, cyclin D3, cyclin E, or cdc25A (15, 45).

Finally, our results demonstrate that v-Jun-transformed fibroblasts are highly susceptible to apoptosis upon mitogen deprivation, and it is this, rather than cell cycle arrest, that primarily limits their multiplication under these conditions. Similar effects have been documented with the Myc, E1A, and E2F oncogenes (29-32), and it seems likely that, as in these other cases, the ability of v-Jun to promote apoptosis will prove to be closely linked to its ability to promote cell cycle progression, although this remains to be demonstrated directly. It will be interesting to determine whether v-Jun-transformed cells die at a specific stage of the cell cycle, whether cell death is p53-dependent, and whether, as with Myc-induced apoptosis, cell death can be suppressed by specific survival factors (30, 46).

Materials and Methods

Cell Culture. Secondary CEFs were cultured in DMEM supplemented with 10% tryptose phosphate broth, 10% NBS, and 2% chicken serum. For serum refedding experiments, cultures were maintained in DMEM supplemented with 10% tryptose phosphate broth and 0.2% NBS for 48 h, followed by the addition of 10% NBS for the indicated time. Culture medium was replenished every 24 h during all experimental protocols.

ASV17 virus was regenerated by transfecting CEFs with ASV17 proviral DNA (pASV17-241; Ref. 47) together with RCAS to provide helper virus functions as described (36, 48). CEFs were infected with ASV17 virus, and the cultures were passaged until uniformly transformed. Normal CEF cultures were infected with RCAS helper virus alone.

Flow Cytometry. For DNA content analysis, adherent cells were detached by trypsinization and washed once with PBS, and the cell suspension was fixed in 70% ethanol. Fixed cells were subsequently resuspended in PBS containing 10 μg/ml propidium iodide and 250 μg/ml RNase A. After 1 h at room temperature, the samples were analyzed using a Becton Dickinson FACScan flow cytometer. To quantitate cells engaged in DNA synthesis, BrdUrd (Sigma) was added to GM to a final concentration of 10-25 μM (depending on labeling time). When chase periods were required, these were carried out either by using conditioned medium from replicate cultures or by preparing the experimental cultures in double the normal volume of GM and removing half before labeling. After labeling,
Acknowledgments

Preparation of Cell Extracts and Western Blotting. Whole-cell extracts were prepared from cultures of normal or ASV17-transformed CEFs as described previously (36). Briefly, cultures (90-mm dishes) were washed twice with PBS at 4°C, drained thoroughly, and lysed by the addition of 100 μl of Buffer E. The extracts were then clarified by Eppendorf centrifugation for 30 min at 4°C, the protein concentration was measured using a Coomassie protein determination kit (Pierce), and the extract was stored at -70°C.

For Western blotting, 50-μg portions of whole-cell extract were resolved by electrophoresis on 10% SDS-PAGE gels, and the proteins were transferred to nitrocellulose. Blots were reacted with primary and secondary peroxidase-conjugated anti-rabbit or anti-mouse antibodies in Blotto [3% w/v nonfat dry milk in Tris-buffered saline containing 0.1% w/v Tween 20 (TBST)], and the stained proteins were visualized using the Abersham enhanced chemiluminescence system. The antisera used were as follows: α-cyclin D1 (rabbit polyclonal; PharMingen 14326), α-pRb (mouse monoclonal; Pharmingen 14326), α-CDK2 (rabbit polyclonal; Santa Cruz sc-163G), α-p27 KIP1 (rabbit polyclonal; a kind gift of S. Coats and J. Roberts; Ref. 39), α-CDC2 (rabbit polyclonal; R8; a kind gift of G. Maridor and E. Nigg; Ref. 49), and α-cyclin A (rabbit polyclonal; R28; a kind gift of G. Maridor and E. Nigg; Ref. 50).

References