p21 *v-ras* Inhibits Induction of *c-myc* and *c-fos* Expression by Platelet-Derived Growth Factor

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The viral oncogene *v-ras* inhibited the platelet-derived growth factor (PDGF)-induced upregulation of *c-myc* and *c-fos* proto-oncogene expression in fibroblast monolayers. These *v-ras*-containing cells proliferated in the absence of *c-myc* induction and no longer required PDGF to support growth. Fibroblasts expressing *v-ras* continued to express the same number of functional PDGF receptors on their surface as uninfected cells, yet the usual induction of transcription of the genes *c-myc*, *c-fos*, and *JE* in response to PDGF stimulation did not occur in the presence of newly introduced *v-ras* or chronic *v-ras* gene expression, and synthesis of *c-myc* protein did not occur. This inhibitory effect on growth factor-mediated induction of cellular proto-oncogenes was specific for PDGF in that induction of the *c-myc* and *c-fos* genes by certain other factors was not impaired.

Serum growth factors stimulate transient induction of the *c-myc* and *c-fos* proto-oncogenes in confluent fibroblast monolayers (6, 7, 16, 19, 21, 26). The level of *c-fos* mRNA peaks about 30 min after addition of serum or platelet-derived growth factor (PDGF) and declines to baseline levels within 2 h. In the same cultures, *c-myc* mRNA is induced 20- to 40-fold within 2 h. Thereafter, *c-myc* mRNA returns to baseline levels in a biphasic fashion, with a rapid decline from 2 to 4 h (to levels about 10-fold above baseline) and a slower decline over the next 24 h. We show here that *v-ras* genes have a profound inhibitory effect on *c-myc* and *c-fos* mRNA levels. Shortly after introduction of a *v-ras* gene into growing fibroblast cultures, *c-myc* mRNA expression was suppressed and remained suppressed in cells chronically expressing the *v-ras* gene. Thereafter, neither *c-myc* nor *c-fos* was induced by exposure to PDGF. The inhibitory effect of activated *ras* genes was specific for the PDGF mitogenic signal in that the induction of *c-myc* and *c-fos* by certain other agents was not impaired. The inhibitory effect of activated *ras* genes was also specific for cell lines in which the *c-myc* and *c-fos* genes are regulated by growth factors. We have analyzed cell lines which constitutively express high levels of the *c-myc* and *c-fos* genes and in which these genes are not regulated by mitogenic signals. In these cell lines, the expression of activated *ras* genes has no effect on *c-myc* and *c-fos* levels. In cells expressing *v-ras*, we detected a constitutive low level of *c-myc* transcription, yet there was no detectable *c-myc* mRNA or protein in the cytoplasm of these cells. These inhibitory effects on *c-myc* and *c-fos* transcript levels occurred rapidly after expression of p21^{*v-ras*} and suggest a role for the cellular homologs of *ras* in the negative feedback regulation of mRNA levels of growth factor-induced genes.

MATERIALS AND METHODS

Cells, cell lines, and viruses. All uninfected cell lines (BALB/c-3T3, NIH 3T3, and L cells) were obtained from the American Type Culture Collection (Rockville, Md.). The rat cell lines NRK and K-NRK were the generous gift of E. Scolnick (Merck, Sharp and Dohme, West Point, Pa.). Virus stocks were derived from cells transfected with cloned

proviral DNAs as previously described (10). Infected or uninfected BALB/c-3T3, NIH 3T3, and L cells and NRK fibroblasts were grown in Dulbecco modified Eagle medium (DME) with 10% calf serum (GIBCO, Grand Island, N.Y.). Sarcoma virus nonproducer cell lines were generated by infection with limiting dilutions of virus stocks or by transfection with sarcoma virus proviral clones as previously described (9). In either case, the integrity of the provirus was ensured by its ability to be rescued by leukemia virus superinfection. Leukemia virus or sarcoma virus titers were assayed by XC and focus-forming assays, respectively, and by reverse transcriptase titers (9). Retrovirus infections were carried out by exposing subconfluent or confluent cell populations to high-titer (>10⁷ XC syncytia-forming units or focus-forming units per ml) virus-containing filtered cell culture supernatants for 1 h at 37°C in the presence of Polybrene (Sigma Chemical Co., St. Louis, Mo.) at 8 µg/ml. Control cultures (mock infections) were made by exposing the cells to Polybrene alone. Cells were harvested at subconfluence during exponential cell growth for some of the experiments, while for other experiments cells were allowed to reach confluence and then incubated overnight in DME containing 5% platelet-poor plasma (and no serum). PDGF (recombinant [Amgen, Thousand Oaks, Calif.] or purified from human platelets), at a final concentration of 5 to 10 ng/ml or 200 U/ml, respectively, of medium, was added to some of the cells at time zero, and the cells were harvested at the times indicated. Purified fibroblast growth factor (FGF), migrating as a single band on protein electrophoresis, was the generous gift of J. Folkman and R. Hannan and was used at a concentration of 5 ng/ml of medium.

Cellular RNA analysis. Cytoplasmic RNA was extracted after cells were lysed in 10 mM Tris (pH 7.4)-1 mM EDTA-0.1% Nonidet P-40 and the nuclei were pelleted. Following phenol-chloroform extraction, the RNA was precipitated in ethanol and quantitated, and 20 µg of RNA was electrophoresed on a 1% agarose gel in the presence of formaldehyde (23). Total cellular RNA was prepared by sequential ethanol precipitations from guanidine as previously described (10). RNA was transferred to nitrocellulose filters with 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) buffer, and the filters were baked in vacuo. The *c-myc*-specific probe was a 0.45-kilobase (kb) *Pst*I fragment from the second exon

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(generously provided by K. B. Marcu [31]). The *c-fos*-specific probe was a 1.6-kb *EcoRI-SstI* fragment from a mouse *c-fos* genomic clone, containing the second, third, and fourth exons (kindly provided by T. Curran). Probes were nick-translated or labeled with ³²P by the oligonucleotide random primer method (12) to a specific activity of greater than 10⁸ cpm/μg of DNA. Hybridizations were carried out in 50% formamide-4× SSC at 42°C as described by Greenberg and Ziff (16), and washes were done under stringent conditions. Autoradiography was performed at -70°C with enhancing screens. Films were scanned with a densitometer. For quantitation, the blots were stripped of probe and rehybridized with a probe specific for actin (660-base-pair *PstI* fragment from a mouse cDNA clone; kindly provided by B. Spiegelman) and probes specific for rat β-tubulin (3) or mouse β₂-microglobulin (41). *c-myc* and *c-fos* transcripts were normalized to the amount of actin or tubulin mRNA per lane. Internal standards for mouse actin, mouse β₂-microglobulin, and rat β-tubulin cDNA probes showed essentially no deviation or induction within the time course of the experiments.

Nuclear runoff analysis. Nuclear transcription of specific genes was compared as described previously (41). Approximately 10⁸ uninfected or chronically infected cells at confluence, after 24 h of serum deprivation, were treated with recombinant PDGF (10 ng/ml) for 1 h or left untreated. Nuclei were prepared, and transcription was allowed to proceed in the presence of ³²P-labeled UTP. Exhaustive hybridization was carried out on filters containing 2-μg spots of DNA specific for exon 1, 2, or 3 of the *c-myc* gene (31), the JE gene (1), the mouse β-actin gene, or the rat β-tubulin gene. The blots were washed at high stringency and exposed to RNase digestion prior to autoradiography, densitometry, and scintillation counting.

Quantitation of cell surface PDGF receptors. Radioreceptor analysis was done with commercially available reagents or [¹²⁵I]-labeled recombinant PDGF and purified or recombinant PDGF as a binding competitor by standard procedures (18).

Detection of v-ras protein. BALB/c-3T3 cells were infected at time zero with a high-titer stock of Ki-MSV (Kirsten sarcoma virus with Moloney murine leukemia virus [Mo-MuLV] helper virus). At time zero and 1, 2, 4, 18, 24, 48, and 72 h, equal portions of the cells were metabolically labeled by exposure to [³⁵S]methionine (New England Nuclear Corp., Boston, Mass.) at a concentration of 150 μCi/ml of methionine-free minimal essential medium (Gibco) for 30 min. Equal numbers of cells were lysed in a buffer containing 0.2M LiCl, 20 mM Tris (pH 8.0), 1 mM EDTA, and 0.5% Nonidet P-40. Lysates were incubated with an antiserum against p21^{ras} protein with a serine substituted in the 12th amino acid position (5) (Cetus Corp., Emeryville, Calif.), and the antibody-antigen complexes were precipitated with Formalin-fixed staphylococcal A and separated on a discontinuous 15% polyacrylamide-sodium dodecyl sulfate gel (22). After the gel was dried, autoradiography was performed at -70°C.

Analysis of cell proliferation. Quadruplicate wells of subconfluent, exponentially growing cells or confluent, serum-starved cells were exposed to a virus-containing supernatant (MuLV-, MSV-, or mock-infected supernatant) for 1 h in the presence of Polybrene (8 μg/ml). The virus-containing or mock-infected supernatants in this case were generated in the absence of serum. The cells were then exposed to serum-free medium containing [³H]thymidine at a concentration of 1 μCi/ml for 24 h. Recombinant PDGF, at a concentration of 10 ng/ml, was added to some of the wells as

FIG. 1. Levels of *c-myc* and *c-fos* transcripts in cells infected with Ki-MSV. The cell lines examined include BALB/c-3T3 (clone A31); BALB/c-3T3 infected with Mo-MuLV clone 1 (11), designated BALB-MuLV; and BALB/c-3T3 infected with Ki-MSV and Mo-MuLV as a helper, designated BALB-KSV. (A) *c-myc* transcripts in exponentially growing cell lines. Lane 1, BALB/c-3T3; lane 2, BALB-MuLV; lane 3, BALB-KSV. The position of the 2.4-kb *c-myc* transcript is indicated. Panel A is overexposed to demonstrate the very low but detectable levels of *c-myc* mRNA in lane 3. (B) PDGF-induced expression of *c-myc* transcripts 1 h after exposure to PDGF. Lane 1, Quiescent BALB/c-3T3; lane 2, BALB/c-3T3 plus PDGF; lane 3, BALB-KSV; lane 4, BALB-KSV plus PDGF. The position of the 2.4-kb *c-myc* transcript is indicated. (C) PDGF-induced expression of *c-fos* transcripts 30 min after PDGF addition. Lanes 1 and 3, BALB/c-3T3 cells at time zero and 30 min, respectively; lanes 2 and 4, BALB-KSV cells at time zero and 30 min, respectively. The position of the 2.2-kb *c-fos* transcript is indicated. All blots were rehybridized with actin and tubulin probes to ensure that equal amounts of RNA were loaded per lane.

a positive control for this period. One well of cells from each treatment group was trypsinized and counted for normalization of the data per 10⁴ cells. The cells in the other three wells for each experimental condition were solubilized with 1% sodium dodecyl sulfate, and the acid-insoluble material was collected on glass fiber filters and assayed by scintillation counting.

RESULTS

Cells containing Ki-MSV express little or no *c-myc* or *c-fos* mRNA during growth. During exponential cell growth in serum-supplemented medium, BALB/c-3T3 cells expressed *c-myc* mRNA at levels 10- to 20-fold higher than that detected in growth-arrested cells. The introduction of a *v-ras*-containing murine sarcoma virus (Ki-MSV) into these cells caused *c-myc* mRNA levels to fall dramatically (greater than 30-fold decrease) despite active cell division during exponential growth (Fig. 1A). Infectious stocks of Ki-MSV contain the nondefective helper virus Mo-MuLV. Infection of BALB/c-3T3 cells with the helper virus alone did not decrease the levels of *c-myc* mRNA (Fig. 1A). Furthermore, nonproducer BALB/c-3T3 cells, which contain the Ki-MSV viral genome in the absence of a helper virus, also failed to express significant quantities of *c-myc* mRNA during active cell growth. When quiescent BALB/c-3T3 cells were exposed to PDGF, induction of *c-myc* and *c-fos* could be detected within 60 and 30 min, respectively (Fig. 1B and C). However, neither *c-myc* nor *c-fos* transcripts were significantly induced by PDGF in Ki-MSV-infected cells (Fig. 1B and C). In rare experiments, very small increases in *c-myc* levels were noted after PDGF treatment of cells newly infected with Ki-MSV (see below). This was most likely due to incomplete infection at these early times, as even such limited induction was not observed in cells chronically infected with the sarcoma virus (see below). Ki-MSV expresses an activated *ras* protein as its only virus-encoded gene product (34, 35). The very low levels or absence of *c-myc* and *c-fos* transcripts in exponentially growing cells and in PDGF-treated cells infected with the *v-ras*-containing Ki-MSV raised the possibility that the levels of these

FIG. 2. Time course of PDGF-induced *c-myc* mRNA levels after infection with Ki-MSV. Quiescent BALB/c-3T3 cells were infected with high-titer virus stocks containing Ki-MSV with Mo-MuLV helper virus or amphotropic 2047 MuLV helper virus or with Mo-MuLV helper virus alone at 37°C for 60 min. PDGF, at a final concentration of 200 U/ml, was added at time zero, and cytoplasmic RNA was extracted at 0, 1, 2, 4, and 24 h and analyzed as described in the text. Time courses are shown for control helper virus-infected cells (MuLV), sarcoma virus plus Mo-MuLV helper virus-infected cells [KSV(M)], and sarcoma virus plus amphotropic MuLV helper virus-infected cells [KSV(A)]. This nitrocellulose blot was hybridized with the *c-myc*-specific probe. The position of the 2.4-kb *c-myc* transcript is indicated. This blot was rehybridized with actin and tubulin probes to ensure that equal amounts of RNA were loaded per lane.

mRNAs were actively suppressed by the presence of the *v-ras* gene.

The time course of *c-myc* transcript expression after the addition of PDGF is shown in Fig. 2. High levels of *c-myc* mRNA were induced in uninfected cells or helper virus (Mo-MuLV)-infected cells within 1 h of exposure of PDGF, and these levels fell to baseline within 24 h. When the same cell line was infected with Ki-MSV 2 h prior to PDGF exposure, the addition of PDGF did not induce *c-myc* expression. *c-fos* expression was similarly inhibited (data not shown). The lack of complete *c-myc* suppression at 1 h in some experiments (two of nine experiments) may have occurred because some cells remained uninfected with Ki-MSV after the relatively brief period of infection. The effect of Ki-MSV on *c-myc* and *c-fos* levels was independent of the type of MuLV helper virus used (ecotropic or amphotropic). Infection of the cells with a helper virus (Mo-MuLV) alone did not affect the *c-myc* and *c-fos* response to PDGF.

The findings of low or absent levels of *c-myc* mRNA in the *v-ras*-containing cells and failure of PDGF to induce *c-myc* transcripts in these cells were supported by examination of the amount of *c-myc* protein in these cells. Immunoprecipitation of metabolically labeled lysates from serum-starved uninfected cells, with or without PDGF stimulation, by *myc*-specific polyclonal antisera or monoclonal antibodies detected a protein of 60,000 daltons, corresponding to the *c-myc* protein (data not shown). No *myc* protein was identified in *v-ras*-containing cells, whether or not they were stimulated with PDGF. Western blot (immunoblot) analysis with the same antisera or *myc*-specific monoclonal antibodies also failed to detect *c-myc* protein in the *v-ras*-containing cells (data not shown).

Despite the inhibition of *c-myc* and *c-fos* expression in response to PDGF, infection with Ki-MSV did not inhibit replication of the cells. BALB/c-3T3 cells at subconfluence continued to grow and incorporate [³H]thymidine at comparable rates whether uninfected or newly infected for 1 h with Mo-MuLV or Ki-MSV and then exposed to [³H]thymidine at 1 μ Ci/ml for 24 h ($43,671 \pm 7,072$, $72,211 \pm 13,657$, and

FIG. 3. p21^{v-ras} expression as a function of time after infection with Ki-MSV. p21^{v-ras} proteins typically migrate as a series of bands at ca. 21,000 daltons, as indicated by the arrows. BALB/c-3T3 cells were infected with a stock of Ki-MSV plus Mo-MuLV, metabolically labeled, and lysed at the indicated times after infection as described in Materials and Methods. p21^{v-ras} proteins were immunoprecipitated, and the products of the immunoprecipitation were separated on a 15% polyacrylamide gel and exposed to film. Lane B is a lysate of BALB/c-3T3 cells immunoprecipitated and run in parallel with a lysate from BALB/c-3T3 cells chronically infected with Ki-MSV, shown as lane K. Because of the large amounts of p21 proteins in the chronically infected cell line, autoradiography of lanes B and K was carried out for only 12 h instead of the 2-day exposure used for lanes depicting 0 to 72 h. Preclearing of the cell lysates with normal sheep serum did not affect the results.

$153,616 \pm 25,123$ cpm of [³H]thymidine incorporated per 24 h per 10^4 cells, respectively). Quiescent, confluent BALB/c-3T3 cells, uninfected or newly infected (for 1 h) with Mo-MuLV or Ki-MSV, were then treated with PDGF. Thymidine incorporation after PDGF treatment was $131,192 \pm 1,211$, $102,894 \pm 6,903$, and $112,769 \pm 1,919$ cpm per 24 h per 10^4 cells, respectively. These data suggest either that *c-myc* and *c-fos* expression is not required for cell division or, more likely, that this requirement is bypassed in the presence of *v-ras*.

Inhibition of *c-myc* induction coincident with *v-ras* protein expression. The effect of Ki-MSV on *c-myc* and *c-fos* expression was quite rapid. Figure 2 demonstrates that inducible levels of *c-myc* mRNA fell dramatically within 1 h after infection with Ki-MSV and were undetectable within 1 to 2 h. This effect was apparent long before any morphological features associated with oncogenic transformation could be seen and most probably even before cellular genomic integration of the Ki-MSV provirus could occur. To determine whether *v-ras* protein was synthesized this rapidly after infection, radiolabeled cell extracts from uninfected or infected BALB/c-3T3 cells were immunoprecipitated with an antiserum specific for the serine-substituted *v-ras* protein encoded by Ki-MSV (5, 38).

As shown in Fig. 3, p21^{v-ras} was detectable within 1 h of infection and reached maximum levels at 2 h. Thereafter, synthesis of viral p21 fell, reaching baseline levels by approximately 72 h. After a lag of several days to several weeks, levels of p21 rose again as the cells became chronically infected and transformed (Fig. 3). This temporal pattern of p21^{v-ras} expression corresponded well to our observed alterations of *c-myc* and *c-fos* expression. PDGF was unable to induce *c-myc* and *c-fos* transcript expression for approximately 48 h after Ki-MSV infection. There followed a period of days during which *c-myc* and *c-fos* could be partially induced and p21^{v-ras} was not detected (data not shown). Finally, with the onset and spread of chronic Ki-MSV infection, *c-myc* and *c-fos* were expressed only minimally during cell growth and were essentially uninducible with PDGF (Fig. 1 and 3).

Effect of *v-ras* specific for PDGF-induced signal. To extend our findings of *v-ras* control of *c-myc* expression to other species, we examined the effects of the *ras* gene of Ki-MSV in a rat cell line (NRK) in which expression of *c-myc* remains closely coupled to proliferation and PDGF exposure. Figure 4A shows the high levels of *c-myc* mRNA in quiescent,

FIG. 4. Levels of *c-myc* transcripts in cells containing Ki-MSV or Ha-MSV. (A) Cytoplasmic RNA was extracted from NRK cells (lane 1) or from NRK cells containing the Ki-MSV genome (NRK-KSV, lane 2) during exponential cell growth in 10% calf serum and probed for *c-myc* transcripts (A) as described in the legend to Fig. 1. Lanes 3 and 4 contain cytoplasmic RNA extracted from confluent NRK or NRK-KSV cells, respectively, which were deprived of serum, grown for 12 h in 5% platelet-poor plasma, and exposed to PDGF (200 U/ml of medium) for 1 h before RNA extraction. (B) Cytoplasmic RNA was extracted from BALB/c-3T3 cells (lane 2) or Ha-MSV-infected BALB/c-3T3 cells (lane 1) during exponential cell growth in 10% calf serum. RNA was also extracted from quiescent, serum-deprived BALB/c-3T3 cells and Ha-MSV-infected BALB/c-3T3 cells both before (lanes 5 and 3, respectively) and 1 h after (lanes 6 and 4, respectively) PDGF treatment. Panel B was hybridized to the *c-myc* probe, and the position of the 2.4-kb *myc* transcript is indicated. Lanes 1 and 2 in panel B represent an 8-h autoradiographic exposure, while lanes 3 to 6 were exposed for 48 h. (C) Cytoplasmic RNA was extracted from confluent, serum-deprived BALB/c-3T3 cells chronically expressing Ki-MSV after no treatment (lane 1), 1 h after addition of PDGF (lane 2), or 1 h after addition of basic FGF at 20 U/ml (lane 3). Panel C was hybridized to the *c-myc* probe, and the position of the 2.4-kb *myc* transcript is indicated. (D) Cytoplasmic RNA was extracted from confluent, serum-deprived murine L cells after no treatment (lane 1), 1 h after addition of PDGF (lane 2), 3 h after infection with Ki-MSV (lane 3), or 3 h after infection with Ki-MSV and 1 h after addition of PDGF (lane 4). Panel D was hybridized to the *c-myc* probe, and the position of the 2.4-kb *myc* transcript is indicated. All blots (A–D) were rehybridized with actin and β_2 -microglobulin probes to ensure that equal amounts of RNA were loaded per lane.

confluent NRK cells 1 h after PDGF stimulation. In comparison, an NRK cell line containing the Ki-MSV genome demonstrated undetectable levels of *c-myc* transcripts after PDGF exposure. Similarly, exponentially growing NRK cells containing the Ki-MSV genome expressed little or no *c-myc* mRNA in comparison to uninfected cells. The effect of a related *v-ras*-containing virus, Harvey sarcoma virus (Ha-MSV), on *c-myc* mRNA expression was also examined. BALB/c-3T3 cells chronically infected with Ha-MSV expressed little or no detectable *c-myc* during active cell growth in comparison with uninfected BALB/c-3T3 cells (Fig. 4B). A long autoradiographic exposure demonstrated very low levels of *c-myc* transcripts in quiescent Ha-MSV-infected cells, but these levels were even lower than those in quiescent uninfected BALB/c-3T3 cells and were not induced by PDGF exposure.

Substances other than PDGF, including FGF (19) and double-stranded nucleic acids (43), have been demonstrated to induce *c-myc* and *c-fos* expression in BALB/c-3T3 cells. FGF treatment induced *c-myc* expression at 1 h in uninfec-

FIG. 5. Nuclear runoff transcription in uninfected cells or cells infected with Ki-MSV. ³²P-labeled transcripts were made from nuclei isolated from uninfected BALB/c-3T3 cells (BALB) and Ki-MSV-infected BALB/c-3T3 cells (K-BALB) which had been serum-starved and treated with PDGF or left untreated. The first two strips represent BALB/c-3T3 cells without (–) and with (+) PDGF, respectively. The third and fourth strips represent Ki-MSV-infected BALB/c cells without (–) or with (+) PDGF, respectively.

ted cells. The presence of *v-ras* did not inhibit this induction (Fig. 4C), suggesting that the p21^{v-ras} effect is specific for the PDGF signal. This specificity was further examined by analysis of *c-myc* expression in cell lines which are unresponsive to PDGF. Murine L cells constitutively expressed *c-myc* transcripts even at confluence during serum deprivation (Fig. 4D). These steady-state levels of *c-myc* were not altered after exposure to PDGF. Furthermore, acute infection of these cells with Ki-MSV had no inhibitory effect on this constitutive *c-myc* expression.

***v-ras* inhibits induction of *c-myc* transcription by PDGF.** Treatment of quiescent BALB/c-3T3 cells with PDGF induced the transcription of the *c-myc* gene, as demonstrated by nuclear runoff studies (Fig. 5). The first exon (leader sequence) was transcribed constitutively, but transcription of the protein-encoding exons 2 and 3 was strongly upregulated by exposure to the growth factor. In the cells containing *v-ras*, however, there was no such induction of transcription by PDGF. A constitutive low level of transcription of all three exons was observed. The induction of another PDGF-inducible gene, called JE, was similarly inhibited in Ki-MSV-containing cells. The transcription of two genes, tubulin and actin, which did not appear to be regulated by PDGF was also unaffected by the presence of *v-ras*.

Despite their lack of response to PDGF, Ki-MSV-containing cells continued to express PDGF-specific receptors on their surface. Competitive binding studies with ¹²⁵I-labeled PDGF revealed the presence of roughly equal numbers of receptors capable of binding PDGF on the surfaces of *v-ras*-expressing cells and uninfected BALB/c-3T3 cells (data not shown).

We next determined whether the failure of PDGF to induce *c-myc* gene transcription in *v-ras*-containing cells was accompanied by a change in the chromatin fine structure of the *c-myc* gene. DNase I hypersensitivity site mapping of chromatin, followed by restriction endonuclease digestion with three different restriction enzymes, demonstrated no differences in the patterns of hypersensitive sites on chromatin derived from PDGF-stimulated cells, whether or not *v-ras* was present. The three major hypersensitive sites were found in DNA from both cell types (data not shown). These studies also indicated that there were no gross rearrangements of the *c-myc* gene due to retrovirus insertion in cells infected with Ki-MSV.

DISCUSSION

Transient increases in mRNA for cellular mitogen-regulated genes, including *c-myc* and *c-fos*, have been strongly associated with subsequent cell division. Following stimulation of quiescent cells with PDGF, *c-myc* and *c-fos* mRNA levels rise and then fall in the period preceding entry into S phase. Expression of *ras* protein also appears to be necessary somewhat later in the cell cycle (just prior to S phase) for proliferation, as microinjection studies with anti-*ras* antisera indicate that *c-ras* protein function is required in the mid- to late G1 phase of the cell cycle (13, 25). The growth-promoting activity of MSV *ras* on various types of cells in vitro is well established, as is its ability to render cells independent of serum or growth factor requirements (10, 17, 24, 32, 33, 40, 42). Microinjection of activated *ras* protein is sufficient to induce proliferation in growth factor-starved cell lines (14, 36). Quiescent cells into which we introduced the Ki-MSV *v-ras* gene via infection were able to undergo division in the absence of PDGF and show little or no stimulation of growth when exposed to PDGF.

Much evidence indicates that the activated *ras* gene product serves the same function as its normal proto-oncogene homolog but in an exaggerated way (4, 15, 28, 36, 39). If *ras* is indeed a late part of the growth factor-*c-fos*-*c-myc* signal pathway for cell division, its ability to profoundly inhibit *c-myc* and *c-fos* expression would suggest that another physiological role for *ras* may be as a regulator of *c-myc* and *c-fos* expression. The transient nature of *c-myc* and *c-fos* responses to PDGF could thus reflect negative feedback by the *c-ras* product on their expression at later times in the cell cycle. Support for this hypothesis will require examination of *c-myc* control in cells constitutively expressing high levels of *c-ras*.

Our finding that *v-ras* controls *c-myc* and *c-fos* expression raises a number of related questions. Do all *v-ras* genes exert the same effect on these cellular proto-oncogenes, and is the act of viral infection necessary to demonstrate this effect? A naturally occurring oncogenic mutant of the cellular Harvey *ras* gene, called EJ, was found to inhibit *c-myc* and *c-fos* expression after transfection into BALB/c-3T3 cells (unpublished observations). The activity of transforming *ras* mutants with mutations involving amino acid residues 59 and 61 is currently being examined.

This report has focused on the early and rapid effects of *v-ras* expression on *c-myc* and *c-fos*, with infection used to efficiently introduce the *v-ras* mRNA into cells. Our ability to study events in signal transduction within several hours of the introduction of a *v-ras* gene into the cell allows us to attribute the effects we observe to the *v-ras* gene rather than to the more obscure event of transformation or biological selection during tumor formation. The stably infected, *v-ras*-expressing cell lines we have also studied here continue to show repression of *c-myc* and *c-fos* transcription. Yet the frequent finding among a wide variety of retrovirus-induced tumors of *c-myc* gene amplification, rearrangement, or deregulation (20, 27, 30, 31, 37) suggests a selective advantage for escape from *v-ras*-induced *c-myc* suppression.

The mechanism by which *v-ras* inhibits induction of *c-myc* and *c-fos* transcripts remains to be defined. The DNase I hypersensitivity studies, together with the finding that other agents are still able to induce *c-myc* gene expression, do not support retrovirus integration and disruption of the *c-myc* gene as a mechanism. More likely would be interference with the PDGF-mediated signal itself. Other investigators have recently reported failure of PDGF to stimulate phos-

pholipase activity or phosphoinositide hydrolysis in cells chronically transformed with a *ras* oncogene (1, 29). Our studies quantitating the binding of [¹²⁵I]PDGF to the cell surface demonstrate that BALB/c-3T3 cells expressing *v-ras* have levels of PDGF receptor on their surface equivalent to levels found on the surface of uninfected or untransformed cells, and this is in agreement with other reports of PDGF receptor levels on *v-ras*-containing cells (29). Furthermore, the PDGF receptor on the cells expressing *v-ras* becomes phosphorylated following binding of PDGF (unpublished observations), suggesting that the receptor remains functionally intact. The subsequent intracellular molecular mechanisms by which PDGF induces *c-myc* and *c-fos* mRNA are incompletely understood. PDGF increases *c-myc* levels in a complex way, affecting transcription as well as altering posttranscriptional mRNA stability (2, 16). The nuclear transcription studies described herein demonstrate that the upregulation of transcription of *c-myc* and JE by PDGF is completely inhibited in the presence of *v-ras*. It is noteworthy that a detectable level of *c-myc* transcription occurs constitutively in the cells chronically infected with Ki-MSV, despite our inability to demonstrate detectable amounts of *c-myc* mRNA or protein. This observation suggests that the *c-myc* mRNA being transcribed in the *v-ras*-expressing cells is rapidly degraded. Thus, neither transcriptional upregulation of the *c-myc* gene nor mRNA stabilization appears to occur in response to PDGF in the *v-ras*-expressing cells. Accurate measurements of *c-myc* mRNA half-life in Ki-MSV-containing cells by standard techniques such as dactinomycin treatment have been impossible to perform owing to the inability to detect any measurable *c-myc* transcripts in these cells.

In *v-ras*-containing cells, the *c-myc* gene remains intact and *c-myc* expression remains inducible by factors such as FGF (which utilizes a membrane receptor distinct from the one which binds PDGF). These findings, together with the fact that p21^{*v-ras*} inhibits the complex induction of the mitogen-regulated genes *c-fos*, *c-myc*, and JE, suggest that *v-ras* is interfering with PDGF-mediated signal transduction at a point after interaction with the PDGF receptor but before *c-myc* and *c-fos* induction.

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