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The Viral Ki-*ras* Gene Must Be Expressed in the G_2 Phase if *ts* Kirsten Sarcoma Virus-Infected NRK Cells Are To Proliferate in Serum-Free Medium†

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NRK cells infected with a temperature-sensitive Kirsten sarcoma virus (*ts*371 KSV) are transformed at 36°C, but are untransformed at 41°C which inactivates the abnormally thermolabile oncogenic $p21^{Ki}$ product of the viral Ki-*ras* gene. At 41°C, *ts*KSV-infected NRK cells were arrested in G_0/G_1 when incubated in serum-free medium, but could then be stimulated to transit G_1, replicate DNA, and divide by (i) adding serum at 41°C or (ii) dropping the temperature to a p21-activating 36°C without adding serum. When quiescent cells at 41°C were stimulated to transit G_1 in serum-free medium by activating p21 at 36°C and then shifted back to the p21-inactivating 41°C in the mid-S phase, they continued replicating DNA but could not transit G_2. Reactivating p21 in the G_2-arrested cells by once again lowering the temperature to 36°C stimulated a rapid entry into mitosis. By contrast, while serum-stimulated quiescent G_0 cells at 41°C replicate DNA and divide, serum did not induce G_2-arrested cells to enter mitosis, indicating that serum growth factors may trigger events in the G_1 phase that ultimately determine G_2 transit. These observations made with the viral *ras* product suggest that cellular *ras* proto-oncogene products have a role in G_2 transit of normal cells.

There is increasing evidence that at least some cellular proto-oncogene products are linked to processes which regulate cell proliferation. The product of the c-*myc* proto-oncogene is a nuclear protein which appears to function near the start of the G_1 phase as a competence-establishing factor (14). The products of other proto-oncogenes, including c-*sis*, c-*erbB*, and c-*neu*, are related to serum growth factors (6, 27) or their receptors (2, 7).

Perhaps the best characterized proto-oncogene products are those of the cellular *ras* genes which are thought to transduce membrane signals necessary for G_1 transit and the initiation of chromosome replication. The *ras* proteins are localized in the plasma membrane (29), bind guanine nucleotides (21, 22), and have weak GTPase activities (11, 16, 26). Unlike c-*myc* expression, c-*ras* expression increases only in the middle to late G_1 phase in a variety of cultured cells (5, 18) and in regenerating rat liver cells (13). Microinjection of purified Ha-*ras* protein (p21) causes quiescent NIH 3T3 cells to transit G_1 and initiate DNA replication (9), whereas the injection of antibody to p21 inhibits the entry of these and other cells into the S phase after serum stimulation (17). The proliferation of NIH 3T3 cells transformed by oncogenes coding for several growth factor or receptor components depends on c-*ras* proteins (25). Mutant activated forms of the c-*ras* proto-oncogenes are found in many human tumors (reviewed in references 12 and 23) and could be responsible for the loss of normal growth control and expression of the transformed phenotype. Indeed, we have previously shown that activating an endogenous $p21^{v\text{-}Ki}$ protein in quiescent NRK cells infected with a temperature-sensitive (*ts*) mutant of the Kristen sarcoma virus (KSV) is sufficient to trigger all the events needed for DNA replication and cell division even in medium without exogenous growth factors (8).

While normal c-*ras* proteins appear to be necessary for, and v-*ras* proteins sufficient for, G_1 progression, their roles in other phases of the cell cycle remain unknown. We can now report that, aside from its role in G_1, $p21^{v\text{-}Ki}$ activity is required for the G_2 transit and multiplication of *ts*KSV-infected NRK cells in serum-free medium.

MATERIALS AND METHODS

Cells and media. *ts*KSV-infected NRK cells (*ts*KSV-NRK cells) were the generous gift of M. Scolnick (Merck Sharp & Dohme, West Point, Pa.). The *ts*KSV-NRK cell line was produced by infecting NRK cells with the temperature-sensitive, transformation-defective KSV mutant *ts*371 (24). Stock cultures were maintained at 36°C in a humidified atmosphere of 5% CO_2 and 95% air in a complete medium consisting of 85% (vol/vol) Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Va.) and 15% (vol/vol) bovine calf serum (Colorado Serum Co., Denver, Colo.) and containing the antibiotic gentamicin.

Cell quiescence and stimulation. *ts*KSV-NRK cells were removed from stock cultures with 0.25% trypsin in phosphate-buffered saline, plated in complete medium at a density of 2.75×10^3 cells per cm^2 in 35-mm dishes containing 25-mm cover slips (Thermanox; Miles Laboratories, Toronto, Ontario, Canada), and incubated at 40°C for 48 h. They were then rendered quiescent in G_0/G_1 by incubation in serum-free medium (Dulbecco modified Eagle medium-F12 [1:1] containing 1 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.2) for 48 h at 41°C. The cells were incubated for the first 48 h at 40°C rather than 41°C because of a transiently reduced plating efficiency at the higher temperature which was caused by trypsinization. The quiescent cells at 41°C were normally stimulated to resume proliferation by either (i) adding serum to the medium to a final concentration of 10% at 41°C or (ii) lowering the temperature from 41°C to a permissive 36°C without adding any exogenous mitogens. Dropping the temperature to 36°C

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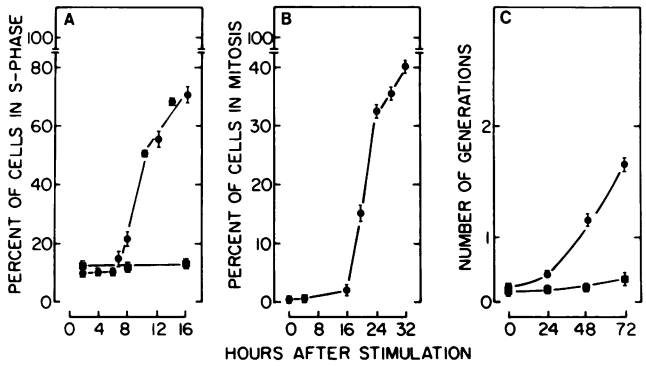

FIG. 1. Activating the viral Ki-*ras* gene product in quiescent *ts*KSV-NRK cells causes the cells to transit G_1 and replicate DNA (A), to enter mitosis (B), and to divide in serum-free medium (C). Cells of logarithmically growing cultures were rendered quiescent in a G_0 state by incubation at 41°C in serum-free medium for 48 h. They were then left unstimulated at 41°C (■) or were stimulated to resume cycling at zero hours by lowering the temperature to a p21-activating 36°C (●). Then: (A) [^{3}H]thymidine was added to the cultures at zero hours and the proportion of cells with labeled nuclei was determined at various times thereafter as described in Materials and Methods; (B) at the indicated times cultures which had been exposed to colchicine for the previous 4 h were analyzed for the proportion of colchicine metaphases as described in Materials and Methods; and (C) cell numbers were measured at various times with a Coulter electronic cell counter. The points are the means ± standard errors of the means (bars) of at least three determinations.

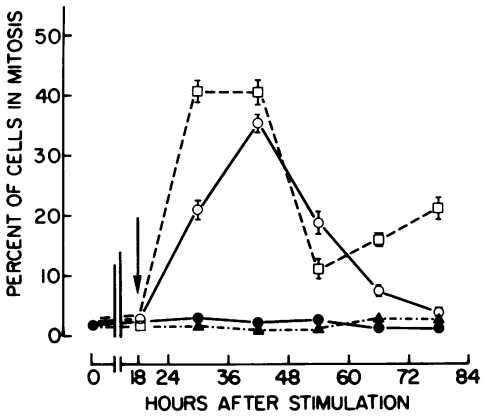

FIG. 2. Inactivation of the viral *ras* protein in the mid-S phase prevents *ts*KSV-NRK cells from entering mitosis. Quiescent G_0 cultures of serum-deprived cells at 41°C were: left unstimulated at 41°C (●); stimulated to resume cycling at zero hours by serum addition at 41°C (○); or stimulated to resume cycling by lowering the temperature to a p21-activating 36°C at zero hours (□, ▲). Some of the p21-stimulated cultures were returned to the nonpermissive 41°C at 18 h (arrow) when the cells were in the middle to late S phase (▲). In all cases the proportion of cells in mitosis was determined at the indicated times after a 12-h exposure to 1 μg of colchicine per ml as described in Materials and Methods. The points are the means ± standard errors of the means (bars) of six determinations.

activates the abnormally thermolabile p21 product, and the cells rapidly resume cycling in serum-free medium (8).

Measurement of DNA-synthetic activity. DNA synthetic activity was indicated by the proportion of cells whose nuclei were labeled during an exposure to [^{3}H]thymidine (5 μCi/ml of medium; specific activity, 20 Ci/mmol; New England Nuclear Corp., Boston, Mass.). Cells were processed by the autoradiographic procedure of Whitfield et al. (28) in which radioactivity is found only in DNA and produces about 500 silver grains in the nuclear track emulsion overlaying each DNA-synthesizing cell. Between 1,000 and 1,500 cells were counted per sample to determine DNA synthetic activity.

Measurement of mitotic activity. The flow of cells into mitosis was measured by the proportion of cells which were arrested in metaphase during an exposure to 1 μg of colchicine per ml. Colchicine was added to the culture medium for the periods indicated in the legends to the appropriate figures, and the cells were then fixed and stained with hematoxylin. Between 800 and 1,000 cells per sample were examined microscopically to determine the proportion of colchicine-induced metaphases.

Cell cycle distribution. Cells were prepared for flow cytometry by the procedure of Boynton et al. (4). The relative DNA content of the propidium iodide-stained cells was measured with a model 50H cytofluorograph from Ortho Diagnostics Systems, Inc. (Westwood, Mass.) equipped with an argon-ion laser tuned to an excitation wavelength of 488 nm. DNA distribution data were analyzed with an Ortho Diagnostics 2150 computer system. The laser power and the photomultiplier gain were the same for all samples.

RESULTS

NRK cells infected with a temperature-sensitive KSV mutant produce an abnormally thermolabile viral Ki-*ras* protein (24) which transforms the cells at 36°C but which cannot transform them at the nonpermissive 41°C (8). Consequently, *ts*KSV-NRK cells arrest in the G_0/G_1 state when cultured at 41°C in serum-free medium (8). These quiescent cells can then be stimulated to resume cycling as nontransformed cells at 41°C by adding serum or as transformed cells by lowering the temperature of incubation to a p21-activating 36°C without adding serum (8). Thus, activating the viral *ras* protein in the absence of exogenously supplied growth factors induced quiescent *ts*KSV-NRK cells to transit G_1 and to start replicating DNA after a delay of about 8 h (Fig. 1A). These p21-stimulated cells began to enter mitosis and divide about 10 h after the onset of DNA replication (Fig. 1B). Once shifted to 36°C, the cells multiplied steadily, although slowly, in the serum-free medium (Fig. 1C), indicating that the $p21^{v\text{-}Ki}$ was able to both initiate and sustain proliferation without serum growth factors.

In serum-free medium, quiescent *ts*KSV-NRK cells typically entered mitosis 18 to 24 h after being stimulated by a lowering of the incubation temperature from 41°C to a p21-activating 36°C (Fig. 2). However, less than 1% of the cells that had been stimulated from the G_0 state by p21 activation and then shifted back to a p21-inactivating 41°C

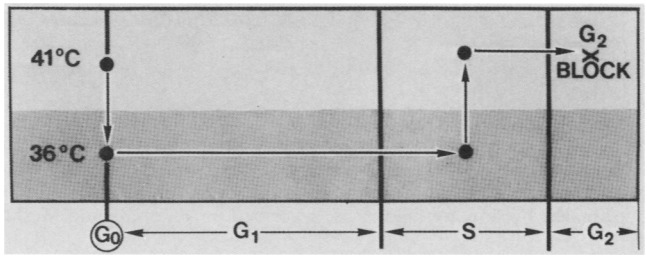

FIG. 3. Diagram illustrating the method used to arrest p21-stimulated *ts*KSV-NRK cells in the G_2 phase.

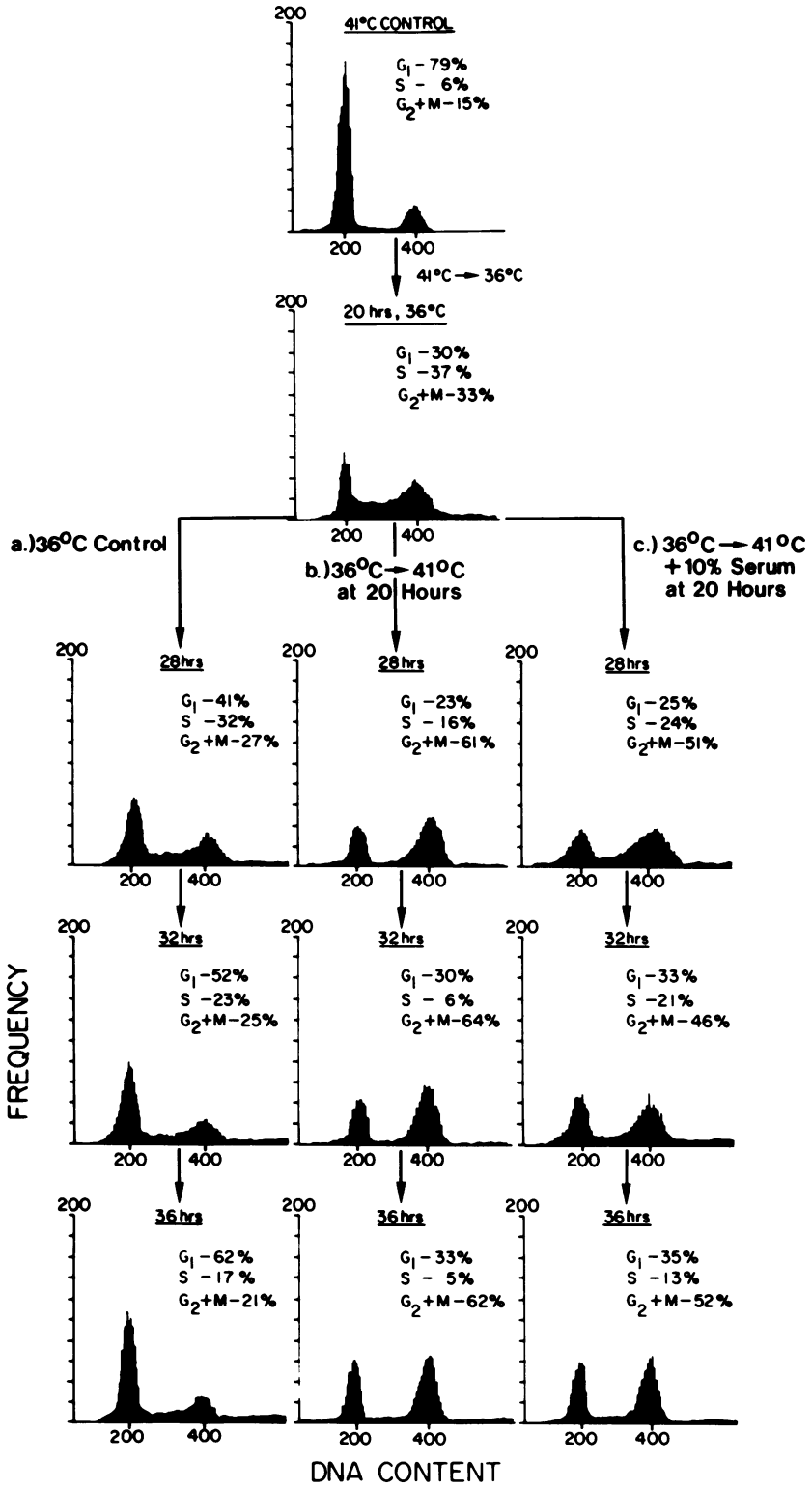

FIG. 4. *ts*KSV-NRK cells need active $p21^{v\text{-}Ki}$ protein to transit G_2 in serum-free medium. Quiescent G_0 cells that had been incubated for 48 h at 41°C in serum-free medium were stimulated to resume cycling by dropping the temperature to a p21-activating 36°C. Twenty hours later, when the cells were in the middle to late S phase, the cultures were either (a) left at the permissive 36°C, or (b) returned to the nonpermissive 41°C, or (c) returned to the nonpermissive 41°C and serum added to 10%. At the indicated times, cell cycle distributions were determined by flow cytometry as described in Materials and Methods.

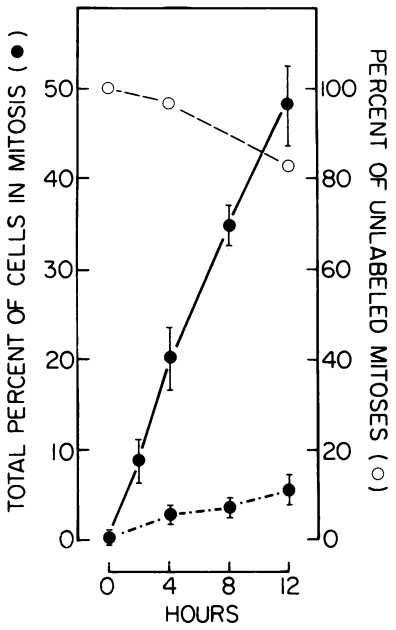

FIG. 5. Cells arrested in the G_2 phase were induced to enter mitosis by reactivating the viral Ki-*ras* protein. Quiescent cells in the G_0 state were stimulated to resume cycling in serum-free medium by activating the Ki-*ras* protein with a 41 to 36°C shift. Eighteen hours later, when the cells were in the middle to late S phase, $p21^{v\text{-}Ki}$ was inactivated by returning the cultures to the nonpermissive 41°C. The cells were allowed to accumulate in G_2 for the next 12 h, and then (i.e., at zero hour) the cultures were either left at 41°C (—●—) or shifted to 36°C to reactivate the viral K-*ras* protein (—●—). Both [^{3}H]thymidine and colchicine were added to the cultures at zero hour to measure the flow of cells into mitosis (●) and the proportion of mitotic cells which did not pass through any part of the S phase (i.e., did not incorporate [^{3}H]thymidine into their chromosomes) on their way to mitosis (○). The points are means ± standard errors of the means (bars) of at least four determinations.

after they had reached the middle to late S phase (16 to 20 h after stimulation; Fig. 3), could enter mitosis (Fig. 2). Moreover, the cell number in these cultures did not increase for at least 3 days after such treatment (data not shown). The block resulted from the inactivation of p21 and not from an intrinsic inability to transit G_2 and enter mitosis at the nonpermissive temperature, because quiescent cells stimulated from G_0 by serum at 41°C entered mitosis nearly as efficiently as cells stimulated by p21 activity at 36°C (Fig. 2). The inhibition also could not have been due to the shock of a sudden 5°C rise in temperature at a critical point in the S phase because quiescent cells which were treated with serum as well as shifted to the p21-activating 36°C transited G_2 and entered mitosis normally after being shifted back to 41°C in the mid-S phase. In these control experiments, more than 70% of the serum-treated cells that entered mitosis at 36°C were still able to enter mitosis during the same 18-h period after the temperature was raised to 41°C in the mid-S phase. These observations suggested that v-*ras* expression is required the G_1 phase as well as at some point between the mid-S phase and mitosis if *ts*KSV-NRK cells are to proliferate in serum-free medium.

To find out when p21 was needed in the later stages of the cell cycle, we resorted to flow cytometry. Inactivating p21 at a time when most cells were in the S phase (i.e., raising the temperature from 36 to 41°C at 20 h) did not inhibit ongoing DNA replication (Fig. 4b), indicating that the cells did not need $p21^{v\text{-}Ki}$ activity to transit the S phase. However, once they had replicated their chromosomes, the majority of cells (~60%) arrested either in the G_2 phase or in mitosis (Fig. 4b). Since the blocked cells were not visibly in mitosis (Fig. 2), they must have stopped in G_2. Thus, *ts*KSV-NRK cells needed $p21^{v\text{-}Ki}$ activity to transit G_2 and initiate mitosis in serum-free medium.

The G_2 block induced in *ts*KSV-NRK cells by inactivating p21 when the cells were in the S phase was readily reversed by reactivating the viral p21 protein. Thus, cells blocked in G_2 began to enter mitosis within 2 h of shifting cultures from 41 to 36°C (Fig. 5). A total of 36% of the cell population (or about 60% of those cells blocked in G_2) initiated mitosis within 8 h after the viral *ras* protein was reactivated (Fig. 5). The cells flowing into mitosis came directly from G_2 and not from some earlier stage because the majority of them did not incorporate [^{3}H]thymidine into DNA when it was added to cultures at the time of the p21-activating temperature shift (Fig. 5).

The flow of G_2-blocked cells into mitosis after p21 reactivation did not depend on conditioning factors which had accumulated in the medium over the course of the experiment because this flow was unaffected by replacing the conditioned serum-free medium with fresh unconditioned serum-free medium immediately before p21 reactivation (data not shown). Moreover, conditioned serum-free medium from *ts*KSV-NRK cultures growing exponentially at 36°C was unable to stimulate G_2-blocked cells to enter mitosis when added at the nonpermissive 41°C. By contrast, this same conditioned medium was able to stimulate quiescent G_0 cells to transit G_1, initiate DNA replication, and divide at 41°C (data not shown). Therefore, the ability of p21 to stimulate the G_2 transit of *ts*KSV-NRK cells at 36°C in serum-free medium is not mediated via the secretion of autocrine factors into the medium.

These data clearly suggest that $p21^{v\text{-}Ki}$ stimulates a G_2 event needed for entry into mitosis. It is well established that transcription and translation are both needed during the G_2 phase (reviewed in reference 22). Indeed, the ability of active $p21^{v\text{-}Ki}$ to trigger G_2 transit and mitosis of *ts*KSV-NRK cells in serum-free medium was wholly dependent on de novo RNA and protein syntheses because both actinomycin D and cycloheximide abolished the p21-induced mitotic response (Table 1).

While the reactivation of $p21^{v\text{-}Ki}$ triggered the release of cells from the G_2 block, the addition of serum at the nonpermissive 41°C did not (Table 1). Moreover, adding serum to cultures at the time of the p21-inactivating 36 to 41°C shift in the mid-S phase did not prevent the cells from being arrested in G_2 (Fig. 4b and c). This was surprising because the addition of serum to quiescent G_0 cultures at

TABLE 1. Actinomycin D and cycloheximide inhibit the flow of G_2-arrested cells into mitosis after p21 activation

Condition[a]	% Mitosis[b]
41°C control (unstimulated)	0.73 ± 1.0
41°C + 10% serum	0.50 ± 0.4
41°C → 36°C	20.3 ± 3.5
41°C → 36°C + actinomycin D[c] (0.5 μg/ml)	0.16 ± 0.22
41°C → 36°C + cycloheximide[c] (1 μg/ml)	0.21 ± 0.17

[a] The cells were blocked in the G_2 phase as described in the legend to Fig. 5 and were then stimulated by serum addition at 41°C or a p21-activating shift to 36°C.

[b] The proportion of colchicine metaphases accumulated in the 12-h period after stimulation ± standard error of the mean.

[c] Added 20 min before stimulation.

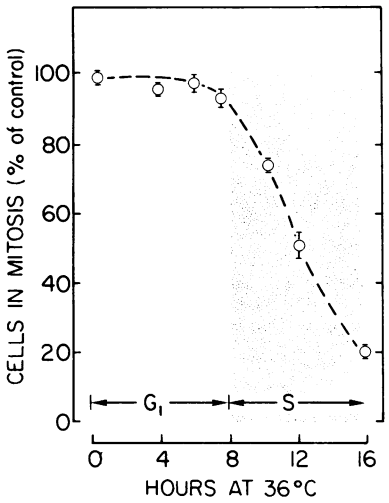

FIG. 6. Serum factors stimulate an event(s) needed for G_2 transit and entry into mitosis at the end of the G_1 phase or in the early S phase but not later in the cycle. Quiescent G_0 *ts*KSV-NRK cells in serum-free medium at 41°C were stimulated to enter the G_1 phase by dropping the temperature to 36°C which reactivated the viral p21 Ki-*ras* gene product. At the indicated times thereafter the thermolabile $p21^{v\text{-}Ki}$ protein was inactivated again by raising the temperature to 41°C, and serum was added to a final concentration of 10%. At 18 h after the initial proliferative stimulation, colchicine was added to the cultures. The total proportion of cells that collected in mitosis between 18 and 36 h was then determined as described in Materials and Methods. The points are means ± standard errors of the means (bars) of at least four determinations.

41°C stimulated cells to both replicate DNA and enter mitosis (Fig. 2). This observation suggested that factors in serum stimulated a process(es) in G_1, or the early S phase, the products of which were needed much later to enable the cells to transit G_2 and enter mitosis. To test this, quiescent G_0 cells at 41°C were first stimulated to enter the G_1 phase by lowering the temperature to 36°C to reactivate p21. At various times after stimulation, the cells were returned to 41°C to inactivate p21, and serum was added to take over from the viral Ki-*ras* protein. The ability of the progressively delayed addition of serum to stimulate G_1 transit was determined. The fraction of cells which entered mitosis between 24 and 48 h after the initial 41 to 36°C shift dropped sharply when serum addition was delayed by more than 8 h (Fig. 6). Thus, serum did stimulate some process(es) needed for G_2 transit when the cells were in the late G_1 or early S phase, a finding that is not entirely without precedent because Rixon and Whitfield (19) have shown that in regenerating rat liver transient events in the early G_1 phase dictate the flow of cells into mitosis some 20 h later.

As stated earlier, adding serum to quiescent cultures at the time of the p21-activating 41 to 36°C shift prevented cells that had been shifted back to 41°C in the mid-S phase from being blocked in the G_2 phase. We then tried to find out which of the many serum components promoted G_2 transit in such cultures. However, several major growth-promoting serum components, such as epidermal growth factor, fibroblast growth factor, insulin, platelet-derived growth factor, transferrin, and retinoic acid, added either separately or in various combinations at the time of the initial 41 to 36°C shift were unable to mimic the ability of serum to prevent G_2 block. Therefore, at this point, we still do not know what serum components are needed for G_2 transit.

DISCUSSION

The ability of the viral *ras* proteins to initiate and sustain the proliferation of murine sarcoma virus-infected cells in serum-free medium is well known (8, 15). Our data show that, besides stimulating G_1 transit, viral p21 activity stimulates some event(s) in the G_2 phase that *ts*KSV-infected NRK cells need to complete the cycle and divide in serum-free medium. Inactivating $p21^{v\text{-}Ki}$ when the cells were in the S phase caused them to arrest in G_2, a blockage which could be reversed only by reactivating the viral Ki-*ras* protein. The addition of conditioned medium from *ts*KSV-NRK cultures (which had been growing logarithmically at 36°C in serum-free medium) to G_2-blocked cells neither induced the G_2-to-M transition at 41°C nor promoted the entry into mitosis after a p21-activating shift to 36°C. Thus, the *ras* gene product appears to stimulate G_2 transit directly by affecting an intracellular mechanism rather than indirectly through the secretion of G_2-stimulating autocrine factors into the medium.

The ability of the viral Ki-*ras* protein to induce NRK cells trapped in G_2 to enter mitosis is supported by recent studies on immature *Xenopus* oocytes which are normally blocked in meiosis I. The microinjection of an oncogenic human Ha-*ras* protein into immature oocytes rapidly induced maturation, that is, the induction of meiosis (3, 20). Microinjection of the wild-type, nononcogenic Ha-*ras* protein also triggered meiosis, albeit at much higher doses (3). The induction of both mitosis and meiosis is dependent on maturation-promoting factors which initiate chromosome condensation and nuclear membrane breakdown (1). Thus, the ability of *ras* proteins to trigger both mitosis in NRK cells and meiosis in oocytes suggests that they are involved in the control of processes that immediately lead up to cell division.

The conclusion that an oncogenic viral *ras* protein acts directly in G_2 to initiate mitosis in *ts*KSV-NRK cells suggests that cellular *ras* proteins play a role during the G_2 phase in normal cell growth. In serum-stimulated BALB/c 3T3 fibroblasts (5, 18) and in regenerating rat liver cells (13), c-*ras* gene expression increases in late G_1 and continues at least into the G_2 phase. Unlike stimulation by other mitogens, activating the viral p21 in quiescent *ts*KSV-NRK cells does not raise the rates of RNA and protein syntheses during the G_1 phase (8). Consequently, the c-*ras* gene product may not accumulate in *ts*KSV-NRK cells induced to proliferate by the viral Ki-*ras* protein in serum-free medium. If so, p21-stimulated cells shifted back to the p21-inactivating 41°C in the mid-S phase would find themselves without functioning *ras* proteins, either viral or cellular, when they reached the G_2 phase and would be unable to progress to mitosis. If c-*ras* gene expression can normally be initiated only in the late G_1 phase, then adding serum to G_2-blocked cells would not stimulate their c-*ras* genes and the p21 production needed for entry into mitosis. Only serum factors acting at a critical point before the G_1/S boundary would be able to induce the production of the *ras* proteins that enable G_2 transit later on (Fig. 6). We are currently attempting to define the role of c-*ras* proteins in G_2 transit and mitosis.

G_2 is a period of intense biochemical activity as the cell readies itself for cell division, and it now appears that *ras* gene expression may play a role in this activity. By the timely inactivation of the viral Ki-*ras* protein we succeeded in reversibly arresting the majority of *ts*KSV-NRK cells in G_2. This simple and effective technique for synchronizing

cells in the G_2 phase should prove valuable in the study of this important though somewhat neglected phase of the cell cycle.

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LITERATURE CITED

1. **Adlakha, R. C., F. M. Davis, and P. W. Rao.** 1985. Role of phosphorylation of nonhistone proteins in the regulation of mitosis, p. 485–513. *In* A. L. Boynton and H. L. Leffert (ed.), Control of animal cell proliferation. Academic Press, Inc., Orlando, Fla.
2. **Bargmann, C. I., M. C. Hung, and R. A. Weinberg.** 1986. The *neu* oncogene encodes an epidermal growth factor receptor-related protein. Nature (London) **319**:226–234.
3. **Birchmeier, C. B., D. Broek, and M. Wigler.** 1985. RAS proteins can induce meiosis in *Xenopus* oocytes. Cell **43**:615–621.
4. **Boynton, A. L., J. F. Whitfield, and L. P. Kleine.** 1983. Ca^{2+}/phospholipid-dependent protein-kinase activity correlates to the ability of transformed liver cells to proliferate in Ca^{2+}-deficient medium. Biochem. Biophys. Res. Commun. **115**:383–390.
5. **Campisi, J., H. E. Gray, A. B. Pardee, M. Dean, and G. E. Sonnenshein.** 1984. Cell cycle control of c-*myc* but not c-*ras* expression is lost following chemical transformation. Cell **36**:241–247.
6. **Doolittle, R. F., M. W. Hunkapiller, L. E. Hood, S. G. Devare, K. C. Robbins, S. A. Aaronson, and H. Antoniades.** 1983. Simian sarcoma virus *onc* gene, *v-sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science **221**:275–277.
7. **Downward, J., Y. Yarden, E. Mayes, C. Scarce, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, and M. D. Waterfield.** 1984. Close similarity of epidermal growth factor receptor and v-*erb* B oncogene protein sequences. Nature (London) **307**:521–527.
8. **Durkin, J. P., and J. F. Whitfield.** 1986. Characterization of G_1 transit induced by the mitogenic-oncogenic viral Ki-*ras* gene product. Mol. Cell. Biol. **6**:1386–1392.
9. **Feramisco, J., M. Gross, T. Kamato, M. Rosenberg, and R. Sweet.** 1984. Microinjection of the oncogenic form of the human H-*ras* protein results in rapid proliferation of quiescent cells. Cell **38**:109–117.
10. **Gelfant, S.** 1977. A new concept of tissue and tumor cell proliferation. Cancer Res. **37**:3845–3862.
11. **Gibbs, J., I. Sigal, M. Poe, and E. Scolnick.** 1984. Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc. Natl. Acad. Sci. USA **81**:5704–5708.
12. **Goldfarb, M. P.** 1985. The role of cellular oncogenes in cancers of non-viral etiology. Pharm. Ther. **29**:205–220.
13. **Goyette, M., C. J. Petropoulos, P. R. Shank, and N. Fausto.** 1984. Regulated transcription of c-Ki-*ras* and c-*myc* during compensatory growth of rat liver. Mol. Cell. Biol. **4**:1493–1498.
14. **Kaczmarek, L., J. K. Hyland, R. Watt, M. Rosenberg, and R. Baserga.** 1985. Microinjected c-*myc* as a competence factor. Science **228**:1313–1315.
15. **Kaplan, P. L., M. Anderson, and B. Ozanne.** 1982. Transforming growth factor(s) production enables cells to grow in the absence of serum: an autocrine system. Proc. Natl. Acad. Sci. USA **79**:485–489.
16. **McGrath, J., D. Capon, D. Goeddel, and A. Levinson.** 1984. Comparative biochemical properties of normal and activated human *ras* p21 protein. Nature (London) **310**:644–655.
17. **Mulcahy, L. S., M. R. Smith, and D. W. Stacey.** 1985. Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH3T3 cells. Nature (London) **313**:241–243.
18. **Pardee, A. B., J. Campisi, H. E. Gray, M. Dean, and G. E. Sonnenshein.** 1985. Cellular oncogenes, growth factors, and cellular growth control, p. 21–29. *In* R. J. Ford and A. L. Maizel (ed.), Mediators in cell growth and differentiation. Raven Press, New York.
19. **Rixon, R. H., and J. F. Whitfield.** 1985. The possible cyclic AMP-dependence of an early prereplicative event that determines mitosis in regenerating rat liver. J. Cell. Physiol. **124**:397–402.
20. **Sadler, S. E., A. L. Schechter, C. J. Tabin, and J. L. Maller.** 1986. Antibodies to the *ras* gene product inhibit adenylate cyclase and accelerate progesterone-induced cell division in *Xenopus laevis* oocytes. Mol. Cell. Biol. **6**:719–722.
21. **Shih, T., A. Papageorge, P. Stokes, M. Weeks, and E. Scolnick.** 1980. Guanine nucleotide-binding and autophosphorylating activities associated with the $p21^{src}$ protein of Harvey murine sarcoma virus. Nature (London) **287**:686–691.
22. **Shih, T., P. Stokes, G. Smythes, R. Dhar, and S. Oroszian.** 1982. Characterization of the phosphorylation sites and the surrounding amino acid sequences of the p21 transforming proteins coded for by the Harvey and Kirsten strains of murine sarcoma viruses. J. Biol. Chem. **257**:11767–11773.
23. **Shih, T. Y., and M. O. Weeks.** 1984. Oncogenes and cancer: the p21 *ras* gene. Cancer Invest. **2**:109–123.
24. **Shih, T. Y., M. O. Weeks, H. A. Young, and E. M. Scolnick.** 1980. p21 of Kirsten murine sarcoma virus is thermolabile in a viral mutant temperature sensitive for the maintenance of transformation. J. Virol. **31**:546–556.
25. **Smith, M. R., S. J. DeGudicibus, and D. W. Stacey.** 1986. Requirement for c-*ras* proteins during viral oncogene transformation. Nature (London) **320**:540–543.
26. **Sweet, R., S. Yokoyama, T. Kamata, J. Feramisco, M. Rosenberg, and M. Gross.** 1984. The product of *ras* is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature (London) **311**:273–275.
27. **Waterfield, M. D., G. T. Scrare, N. Whittle, P. Stoobant, A. Johnsson, A. Wasteson, B. Westermark, C. H. Heldin, J. S. Huang, and T. Deuel.** 1983. Platelet-derived growth factor is structurally related to the putative transforming protein p28 *sis* of simian sarcoma virus. Nature (London) **304**:35–39.
28. **Whitfield, J. F., J. P. MacManus, T. Youdale, and D. J. Franks.** 1971. The roles of calcium and cAMP in the stimulatory action of parathyroid hormone on thymic lymphocyte proliferation. J. Cell. Physiol. **78**:355–368.
29. **Willingham, M. C., I. Pastan, T. Y. Shih, and E. M. Scolnick.** 1980. Localization of the *src* gene product of the Harvey strain of MSV to the plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell **19**:1005–1014.