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Alterations in Differentiation and Behavior of Monocytic Phagocytes in Transgenic Mice That Express Dominant Suppressors of *ras* Signaling

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To address the role of *ras* signaling in monocytic phagocytes in vivo, the expression of two dominant suppressors of in vitro *ras* signaling pathways, the carboxyl-terminal region of the GTPase-activating protein (GAP-C) and the DNA binding domain of the transcription factor ets-2, were targeted to this cell compartment. A 5-kb portion of the human c-*fms* proximal promoter was shown to direct expression of the transgenes to the monocytic lineage. As a result of the GAP-C transgene expression, *ras*-GTP levels were reduced in mature peritoneal macrophages by 70%. The terminal differentiation of monocytes was altered, as evidence by the accumulation of atypical monocytic cells in the blood. Mature peritoneal macrophages exhibited changes in colony-stimulating factor 1-dependent survival and structure. Further, expression of the colony-stimulating factor 1-stimulated gene urokinase plasminogen activator was inhibited in peritoneal macrophages. The results indicate that *ras* action is critical in monocytic cells after these cells have lost the capacity to traverse the cell cycle.

The *ras* gene products are GTP-dependent switches essential for growth of cultured cells (3, 42). Recent work has clarified how these molecular switches are regulated and has identified downstream effectors necessary for their action. Genetic experiments with the invertebrate organisms *Drosophila melanogaster* and *Caenorhabditis elegans* (5, 39) and biochemical experiments performed with mammalian cells (10, 19, 37) reveal a mechanism for *ras* activation by tyrosine kinase receptors. Two genes highly conserved through evolution, *grb2* and *sos* (9, 30), encode gene products that form a signal transduction complex with activated tyrosine kinase receptors. This complex catalyzes the conversion of membrane-associated *ras* to the active GTP conformation (reviewed in reference 20).

Analyses of the invertebrate *ras* pathways also pointed to a role for *raf* protein kinases as downstream effectors of *ras* action (17, 24). The mammalian *raf* counterparts have been demonstrated to directly interact with *ras* in a GTP-dependent manner (33, 44, 49), although direct activation of *raf* kinase activity by *ras* was not demonstrated. The experiments implicate *raf* kinase as a long-sought effector of *ras* action.

These rapid advances have provided a framework for understanding the role of *ras* in signal transduction in cultured cells, but fundamental issues concerning the specificity and biology of *ras* signaling pathways in mammalian cells remain to be addressed. The activation of *ras* appears to be a prerequisite for the action of many tyrosine kinase receptors, but the consequences of *ras* activation can be diametrically opposed, dependent on the particular receptor from which the signal emanates. For example nerve growth factor and epidermal growth factor receptors both activate *ras* signaling pathways in the PC12 cell line, but *ras* activation results in either differentiation or mitogenic cell growth (34). Likewise, how the *ras* signaling pathway described by the tissue culture and biochemical assays relates to the role of *ras* signaling in mammalian organisms remains to be determined.

Our work has demonstrated that *ras* signaling is coupled to the action of c-*fms* when this gene is expressed in NIH 3T3 fibroblasts (7, 8, 28). Two reagents that dominantly suppress colony-stimulating factor 1 (CSF-1) receptor and *ras* action were developed for these studies: the carboxyl-terminal catalytic domain of the GTPase-activating protein (GAP-C [8]) and the DNA binding domain of human c-*ets*-2 fused in frame with the bacterial *lacZ* gene (*ets-lacZ* [28]). These reagents were critical in defining a *ras* signaling pathway efficiently activated by c-*fms* and necessary for mitogenic growth of NIH 3T3 cells.

The c-*fms* proto-oncogene encodes the tyrosine kinase receptor for CSF-1 and is usually expressed in monocytic phagocytes and in placental trophoblasts (38). Two distinct promoters appear to be responsible for the tissue-specific expression of the human gene in monocytes or placenta (36). A classic mouse mutation, osteopetrotic (*op* [31]), reveals the essential roles of CSF-1 signaling in vivo. The *op* mutation results from an in-frame stop codon in the CSF-1 gene that precludes translation of a functional growth factor (47). Because of the lack of CSF-1 production, the *op*/*op* mice have drastically reduced numbers of circulating monocytes and have decreased numbers of some classes of tissue macrophages and of osteoclasts and thus exhibit the observed phenotype (25, 47). Treatment of *op*/*op* mice with CSF-1 can partially rescue the mutant phenotype (23, 46). Thus, in the presumed absence of CSF-1/c-*fms* signaling, defects in macrophage and osteoclast differentiation and survival occur. Therefore, the normal biological milieu for c-*fms* action is quite distinct from the fibroblast model system used in our previous experiments.

To address the role of *ras* signaling in the biological action of c-*fms* in a more relevant setting, we set out to target expression of the dominant suppressors of *ras* action, GAP-C and *ets-lacZ*, to monocytes and their committed precursors in transgenic

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mice with the expectation that expression of the transgenes would result in at least a partial phenocopying of the *op/op* mutant. To accomplish this, we used the human c-*fms* proximal promoter present in intron 1 (35, 36) to direct transgene expression to the monocytic compartment. Mice bearing these transgenes contained elevated levels of unusual monocytic cells in their circulation, while other lineages were normal. Biochemical experiments performed with the transgenes that express GAP-C demonstrated that the amount of *ras* present in the active GTP form is significantly reduced in elicited peritoneal macrophages. Further analysis of elicited peritoneal macrophages isolated from transgenic mice demonstrated alterations of CSF-1 action in these cells, at both a morphological and a molecular level, including deregulation of the urokinase plasminogen activator (uPA) gene. These experiments indicate that *ras* signaling pathways are necessary for the terminal differentiation as well as for select biological properties of monocytic cells.

MATERIALS AND METHODS

Generation of transgenic mice. A 5-kb fragment of c-*fms* promoter was fused to the C-terminal portion (amino acids 673 to 1047) of human GAP cDNA (8) or to the C-terminal portion (amino acids 334 to 466) of human c-*ets*-2 cDNA, which was linked to the prokaryotic β-galactosidase gene (28). A simian virus 40 T-antigen intron and poly(A) addition signal used successfully in transgenic projects (2) were inserted downstream of c-*fms* and *ras* suppressor genes. The intended transgenes were freed from prokaryotic vector sequences by digestion with *Sal*I and *Not*I and purified by a glass bead method (Geneclean; Bio 101, LaJolla, Calif.).

The transgenic mice were made by the Duke Comprehensive Cancer Center Transgenic Facility. Embryos were collected from B6/SJL F_1 mice, and microinjection of transgenes and reimplantation of embryos into foster mothers were performed by standard protocols (27). DNA was extracted from mouse tails and was analyzed by Southern blotting or slot blotting in order to identify transgenic mice. The mice were maintained in a virus-free barrier facility.

Histological and hematological analyses of transgenic mice. Mice were sacrificed at several ages (15 days to 1.5 years) and dissected, and their organs were examined. Tissues were preserved (10% neutral buffered formalin) and subsequently embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin prior to evaluation by light microscopy. Bone was decalcified (S/P decalcifying solution; Baxter, McGaw Park, Ill.) prior to embedding and sectioning. Blood, obtained from anesthetized animals, was collected by cardiac puncture, spread onto glass slides, air dried, and fixed and stained with a Diff-quik kit (Baxter). Nonspecific esterase staining of monocytes was performed by using a kit (Sigma, St. Louis, Mo.). The reaction product is a red-brown complex of insoluble chromogenic α-naphthyl-hexazotized pararosaniline.

Indirect immunofluorescence. For immunofluorescence, dried blood or bone marrow smears on glass slides were fixed by being briefly submerged into 100% methanol (15 s, room temperature), air dried, and then fixed in ice-cold 4% paraformaldehyde prepared in phosphate-buffered saline (PBS) for 15 min. Slides were incubated in blocking solution containing 0.1% Nonidet P-40 and 0.4% bovine serum albumin (BSA; Sigma) for 1 h at room temperature and reacted with polyclonal rabbit anti-β-galactosidase antibody (5′-3′ Inc., Bountiful, Utah) diluted 1:100 in PBS–0.4% BSA for 2 h at room temperature. Slides were washed five times with PBS and incubated for 1 h with donkey anti-rabbit dichlorotriazinyl amino fluorescent (DTAF) antibody (Chemicon, Temecula, Calif.) diluted 1:50 in PBS–0.4% BSA at room temperature. Again slides were washed five times in PBS, and cell nuclei were stained with 10 μM bisbenzamide for 5 min. The same procedure was used for the monocyte/macrophage cell surface marker F4-80 (1) except that an anti-rat fluorescent second antibody was employed. Fluorescence microscopy was performed with a Zeiss Axioskop fluorescent microscope. Peritoneal macrophages were analyzed in the same manner except the cells were not allowed to air dry prior to fixation.

RNA analysis. Cellular RNA was extracted from the various murine tissues by a guanidine isothiocyanate-cesium chloride method as previously described (28). Northern (RNA) analysis was also performed as described previously (28).

For reverse transcription coupled with PCR (RT-PCR), 10 μg of total RNA was digested with 0.5 U of DNase in 5 mM $MgCl_2$–0.5 mM dithiothreitol–2 U of RNasin at 37°C for 20 min, extracted with phenol-chloroform, and precipitated with ethanol. Reverse transcription reaction mixtures contained 0.5 μg of RNA in 10 mM Tris (pH 8.3)–50 mM KCl–5 mM $MgCl_2$–1 U of RNasin–1 mM deoxynucleoside triphosphates, 2.5 mM oligo(dT)–2.5 U of reverse transcriptase. The reaction mixtures were incubated at 42°C for 15 min, boiled at 99°C for 5 min, and cooled on ice. The PCR mixture contained primers previously described for the GAP-C and *ets-lacZ* genes (8, 26), primers for the mouse γ-actin gene, and 0.5 U of *Taq* polymerase. Amplification was performed with a Programmable Thermal Controller (MJ Research, Watertown, Mass.) for 30 cycles (94°C for 1 min, 60°C for 2 min, and 72°C for 3 min).

Preparation and analysis of peritoneal macrophages. Peritoneal macrophages were obtained by the method outlined by Conrad (16). Mature mice were injected with 1 ml of thioglycolate into the peritoneal cavity. Four days following injection, animals were sacrificed, and peritoneal cells were harvested by flushing the peritoneal cavity with 10 ml of PBS with 10 U of heparin per ml. Cells were centrifuged at 250 × *g* for 5 min at 4°C and washed with serum-free RPMI. After the wash step was repeated, cells were counted on a hemacytometer and further analyzed. Usually, normal mice yielded 2×10^7 elicited macrophages, while 0.7×10^7 to 1.5×10^7 cells were obtained from transgenic mice.

Generally, cells were plated in 100-mm-diameter dishes at 0.5×10^7 to 1×10^7 cells per plate. Cells were cultured in RPMI with 10% fetal calf serum and 2,000 U of recombinant CSF-1 (Genetics Institute) per ml. For the analysis with different amounts of CSF-1, cells were plated on printed, 10-well slides (Roboz Surgical Instrument Co.) that had been coated with 1% gelatin in PBS. Then 2,000 cells per well were added in RPMI that contained 0.1% fetal calf serum with exogenous CSF-1 as indicated. Cells were fixed with a 2% paraformaldehyde–0.2% glutaraldehyde solution in PBS prior to light microscopic investigation or as described above for immunofluorescence.

***ras*-GTP assays.** Determination of the level of *ras* in the GTP complex was essentially as described previously (18). Briefly, 7×10^6 to 8×10^6 elicited peritoneal macrophages were placed in a 100-mm-diameter dishes in medium containing 2,000 U of CSF-1 for 16 h. Nucleotide pools were labeled with radioactive P_i in phosphate-free medium for 3 h, and the cells were lysed with a high-salt buffer that contained 5 μg of *ras* antibody Y13-259 (Oncogene Science). This amount of antibody was determined to be fivefold in excess of the amount needed to block stimulation of *ras* GTPase activity in the peritoneal macrophage extracts. Nucleotides were extracted and analyzed by thin-layer chromatography in parallel with unlabelled nucleotide standards. Radioactivity was quantitated with a Betascope 603 phosphoimager (Betagen Corp., Waltham, Mass.), and the results are expressed as 2GTPdpm/(2GTPdpm + 3GDPdpm), where dpm is disintegrations per minute.

RESULTS

Construction of transgenic lines expressing suppressors of *ras* and c-*fms* action. To test the hypothesis that *ras* signaling pathways are necessary for monocytic phagocyte differentiation and function, we constructed the recombinant plasmids shown in Fig. 1a and microinjected them into fertilized mouse eggs. These plasmids included 5 kb of sequence derived from the first exon of the human c-*fms* gene that contained elements necessary for monocyte-specific expression (35, 36) and a gene encoding GAP-C (8) or *ets-lacZ* (28). Potential transgenic animals were screened by examining tail DNA by Southern blotting. Of six founders obtained, two c-*fms* promoter/GAP-C founder mice, containing 15 and 30 copies of the transgene, respectively, were chosen for further analysis (data not shown). For c-*fms* promoter/*ets-lacZ* DNA, three founders, containing 2, 10, or 15 copies of the transgene (data not shown), were obtained, and all three were used for further analysis.

Expression of the transgenes was assayed in various tissues, using RT-PCR. The results of the analysis of expression in two GAP-C transgenic mice from one of the founders are presented in Fig. 1b (ethidium bromide-stained gel) and c (Southern blot of the gel shown in Fig. 1b). The data demonstrated that the transgene was expressed in peritoneal macrophages and bone marrow, as expected (43). The transgene was also expressed in liver, a tissue that contains CSF-1-dependent tissue macrophages (45). Other tissues known to contain monocytes and/or macrophages and their precursors, such as spleen and lung, expressed the transgene (data not shown). In contrast, two tissues that contain relatively few CSF-1-dependent macrophages (45), thymus and skeletal muscle, did not express the transgene (Fig. 1c). Analysis of the *ets* transgenic lines indicated a similar pattern of expression (data not shown). These results suggest, but do not prove, that expression of the transgene is monocyte/macrophage specific.

Abnormal monocytes appear in the blood of transgenic animals. On gross observation, the transgenic animals from all five founder lines were largely unremarkable. However, from

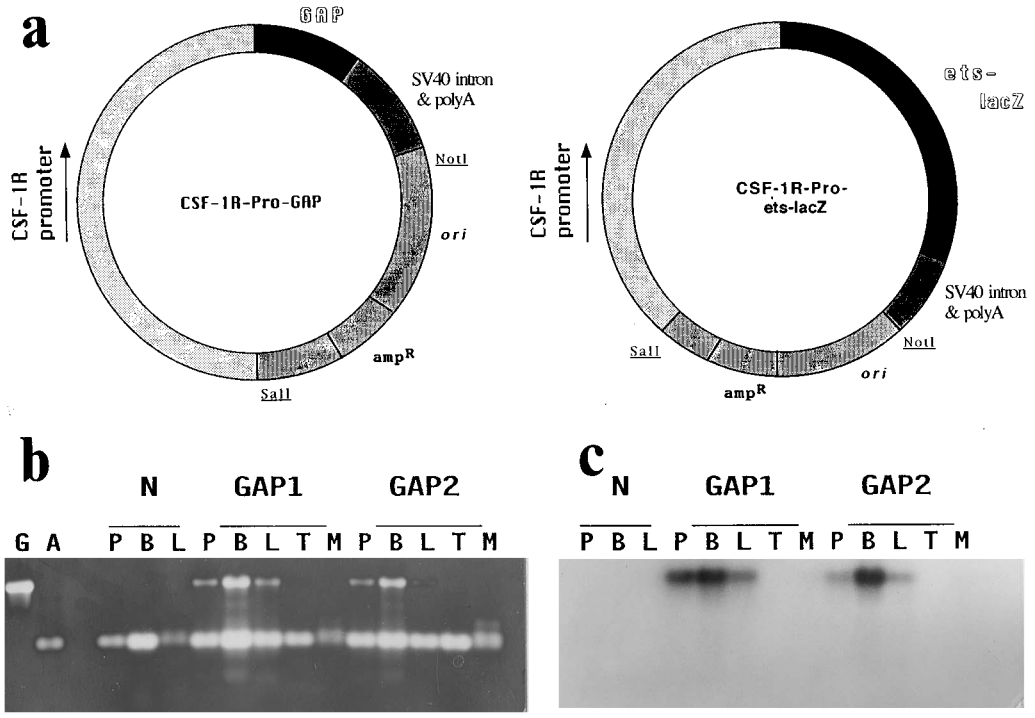

FIG. 1. Schematic representation of transgenic constructs and expression pattern of the transgenes in mice. (a) Vectors were digested with *Not*I and *Sal*I and the resulting c-*fms*/GAP-C and c-*fms*/*ets-lacZ* fragments were purified for microinjection. CSF-1R, CSF-1 receptor; SV40, simian virus 40. (b) RT-PCRs from various tissues of a nontransgenic animal (N) and two GAP transgenic animals (GAP1 and GAP2). Probes that were specific for the GAP-C transgene and the γ-actin gene were included in each reaction. Tissues were taken from peritoneal macrophages (P), bone marrow (B), liver (L), thymus (T), and muscle (M). Lane 1 is a GAP plasmid control (G), and lane 2 is a γ-actin control (A). (c) Southern blot of RT-PCRs from panel b. The blot was probed with a GAP-specific probe. Lanes are labeled as in panel b.

day 8 postpartum until weaning, they were 30 to 50% smaller than normal littermates. Their small size has been a reliable method to distinguish transgenic mice from normal littermates. The eyes of the transgenic mice open 1 day later than normal, and incisors often erupt with a delay of 1 day. After weaning, the difference in size between transgenic and normal animals diminishes, but the transgenic mice attain only 80 to 90% of wild-type weight.

Observation of blood smears from transgenic animals at 15 to 40 days of age provided the first strong indication of an abnormal phenotype in these animals (Fig. 2). Normal monocytes usually constitute about 5 to 10% of leukocytes in mouse blood. In all five transgenic lines (GAP-C and *ets-lacZ* animals), 30 to 50% of leukocytes had the appearance of cells shown in Fig. 2. These cells had large, immature, blast-like nuclei that often contained multiple nucleoli. The foamy cytoplasm was extensive and often appeared highly vacuolated. These cells adhered to one another, as they were often found in clumps (Fig. 2A and C). Sometimes these clumps formed multinucleated syncytia (Fig. 2A), but usually cell boundaries were distinct (Fig. 2C). Monocytes with normal morphology were generally difficult to locate in blood smears such as those depicted in Fig. 2. However, as the animals matured beyond 40 days, normal monocytes could be found intermittently along with the abnormal monocytes. The exact explanation for this phenomenon is unclear. However, in *op*/*op* mice, osteopetrosis and hematopoietic deficiencies are progressively corrected with age (4, 31). Thus, it may be that alternate pathways can eventually override deficiencies in *ras* signaling.

The unusual cells reacted positively with a histochemical stain that detects nonspecific esterase activity (Fig. 2D) and also expressed the monocytic phagocyte-specific antigen F4-80 (1), as determined by immunofluorescence analysis (data not shown), demonstrating that they are monocytic. In comparison with the monocytic cells, other classes of leukocytes, including lymphocytes, neutrophils, and megakaryocytes, were of unremarkable morphology and were present in normal counts. The data corroborate that transgene expression is tissue restricted.

Indirect immunofluorescence utilizing a polyclonal antibody specific to the bacterial *lacZ* gene revealed the expression of *ets-lacZ* gene in cells present in blood smears obtained from transgenic animals (Fig. 3). Expression of *ets-lacZ* was localized primarily, but not exclusively, to the nuclei of the unusual monocytes (Fig. 3B and D), as expected from previous studies performed with established cell lines (28). Expression was not observed in any other cell type present in blood, including neutrophils (Fig. 3F). Monocytes from nontransgenic mice were negative in the analysis (data not shown). We have analyzed bone marrow smears obtained from the *ets-lacZ* transgenic mice and find that only cells that counterstained with the F4-80 marker were positive for transgene expression (data not shown). These results provide the most convincing evidence that expression of the transgene is directed to monocytic cells by the c-*fms* proximal promoter.

Histological analyses of other tissues and organs of mice between the ages of 15 and 40 days were uninformative, including the number and morphology of macrophages resident in many tissues. In particular, bone marrow appeared normal, while in spleen, only an occasional unusual monocyte as depicted in Fig. 2 was encountered. These data indicate that the unusual cells found in the circulation did not occur as the result of abnormal differentiation of monocytic stem cells resident in

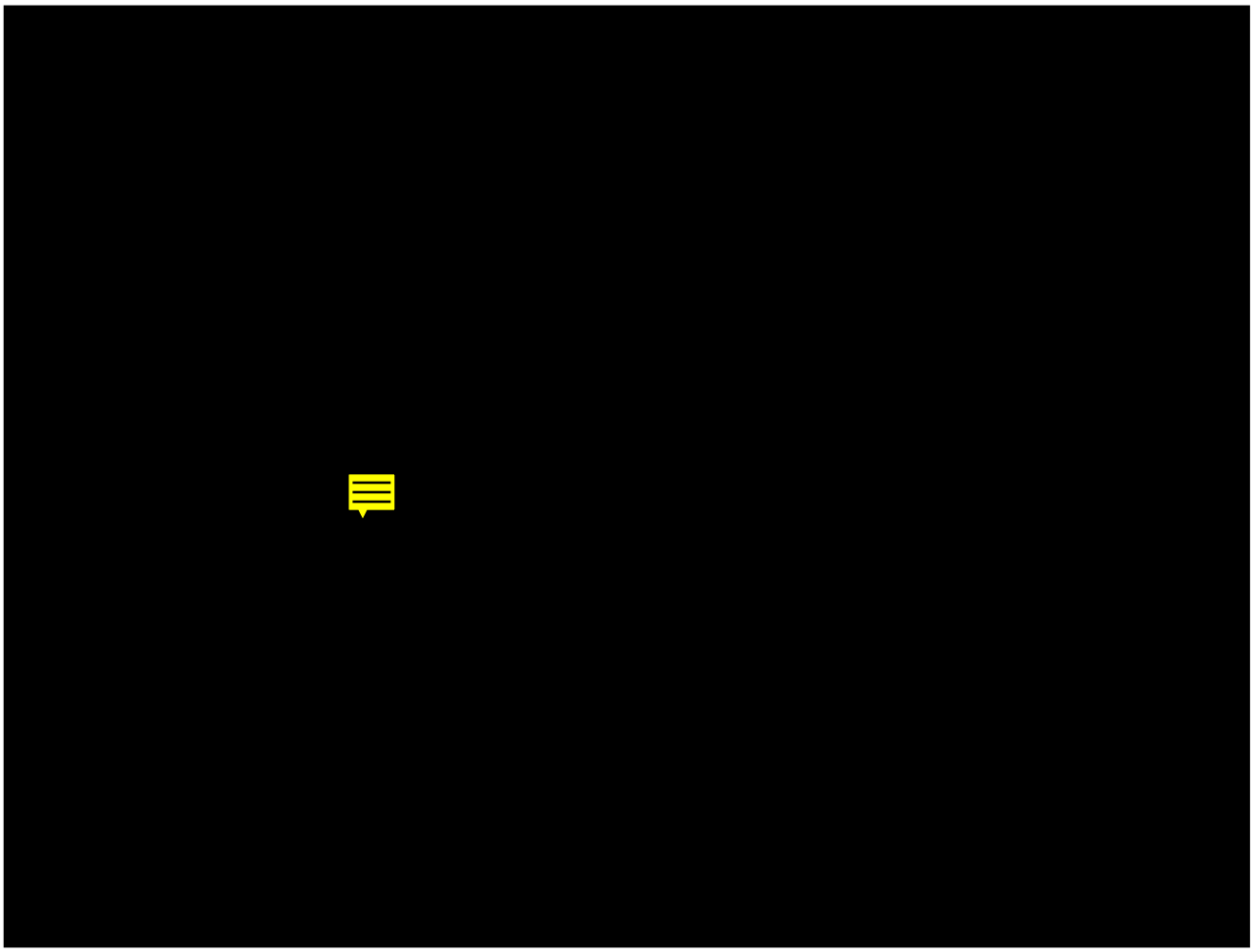

FIG. 2. Abnormal monocytes present in blood smears prepared from transgenic mice. (A) Blood smear from a mouse with the *ets-lacZ* transgene. The feathered edge of the smear shows a giant cell containing two round nuclei and one oval nucleus exhibiting prominent nucleoli and abundant cytoplasm. Cellular boundaries are indistinct. Numerous erythrocytes, one segmented granulocyte (G), and one lymphocyte (L) are also present. (B) Feathered edge of a blood smear from a mouse with the *ets* transgene. Nuclei of cells are large, open, and round or oval, and there is abundant cytoplasm. Erythrocytes and a cluster of platelets are present in the field. (C) Blood smear from a mouse with the GAP-C transgene. The feathered edge of the smear with a cluster of monocytoid cells exhibits round nuclei, prominent nucleoli, and abundant cytoplasm with some vacuolization and more distinct cellular boundaries. (D) Blood smear from a mouse with the GAP transgene stained for nonspecific esterase activity. The brown nonspecific esterase reaction product is evident in the cytoplasm.

the bone marrow, as such cells would have been expected to be found on examination of the marrow. Bone marrow-derived macrophages could be readily produced by culturing bone marrow derived from transgenic animals in vitro with CSF-1. The number of cells found after 6 days in culture was virtually identical between transgenic (both GAP-C and *ets-lacZ*) mice and controls (data not shown). Further, thymidine incorporation by bone marrow cells that were cultured for 24 or 48 h in the presence of either CSF-1 or granulocyte-macrophage colony-stimulating factor was the same for both transgenic mice (both GAP-C and *ets-lacZ*) and normal controls (data not shown). Thus, we could not determine an obvious effect on CSF-1-dependent differentiation of bone marrow stem cells derived from the transgenic mice.

Demineralized bone from the transgenic animals also appeared normal. We have not yet undertaken a careful developmental analysis of bone that had not been demineralized, and in *op*/*op* mice, bone abnormalities disappear as the mice mature (4, 31). These results may mean that we have not examined the transgenic mice at the correct stage of bone development. Alternatively, our findings may indicate that *ras* signaling is not critical for osteoclast differentiation and function. However, preliminary results, obtained in assays using the murine tartrate-resistant acid phosphatase promoter (13) to direct *ets-lacZ* transgene expression to later stages of macrophage and osteoclast differentiation, revealed bone abnormalities (27a), suggesting that the c-*fms* promoter may not be optimal for experiments with osteoclasts.

Reduced *ras*-GTP levels in elicited peritoneal macrophages isolated from GAP-C mice. The results described above indicated that monocyte differentiation was affected by the expression of GAP-C or *ets-lacZ* during the later, terminal stages after the cells had entered the circulation. To examine directly whether *ras* signaling was affected in mature macrophages, we measured the level of *ras* in the active GTP complex (18) in thioglycolate-elicited peritoneal macrophages. These cells were chosen because cells isolated from a single animal were sufficient for performing the biochemical assay. The results of typical analyses are documented in Fig. 4. Peritoneal macrophages from nontransgenic animals had *ras*-GTP levels of

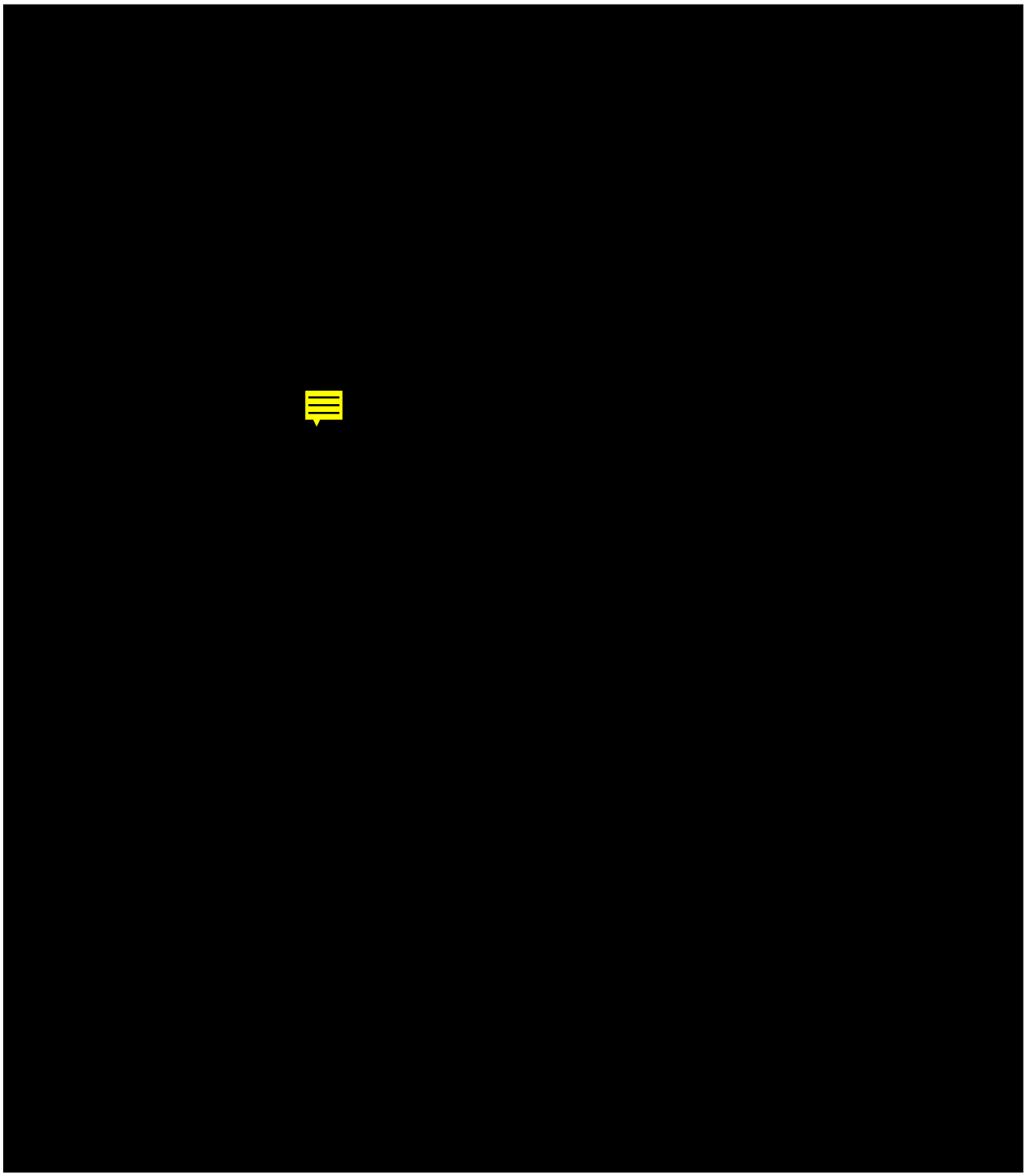

FIG. 3. Expression of the *ets-lacZ* transgene in monocytic cells detected by indirect immunofluorescence. Blood smears from *ets-lacZ* transgenic mice were fixed and analyzed as described in Materials and Methods. (A to D) Monocytic cells from two different transgenic lines; (E and F) granulocytes from the same smear as in panels A to D. Panels A, C, and E show nuclear staining of cells with bisbenzamide; panels B, D, and F were labeled with the *lacZ* polyclonal antibody.

greater than 30%, while *ras*-GTP levels in the four GAP-C animals were reduced to approximately 12%. Controls in which the *ras* antibody was not added lacked the GTP and GDP signals but still had the ATP signal (data not shown). The difference in ATP signal between control and transgenic mice may reflect a difference in metabolic or respiratory rate or in the size of the ATP pools in the cells. A total of six normal and eight GAP-C transgenic animals exhibited the same results as

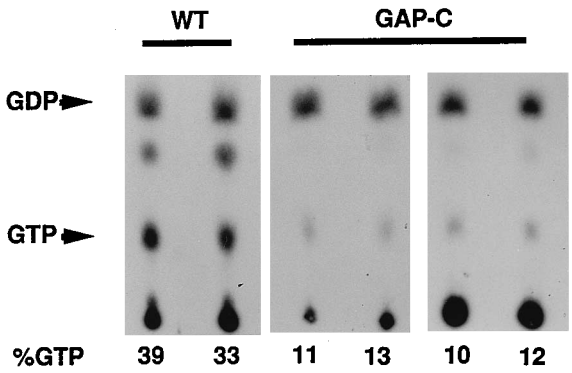

FIG. 4. The level of the *ras*-GTP complex is reduced in peritoneal macrophages isolated from transgenic mice. The amount of *ras* in the GTP form was determined as described in Materials and Methods. Shown are autoradiographs of the thin-layer separation of nucleotides extracted from two nontransgenic (wild-type [WT]) littermates and four GAP-C transgenic animals, as indicated. The radioactivity present in the GTP and GDP spots (marked with arrows) was measured, and the percent GTP form of *ras* is expressed as 2GTPdpm/(2GTPdpm + 3GDPdpm). The extra spot located intermediate to the guanine nucleotides is ATP.

shown in Fig. 4. A control experiment in which macrophages from normal and transgenic mice were mixed and then subjected to this analysis demonstrated that the wild-type *ras*-GTP levels were dominant, indicating that GTP hydrolysis during the procedure could not explain the differences observed between normal and transgenic mice (data not shown). Two *ets-lacZ* mice were analyzed by the procedure and had high (30%) *ras*-GTP signals (data not shown). Thus, *ras* signaling was significantly impaired in mature peritoneal macrophages from transgenic GAP-C mice.

Morphological differences in peritoneal macrophages isolated from transgenic animals. One consequence of reduced *ras* signaling in peritoneal macrophages isolated from transgenic animals was their appearance after 3 days in culture (Fig. 5). Macrophages from transgenic animals did not exhibit the characteristic morphological features readily apparent in cultures obtained from normal mice, even after prolonged culture in the presence of CSF-1 (Fig. 5A and B, *ets-lacZ* line). Indirect immunofluorescence demonstrated that 100% of macrophages in culture expressed the *ets-lacZ* transgene independent of CSF-1 status (Fig. 5D). The *ets-lacZ* transgene was expressed predominantly, albeit not exclusively, in the nucleus and perinuclear space of peritoneal macrophages.

To further examine morphological responses to CSF-1 in the transgenic mice, small numbers of peritoneal macrophages

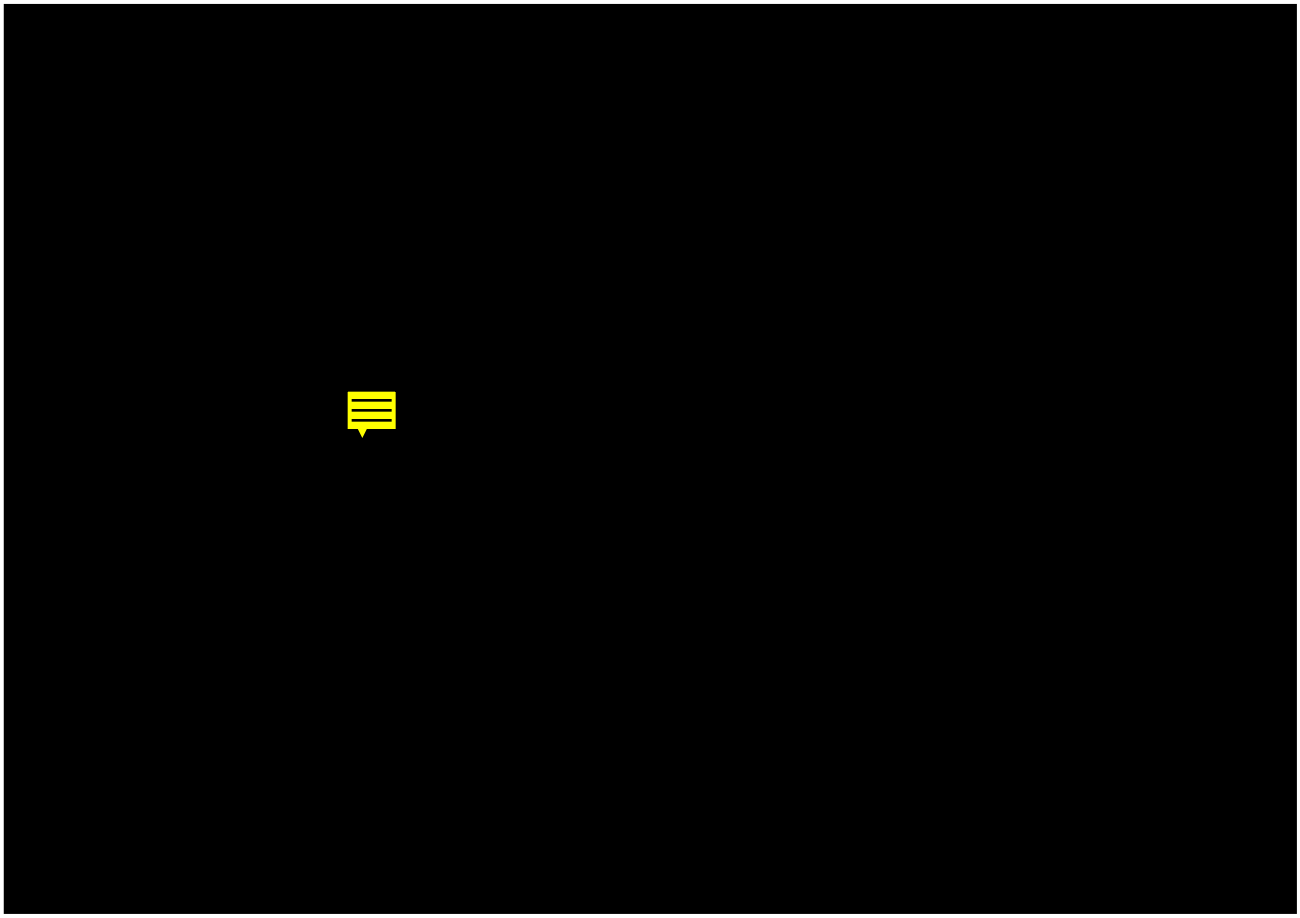

FIG. 5. Characterization of morphology and *ets-lacZ* expression in peritoneal macrophages. Peritoneal macrophages from a nontransgenic animal (A) and an *ets-lacZ* transgenic animal (B) were treated with 2,000 U of CSF-1 per ml for 72 h, fixed, stained, and photographed. Peritoneal macrophages from an *ets-lacZ* transgenic animal were again fixed, permeabilized, and stained with a polyclonal anti β-galactosidase antibody and a secondary fluorescein-conjugated donkey anti-rabbit immunoglobulin (D). Cells were then treated with bisbenzamide nuclear counterstain (C). Magnification is 2.5× higher for panels C and D.

(2,000 cells) were cultured on glass slides in low-serum medium that contained different concentrations of CSF-1 (Fig. 6). The addition of small amounts of CSF-1 caused marked morphological changes in macrophages from normal animals (Fig. 6A to D). In contrast, the fate of macrophages obtained from GAP-C (depicted in Fig. 6E to H) or *ets-lacZ* (not shown) mice differed from those of littermates in two characteristic manners. First, these cells were very sensitive to withdrawal of CSF-1, undergoing what appeared to be programmed cell death (Fig. 6E). Preliminary experiments indicated that DNA isolated from these cells exhibited the nuclear condensation and DNA cleavage pattern characteristic of programmed cell death (data not shown). When CSF-1 was added to the culture medium, cell survival increased in proportion to the concentration of growth factor (Fig. 6F to H). However, even when the concentration of CSF-1 was increased over 2 log units, only minimal morphological responses were elicited in these cells. Macrophages derived from more than 30 GAP-C and *ets-lacZ* transgenic mice, representing all 5 founders, were analyzed in this manner with the same result, i.e., hypersensitivity to CSF-1 withdrawal and abrogation of typical morphological changes.

Expression of the uPA gene is altered in transgenic mice. To extend these morphological observations, the expression of uPA mRNA (14) was monitored in peritoneal macrophages. Recent work in our laboratory (46a) has confirmed that the uPA gene contains a *ras*-responsive enhancer element composed of binding sites for *ets* and AP1 factors (6); further CSF-1 stimulation of uPA mRNA expression in NIH 3T3 cells expressing human c-*fms* can be blocked by expression of either GAP-C or *ets-lacZ* (42a). Results from two varieties of experiments are presented (Fig. 7). In one procedure, elicited peritoneal macrophages were isolated and placed in culture with CSF-1 for about 1 h until the macrophages adhered to the plastic dishes. Subsequently, RNA was prepared and analyzed by Northern blotting (Fig. 7B). The expression of uPA mRNA was reduced by 80 to 90% in macrophages prepared from the *ets-lacZ* or GAP-C transgenic mice.

In the second procedure, elicited macrophages were cultured in duplicate dishes in the presence of CSF-1 for 3 days. Following this, CSF-1 was removed from the medium in both cultures for 24 h. CSF-1 was then added back to the medium of one culture, and after 6 h, RNA was prepared from both cultures and analyzed by Northern blotting (Fig. 7A). Expression of uPA mRNA is already high in peritoneal macrophages isolated from normal mice (Fig. 7A) and is induced two- to threefold by treatment with CSF-1, when expression is normalized to the level of γ-actin mRNA (Fig. 7A, bottom row). In macrophages isolated from transgenic mice, the basal expression of uPA mRNA is 10 to 20% of the level in nontransgenic littermates, and CSF-1 treatment has little effect on these levels, after normalization to γ-actin expression (Fig. 7A). We have studied uPA expression in peritoneal macrophages from 45 animals (12 normal, 18 GAP-C, and 15 *ets-lacZ*), with results identical to those presented in Fig. 7. These results demonstrate that *ras*-mediated signaling to the nucleus is deregulated in the transgenic mice.

The RNA from peritoneal macrophages was also analyzed for expression of either c-*fms* mRNA or tartrate-resistant acid phosphatase mRNA. The latter is a macrophage and osteoclast marker that is expressed in terminally differentiated cells (13), but this gene is not directly regulated by the c-*fms*/*ras* pathway (27a). This analysis revealed that the levels of c-*fms* mRNA are equivalent in normal and transgenic animals (Fig. 8A). Therefore, the results presented above cannot be attributed to negative regulation of c-*fms* mRNA expression by the transgenes. Expression of tartrate-resistant acid phosphatase mRNA was not affected by expression of either GAP-C or *ets-lacZ* transgenes (Fig. 8B), further indicating that the inhibition of uPA expression was selective.

DISCUSSION

Genetic experiments with the invertebrate organisms *D. melanogaster* and *C. elegans* have identified new genes, *sem5*/*grb2* and *sos*, that are components of a complex required for coupling the *ras* signaling pathway to the action of tyrosine kinase receptors (15, 39). While *ras* is an essential gene, the experiments succeeded because the *ras* pathway could be studied in nonessential organ systems in these invertebrates. In the approach described here, we have attempted to define roles for the *ras* signaling pathway by using monocytic phagocytes, a system that the *op*/*op* mutation demonstrates is not essential in the mouse (4, 45, 47). Our work defines a mammalian genetic system that will allow definition of the *ras* pathway in vivo. For example, the 42- and 44-kDa mitogen-activated protein kinases are necessary for mitogenic *ras* signaling in mouse fibroblasts (reviewed in reference 20); however, these molecules appear dispensable for c-*fms*/*ras*/*raf* signaling in the CSF-1-dependent macrophage cell line BAC-1.2F5 (11). Complementation of the various defects described here with various mitogen-activated protein kinase genes should be possible by infection of transgenic mice with appropriate retrovirus vectors and should allow the relevance of such molecules to *ras* signaling in vivo to be determined.

Embryonic stem cell technology (12) provides one approach to experimentally comparing the relevance of in vitro observations with the action of *ras* genes in monocytes in vivo. However, multigene families like the *ras* family represent a challenging target for embryonic stem cell-generated knockout mutations. Additionally, such mutations would delete the entire spectrum of *ras* signaling during murine development and likely prove lethal to embryos. This is a general problem expected with the knockout approach for studying intracellular signal transduction pathways in the mouse. An alternate approach used in the present study is to employ a promoter presumed to be tissue specific to drive the expression of dominant suppressors of *ras* action in a restricted cell compartment. The choice of promoter and especially the choice of dominant suppressors are critical for the interpretation of such experiments. A novel aspect of our work is the apparent monocyte specificity of the human c-*fms* proximal promoter in vivo, demonstrated by indirect immunofluorescence on blood smears, bone marrow smears, and mature peritoneal macrophages, as well as the lack of phenotype in other hematopoietic lineages.

The GAP-C gene fits well the role of specific *ras* suppressor, since the primary identified activity of this region of GAP is stimulation of *ras* GTPase activity (32). That *ras*-GTP levels are reduced in macrophages isolated from the GAP-C transgenic mice demonstrates that *ras* signaling is directly impaired. The specificity of the *ets-lacZ* suppressor is less certain. A suppressor based on the DNA binding activity of a transcription factor may be expected to be promiscuous. However, the phenotypes in monocytes and peritoneal macrophages detected in multiple GAP-C and *ets-lacZ* transgenic lines were indistinguishable, arguing for specificity of this dominant-negative gene for the *ras* pathway as well.

One consequence of interrupting *ras* signaling in monocytic cells appeared during the differentiation of this cell type after cells enter the circulation. An accumulation of abnormal monocytic cells was not detected in bone marrow or spleen, revealing that changes in differentiation occurred at the stage

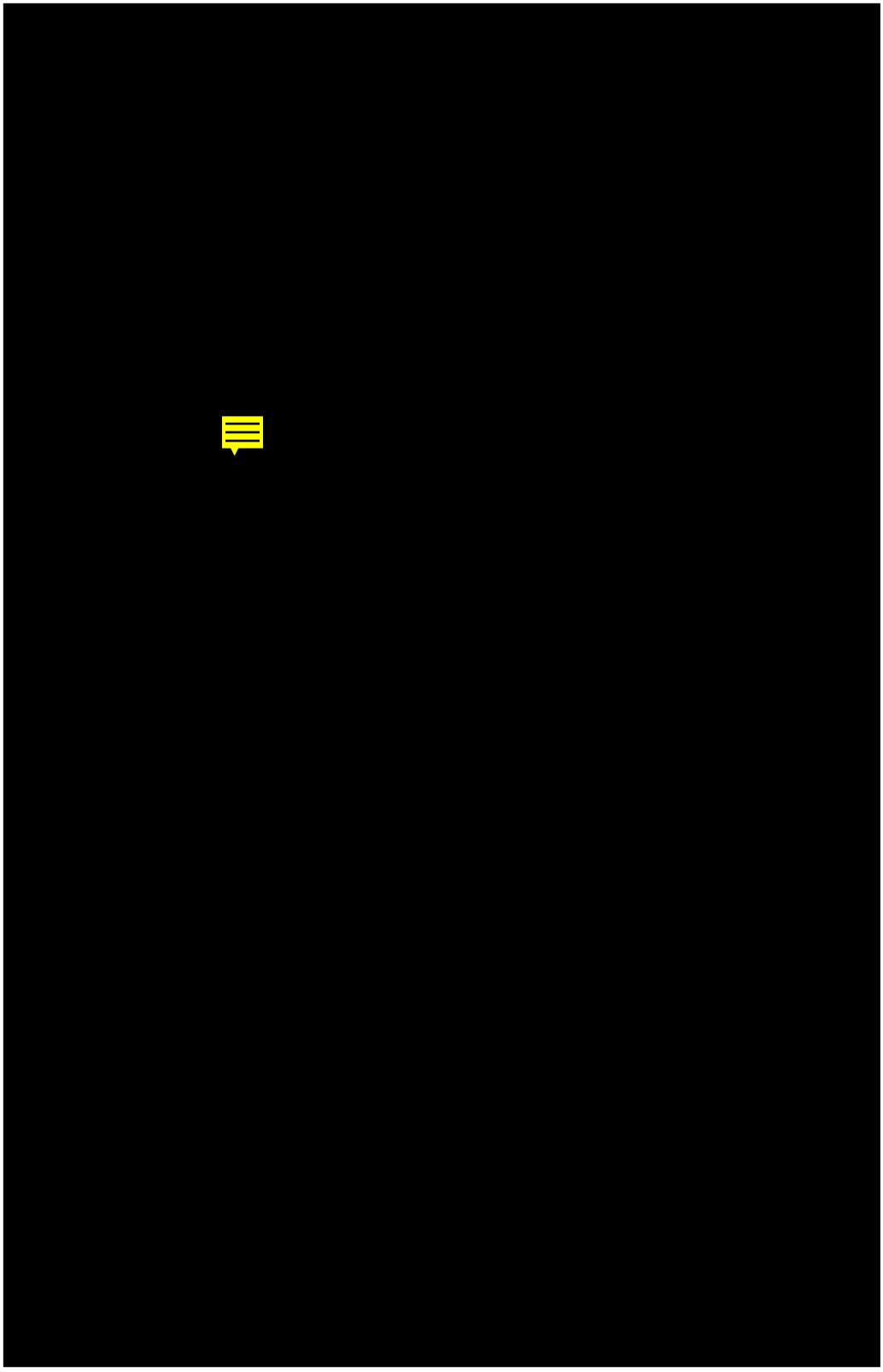

FIG. 6. Morphological characterization of peritoneal macrophages in response to CSF-1. Adherent peritoneal macrophages from wild-type (A to D) and GAP-C (E to H) mice were treated with CSF-1 (A and E, 0 U/ml; B and F, 20 U/ml; C and G, 200 U/ml; D and H, 2,000 U/ml) for 72 h.

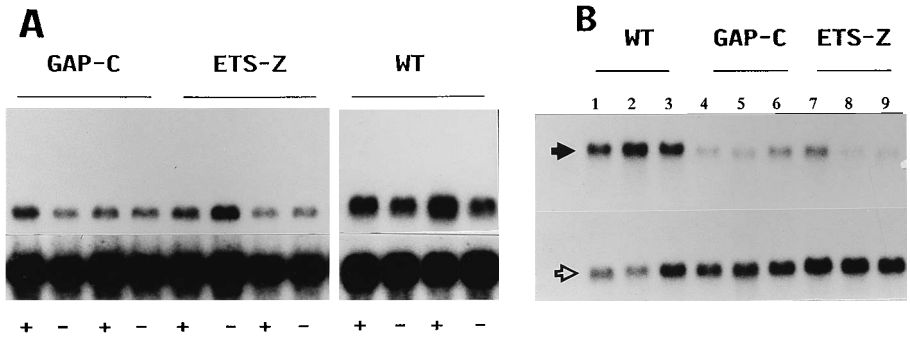

FIG. 7. Analysis of uPA mRNA expression in transgenic mice. (A) Macrophages were placed in duplicate cultures for 72 h in the presence of 2,000 U of CSF-1 per ml and then withdrawn from growth factor for 24 h. After restimulation of one culture with 2,000 U of CSF-1 per ml for 6 h, total RNA was prepared, and 10 μg per lane was analyzed by Northern blotting. Filters were probed with a ^{32}P-labeled murine uPA probe (upper row) or rehybridized with a γ-actin probe (lower row). Unstimulated (−) or CSF-1-stimulated (+) samples are displayed. RNAs from two GAP-C transgenic animals (GAP-C), two *ets-lacZ* transgenic animals (ETS-Z), and two nontransgenic animals (wild type [WT]) were analyzed. (B) Comparison of the levels of uPA in peritoneal macrophages that attached to plastic after 1 h in the presence of 2,000 U of CSF-1 per ml. RNAs derived from three nontransgenic animals (WT), three GAP-C transgenic mice (GAP-C), and three *ets-lacZ* mice (ETS-Z) are displayed. Filters were probed as described above.

of the circulating monocyte and not earlier. Consistent with this notion are preliminary experiments in which the promoter for the tartrate-resistant acid phosphatase gene, a gene that turns on during the terminal stages of macrophage and osteoclast differentiation (13), was used to drive expression of the *ets-lacZ* suppressor. The monocyte phenotype observed to date in such transgenic lines corresponds to what was observed with the c-*fms* promoter (27b). The lack of apparent phenotype of bone marrow progenitors, either in vivo or in vitro, was an unexpected result. A likely explanation for this phenomenon is that the c-*fms* promoter has relatively low activity in immature cells present in the bone marrow and thus is incapable of driving the relatively high levels of dominant-negative gene products likely required to elicit a biological effect. For example, recent experiments in which the activity of the c-*fms* promoter was studied during differentiation of M1 leukemia cells to macrophages in vitro demonstrated that the promoter was much more active after differentiation had been initiated, at least in part because of the higher expression of the c-*myb* proto-oncogene in the more immature cell type (35).

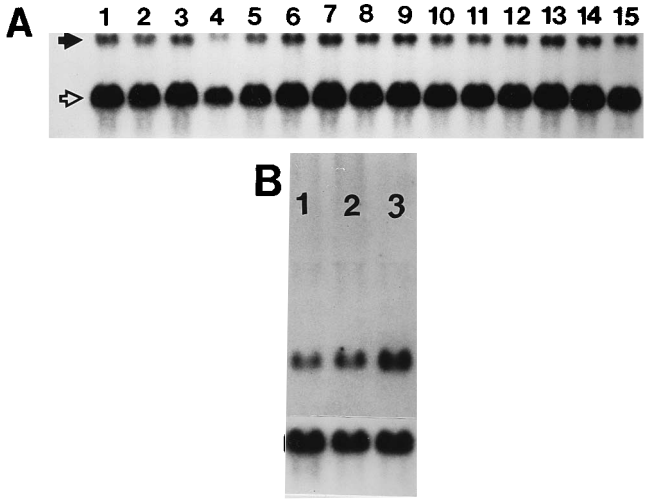

FIG. 8. Analysis of c-*fms* and TRAP mRNA expression in transgenic mice. Total RNAs (10 μg) from peritoneal macrophages were electrophoresed, transferred to a Nytran membrane, and hybridized with cDNA probes. (A) The membrane was hybridized with a murine c-*fms* probe (upper bands) and a murine γ-actin probe (lower bands). Total cellular RNAs from five individual nontransgenic mice (lanes 1 to 5), five GAP-C transgenic mice (lanes 6 to 10), and five *ets-lacZ* transgenic mice (lanes 11 to 15) were analyzed. (B) The membrane was hybridized with a TRAP probe (upper band) and a γ-actin probe (lower band). RNAs from nontransgenic (lane 1), GAP-C (lane 2), and *ets-lacZ* (lane 3) mice were analyzed.

Another result of interfering with *ras* signaling in the transgenic mice is the decreased survival and of peritoneal macrophages in vitro when CSF-1 levels are depleted. The cell death has the hallmarks of apoptosis, including nuclear condensation and nuclease laddering of DNA (42b). Therefore, the *ras* pathway may be opposed to an apoptotic pathway in the mature peritoneal macrophage and thus contribute to the ability of c-*fms* to promote macrophage survival. Additionally, obvious alterations in cell morphology and inhibition of the expression of the CSF-1 and *ras*-inducible uPA gene, a gene that is involved in macrophage migration (21), were observed. Cellular properties necessary for macrophage competence in immune responses may be altered in the transgenic mice. Experiments are currently under way to study the effect of suppressed *ras* signaling on inflammation and antimicrobial responses in the GAP-C and *ets-lacZ* transgenic mice.

Notably, the abnormalities observed in the transgenic mice used in these studies occurred during the later stages of monocyte/macrophage differentiation when more than 90% of these cells had lost the capacity for cell division (43). In contrast, in tissue culture cells, *ras* action has been demonstrated to be coupled intimately to cell cycle progression (3, 20, 42). Previous studies have indicated a role for *ras* during monocyte differentiation. For example, FDC-P1 cells can be induced to differentiate to macrophages in vitro by a c-*fms*/*ras* signaling pathway (29) or by activated *ras* alone (26). The macrophage expression of class II histocompatibility antigens has been reported to be controlled by *ras* signaling (41). Treatment of human bone marrow with antisense oligonucleotides directed to the N-*ras* gene can block CSF-1-dependent differentiation of macrophages in vitro (40). Finally, acute myelomonocytic or monocytic leukemia in humans is associated with a high rate (36 to 56%) of *ras* gene mutations (22, 48), suggesting a relationship between *ras* action and monocyte differentiation. Our results are uninformative about the role of *ras* in the earlier stages of differentiation when mitogenic growth still occurs; however, they do indicate that *ras* signaling is necessary for distinct aspects of monocyte terminal differentiation and function, consistent with the work cited above. This transgenic

approach should allow us to understand in more detail how *ras* signaling is involved in the various aspects of monocyte biology.

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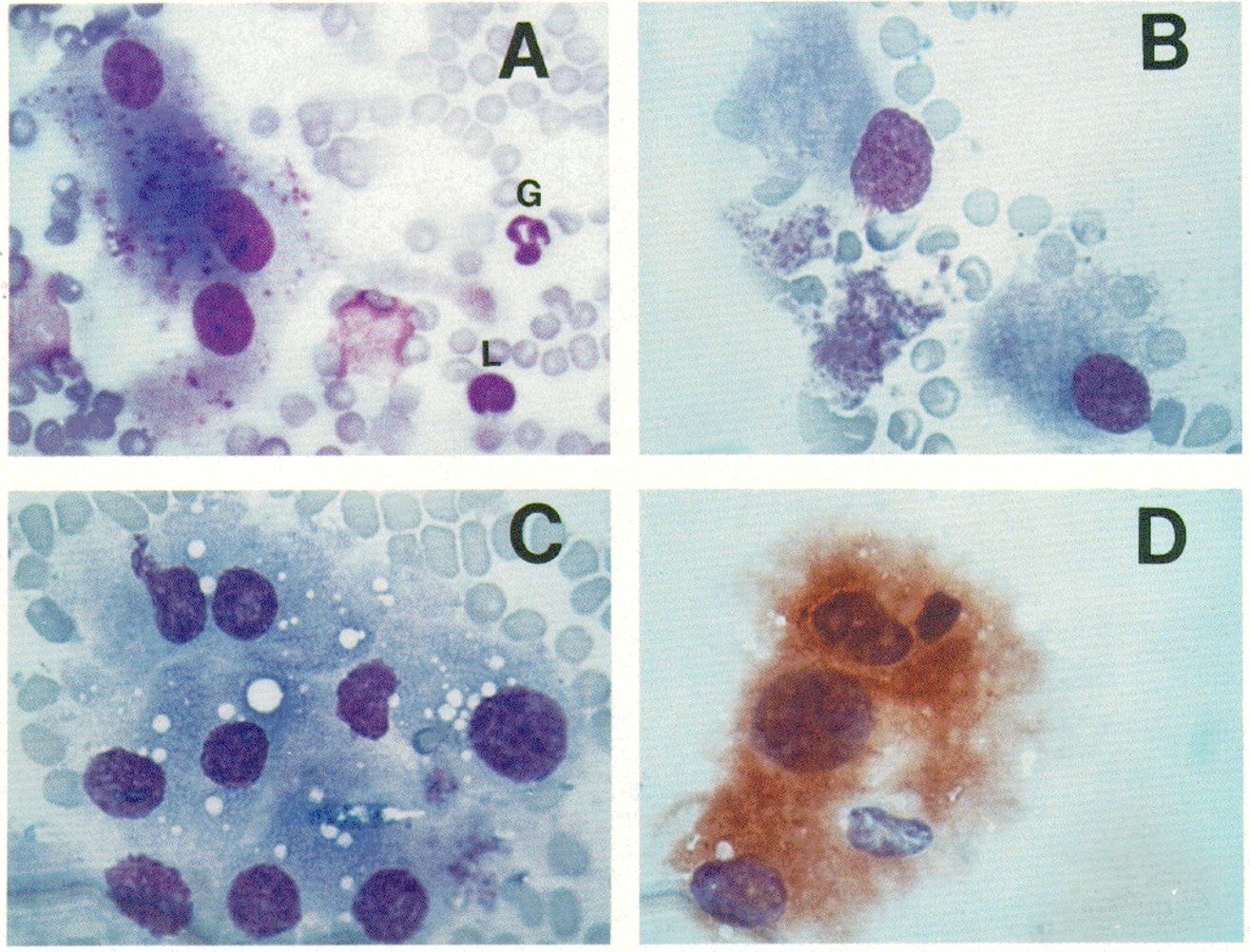
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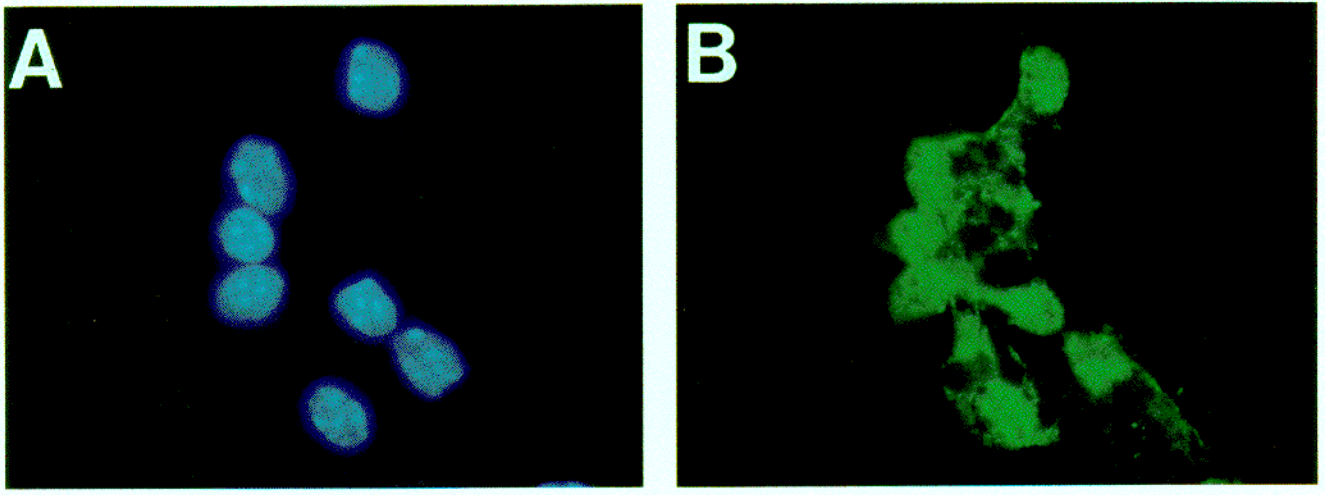
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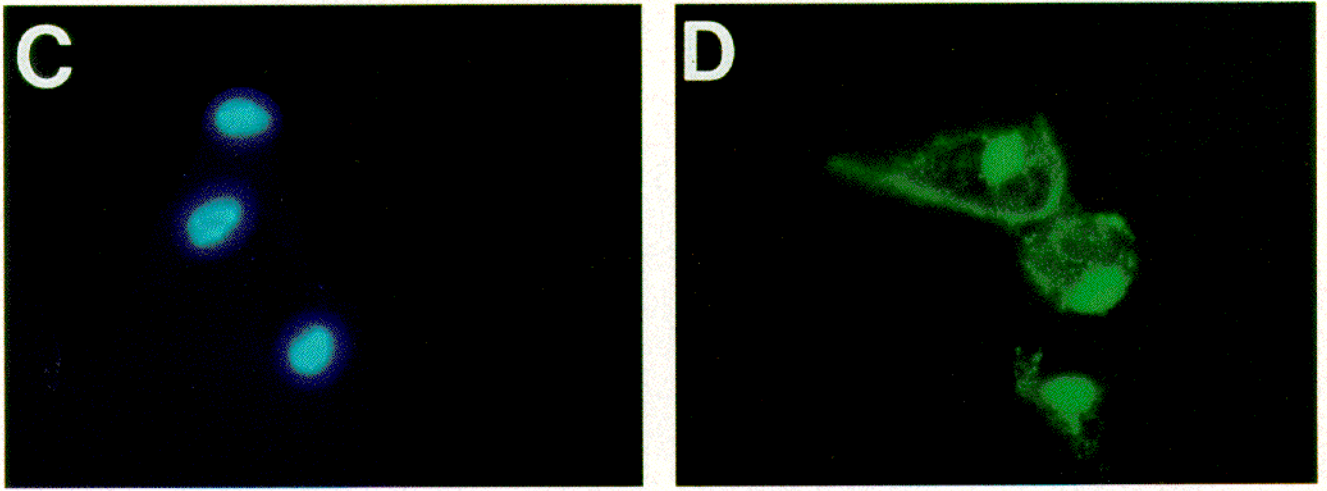
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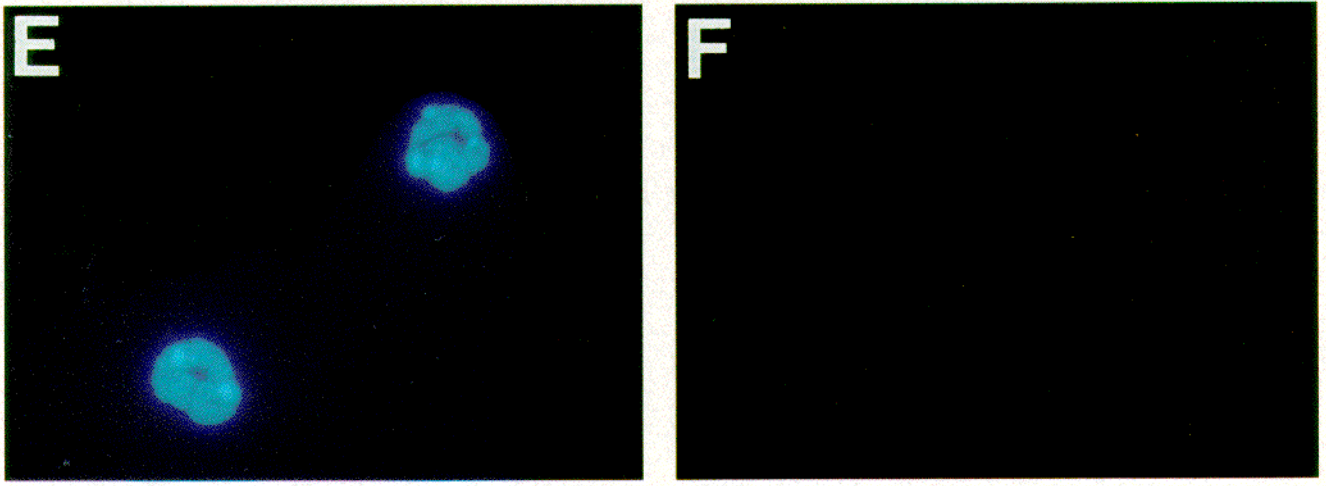
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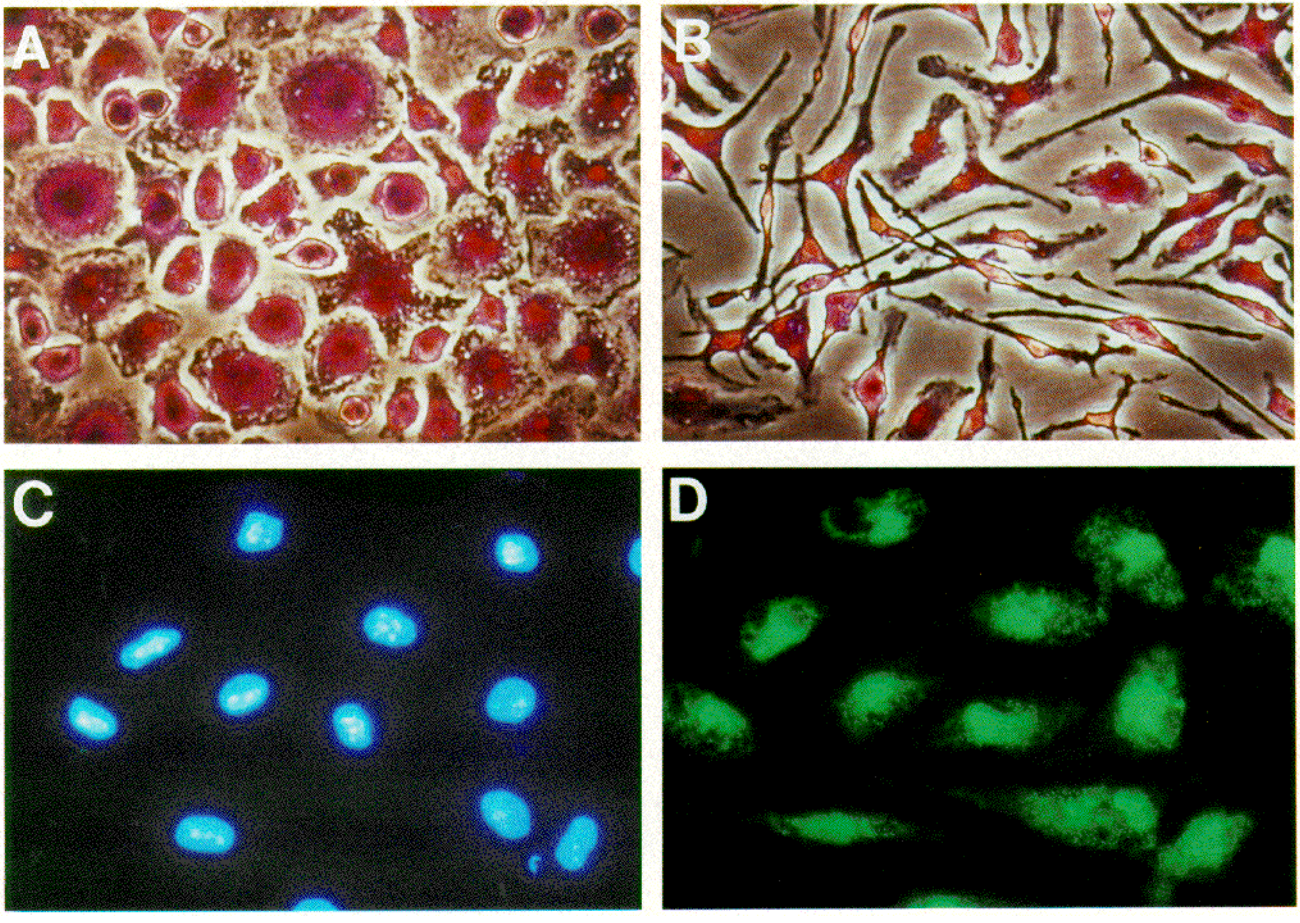
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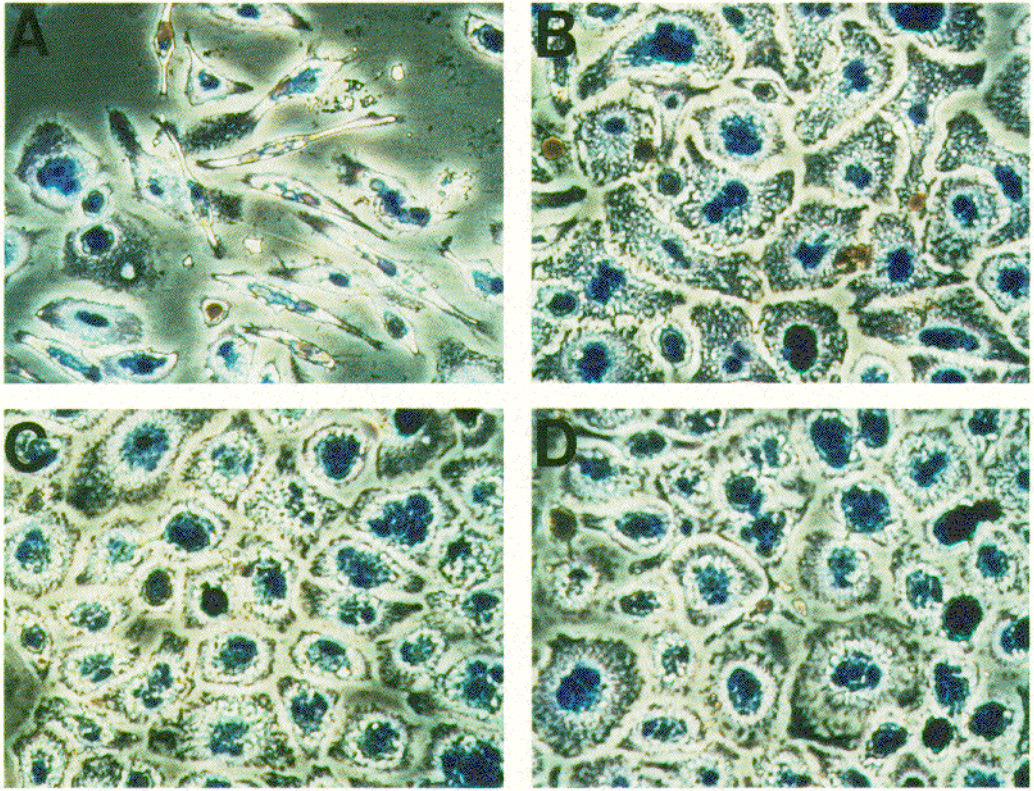
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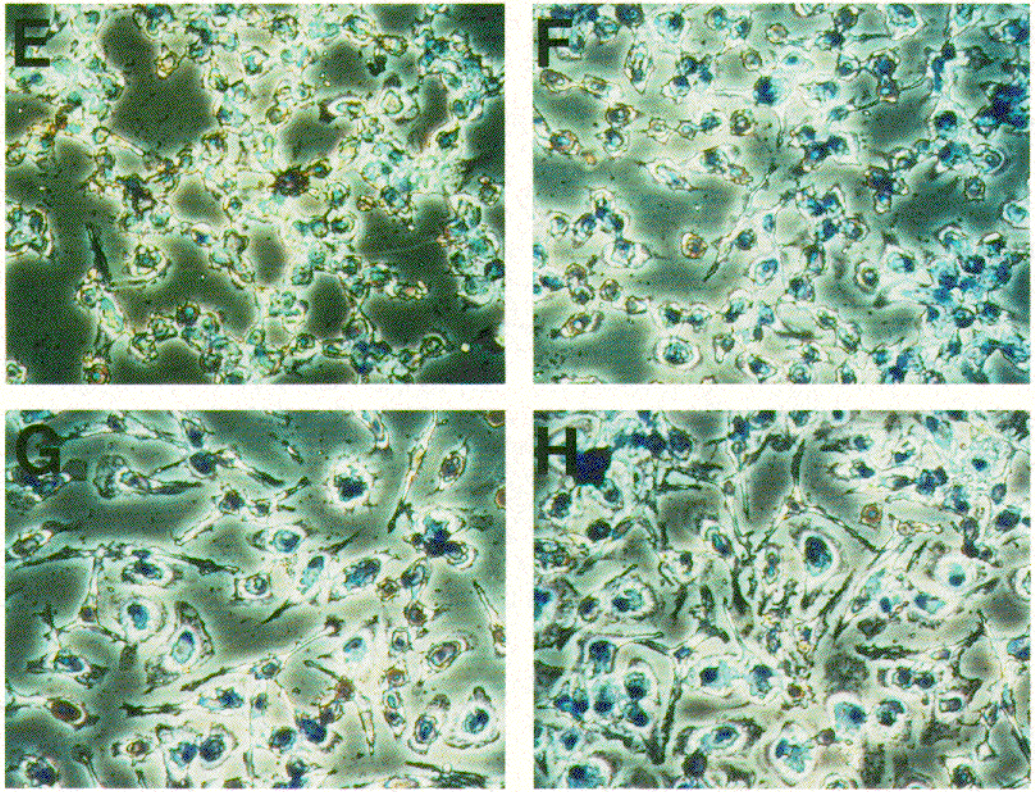
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