MAPK Mediates RAS-induced Chromosome Instability*

(Received for publication, May 25, 1999, and in revised form, October 7, 1999)

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The generation of micronuclei is a reflection of DNA damage, defective mitosis, and loss of genetic material. The involvement of the MAPK pathway in mediating v-ras-induced micronuclei in NIH 3T3 cells was examined by inhibiting MAPK activation. Conversely, the MAPK pathway was constitutively activated by infecting cells with a v-mos retrovirus. Micronucleus formation was inhibited by the MAPK kinase inhibitors PD98059 and U0126, but not by wortmannin, an inhibitor of the Ras/phosphatidylinositol 3-kinase pathway. Transduction of cells with v-mos resulted in an increase in micronucleus formation, also consistent with the involvement of the MAPK pathway. Staining with the anti-centromeric CREST antibody revealed that instability induced by constitutive activation of MAPK is due predominantly to aberrant mitotic segregation, since most of the micronuclei were CREST-positive, reflective of lost chromosomes. A significant fraction of the micronuclei were CREST-negative, reflective of lost acentric chromosome fragments. Some of the instability observed was due to mitotic events, consistent with the increased formation of bi-nucleated cells, which result from perturbations of the mitotic spindle and failure to undergo cytokinesis. This chromosome instability, therefore, is a consequence of mitotic aberrations, mediated by the MAPK pathway, including centrosome amplification and formation of mitotic chromosome bridges.

The Ras proteins are small (21 kDa) GTP-binding, membrane-associated proteins (1). They are in their activated state when bound to GTP, and are inactivated by GTP hydrolysis. This intrinsic GTPase activity is enhanced by association with GTPase-activating protein (1). The Ras proteins transduce signals from ligand-activated tyrosine kinase receptors to downstream effectors (2). Activating mutations, such as those in EJ ras, can impair GTP hydrolysis and lead to constitutively activated Ras that impacts the cellular phenotype (3). Oncogenic Ras can lead to cellular transformation (4), presumably by perturbing its signal transduction pathways.

One major downstream target of the MAPK pathway is the AP-1 transcription complex, and the constitutive activation of AP-1 results in altered transcription of AP-1-regulated genes (8). The Ras signal transduction pathway is complex with multiple intersections and bifurcations (9–11). Cells utilize the various Ras-mediated signal transduction pathways to regulate a plethora of phenotypes such as cell growth (12, 13), the differentiation of certain cell types (i.e., PC-12) (13, 14), and morphological transformation via Rac1, RhoA, Cdc42, and c-Jun NH2-terminal kinase kinase (15–18). Ras also can mediate responses to hypoxia via NFkβ (19) and responses to a variety of environmental stresses via c-Jun NH2-terminal kinase kinase (20–23), as well as apoptosis in response to FAS (24, 25), and tumor necrosis factor (26). Ras can also stimulate the PI3K pathway (27) to induce cellular transformation and control the actin cytoskeleton (28).

In the absence of p53, a protein that monitors changes in the stability of the genome (29–31), overexpression of the RAS oncogene leads to chromosomal instability. In NIH 3T3 cells, selective induction of the human EJ Ha-ras oncogene expression leads to potentially deleterious cellular phenotypes such as premature entry of cells into S phase (32, 33) and increased permissivity for gene amplification (34, 35). Oncogenic Ras also induces the generation of chromosome aberrations such as dicentric chromosomes, acentric chromosomes, and double minute chromosomes (33, 36). Chromosome aberrations induced by oncogenic Ras lead to improper segregation of chromosomes and the consequent exclusion of chromosomes from daughter nuclei (37, 38). Overexpression of oncogenic ras also produces chromosome aberrations in rat mammary carcinoma cells (39), in rat prostatic tumor cells (40), and in a human colon carcinoma cell line (41). Thus, one of the major consequences of oncogenic ras in carcinogenesis is destabilization of the karyotype.

Since Ras serves as a focal point for multiple signal transduction pathways, we have examined whether activation of the Ras/MAPK pathway is sufficient to induce chromosome instability. To this end, we have expressed constitutively activated ras, and mos (a serine threonine kinase known to activate primarily the MAPK pathway) in NIH3T3 cells, and have used specific inhibitors of MAPKK-1 and -2 to assess the role of the MAPK pathway in inducing chromosome instability. Overexpression of oncogenic ras and mos resulted in chromosome instability, as measured by a standard micronucleus assay (42). This chromosome instability is mediated by activation of the MAPK pathway, and was characterized, in part, by whole chromosome loss, as well as chromosome fragment loss.

**EXPERIMENTAL PROCEDURES**

Retroviral Vectors and Retroviral Infection—Infectivity of ecotropic cell lines producing empty Murine leukemia virus (MuLV), v-ras (MSV-}

phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; DAPI, 4',6-diamidino-2-phenylindole.

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* This work was supported by National Institutes of Health Grant CA65769. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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¶ The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; DAPI, 4',6-diamidino-2-phenylindole.

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Ha-ras, and v-mos has been calculated to be between 5 and 10 viral particles per cell (43). Between 1 and 2 × 10⁶ NIH 3T3 cells were plated in 6-well plates in Dulbecco's modified Eagle's high glucose medium containing penicillin/streptomycin and 10% fetal bovine serum. Infections with MuLV, as with v-mos and v-ras retroviruses were carried out with Mouse Polybrene for 4–8 h. For experiments involving the MAPKK inhibitors PD98059 and U0126, cells were infected with v-ras as described above and the inhibitors were applied at concentrations of 75 μM for PD98059 and 50, 80, and 100 μM for U0126. Wortmannin was applied at a concentration of 1 μM. Fresh inhibitor was applied every 2 days.

Western Blots—Cellular proteins were isolated by lysing cells in RIPA solution (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) in the presence of protease inhibitors (aprotinin, 0.1 μg/ml; leupeptin, 0.5 μg/ml; pepstatin, 1 μg/ml; phenylmethylsulfonyl fluoride, 20 μg/ml; N-α-tosyl-l-lysine chloromethyl ketone, 50 μg/ml; and t-1tosylamido-2-phenylethyl chloromethyl ketone, 100 μg/ml) and phosphatase inhibitors (5 mM sodium fluoride and 0.1 mM sodium orthovanadate) and incubating at 4 °C for 15 min (44). Protein lysates (50 μg) were denatured in 2% SDS, 10 mM dithiothreitol, 60 mM Tris, pH 6.8, and loaded onto a 12% polyacrylamide/SDS gel. The separated proteins were then transferred by electrophoret (100 mA, 1 h) onto a PVDF-Plus membrane (Micron Separations Inc., Westborough, MA). The blot was blocked in TBS (0.2 M Tris, pH 7.6, 150 mM NaCl, 0.005% Tween 20, 1% BSA) for 2 h at room temperature, washed with TBS, 0.1% Tween 20. The ECL non-radioactive detection kit (Amersham Pharmacia Biotech) was used to detect the antibody-protein complexes by exposure of the membrane to a Kodak X-Omat autoradiography film.

Microcule Assay—The microcule assay was performed using a standard procedure (42). Briefly, cells were trypsinized 5–7 days after infection, centrifuged at 1500 rpm for 3 min in a table top centrifuge, and lysed in 600 μl of solution I (10 mM NaCl, 3.4 mM sodium citrate, 0.001% (w/v) RNase A, 0.03% Nonidet P-40) supplemented with 0.2 μl of 5 mM sytox (Molecular Probes Inc., Eugene, OR) and incubated at 25 °C for 1 h. This incubation was followed by addition of 600 μl of solution II (1.4% citric acid, 0.25 mM sucrose) supplemented with 2 μl of propidium iodide (PI) (10 μg/ml). Micronuclei (particles stained with both sytox and PI with a size range of 1/100–1/10 the mean size of a G1 nucleus) were analyzed using a Coulter EPICS XL flow cytometer (Miami, FL) at an excitation range of 488 nm (argon laser) and a 525 band-pass filter for sytox and 620 band-pass for PI. Quantitation of micronuclei was based on 10,000 cells counted. The frequency of micronuclei was based on 10,000 cells counted. The frequency of micronuclei formation was confirmed by visual observation of PI-stained nuclei and counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) at 0.5 μg/ml. Cells were visualized using a standard fluorescence microscope and a ×100 objective.

RESULTS

RAS-induced Cellular Transformation Is Dependent upon MAPK Phosphorylation—NIH 3T3 cells were infected with either virus harboring oncogenic ras (MSV-Ha-ras, in short, v-ras) or MuLV, the parental virus. To test for expression of Ras, pools of NIH 3T3 cells infected with MuLV or v-ras were first analyzed by Western blots using anti-Ras antibody as a probe (Fig. 1). Cells infected with v-ras (Fig. 1, lane 2), but not those infected with control MuLV (Fig. 1, lane 1), expressed an increased level of Ras. Equal loading was monitored by using an antibody against MAPK-2 (Fig. 1).

Since the MAPK pathway is a major signal transduction pathway activated by Ras (5–7), we have assessed the extent to which v-ras is able to induce MAPK activation in the infected cells. Phosphorylation of MAPK was monitored by Western blots, using an antibody directed against phosphorylated p42/p44 MAPK. Transduction of exponentially growing cells with v-ras (Fig. 2A, lane 3) or v-mos (Fig. 2A, lane 2) induced higher MAPK activation than in cells transduced with MuLV (Fig. 2A, lane 1). Treatment of ras-transduced cells with the specific MAPKK inhibitors PD98059 and U0126 resulted in reduction of MAPK phosphorylation (Fig. 2A, lanes 4–6). The inhibitor concentration resulting in 50% inhibition of MAPK activation was 10 μM for PD98059 and 50, 80, and 100 μM for U0126. Wortmannin was applied at a concentration of 1 μM. Fresh inhibitor was applied every 2 days.

Cell Cycle Analysis by Flow Cytometry—Cells (10⁵) were plated into each well of a 6-well tissue culture dish and infected with MuLV or v-ras in the presence or absence of PD98059 and U0126, or wortmannin. Cells were collected and fixed in 70% ethanol at 4 °C, washed in 1 × PBS, resuspended in a solution containing 100 μg of PI, and 1 mg of RNase A, 10 μl of PBS, and analyzed by flow cytometry (53) using a Coulter EPICS XL flow cytometer (Miami, FL) at an excitation range of 488 nm (argon laser), and a 250 band-pass filter for PI. The percentage of cells in G1, S, and G2/M was based on 10,000 cells counted.

Staining of the Mitotic Spindle and Centrosomes—Cells (5 × 10⁴) were plated on gelatinized (0.1% gelatin in PBS) 2-well plastic culture chambers (Fisher, St. Louis, MO). Cells were infected with MuLV and v-ras as described above in the presence or absence of PD98059, U0126, or wortmannin. Five days after infection, cells were stained as follows (54): cells were rinsed with PHEM (60 mM Pipes, 1.4% citric acid, 0.25 M sucrose) supplemented with 2 μl of propidium iodide (PI) (10 μg/ml). Micronuclei (particles stained with both sytox and PI with a size range of 1/100–1/10 the mean size of a G1 nucleus) were analyzed using a Coulter EPICS XL flow cytometer (Miami, FL) at an excitation range of 488 nm (argon laser) and a 525 band-pass filter for sytox and 620 band-pass for PI. Quantitation of micronuclei was based on 10,000 cells counted. The frequency of micronucleus formation was confirmed by visual observation of PI-stained nuclei and counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) at 0.5 μg/ml. Cells were visualized using a standard fluorescence microscope and a ×100 objective.
activation of MAPK, which is inhibited by MAPKK inhibitors, and to activation of the PI3K pathway, which is inhibited by wortmannin. Panel A, activation of the MAPK pathway by v-ras and v-mos. NIH 3T3 cells were infected with MuLV (lane 1), v-ras (lane 2), or v-mos (lane 3). v-ras plus 75 μM PD98059 (lane 4), v-ras plus 50 μM U0126 (lane 5), or v-ras plus 80 μM U0126 (lane 6). Western blots were done 5 days after infection and probed with antibodies against phosphorylated MAPK (p-MAPK) or with an antibody against MAPK-2 to monitor equal loading. Panel B, activation of the PI3K/AKT pathway by RAS. NIH 3T3 cells were infected with MuLV (lane 1), or v-ras in the absence (lane 2) or presence of 1 μM wortmannin (lane 3). Lysates were subjected to polyacrylamide gel electrophoresis and probed with antibody against phosphorylated AKT or against MAPK-2 to monitor loading.

able activation of the JNK or p38 proteins (data not shown). Transduction with v-ras or v-mos resulted in a change in cellular morphology characteristic of transformation (Fig. 3, B and C). Cellular transformation induced by v-ras was dependent on phosphorylation of MAPK by MAPKK as indicated by the capacity of the MAPKK inhibitor U0126 to revert cells to a flat morphology (Fig. 3D). Treatment with wortmannin had no effect on cellular morphology (not shown).

The ras and mos Oncogenes Induce Micronuclei Formation—One consequence of oncogenic ras expression or mos expression and the activation of downstream signaling depends on the induction of chromosome instability (33–37, 43, 55). To confirm that ras and mos induce genomic instability in NIH 3T3 cells, chromosome instability was assessed by the generation of micronuclei. This measure has been used as a marker of whole chromosome loss or chromosome fragment loss induced by DNA-damaging agents such as ionizing radiation, or by agents that interfere with the proper functioning of the mitotic spindle and lead to aneuploidy (45–52). Immunocytochemical observation of cells infected with MuLV, v-ras, or v-mos (Fig. 4) revealed that overexpression of v-ras (Fig. 4, B and D) and v-mos (Fig. 4C) induced loss of chromosomes, revealed by the appearance of micronuclei in the cytoplasm. Analysis of micronucleus formation by flow cytometry (Fig. 5) and independently by immunocytochemistry (Fig. 6) showed that overexpression of oncogenic ras and mos induced the formation of micronuclei. At the high levels of active MAPK induced by v-ras, the frequency of micronucleus formation rose to 8.6-fold (Figs. 5 and 6), whereas transduction with v-mos resulted in a 2.1-fold increase in micronucleus formation (data not shown). Since increases in growth rates can potentially lead to increases in genomic instability, we calculated doubling times for cells infected with v-ras and vector controls. In cells overexpressing v-ras, there was a significant increase in the doubling time (Table 1).

Oncogenic ras Induces Micronuclei Formation by the MAPK Pathway—Although the generation of micronuclei correlated with MAPK activation by v-ras and v-mos (which stimulates only the MAPK pathway), we further tested the involvement of the MAPK pathway in inducing chromosome loss by treating the v-ras cells with PD98059 and U0126. As a control, we treated cells with wortmannin. Treatment of NIH 3T3 cells with MAPK inhibitors resulted in suppression of micronucleus formation (Fig. 6). Expression of v-ras resulted in an 8.5-fold increase in micronucleus formation (Figs. 5 and 6). Treatment of cells that overexpress v-ras with 75 μM PD98059 resulted in suppression of micronucleus formation by 60%, whereas treatment of cells with 50 and 80 μM U0126 resulted in a 65 and 67% reduction, respectively (Fig. 6). Total inhibition of micronucleus formation could not be accomplished, even at doses that approached undetectable activation of the MAPK pathway such as 100 μM U0126. Treatment of cells with wortmannin did not result in inhibition of micronucleus formation. At the concentrations used, PD98059, U0126, or wortmannin did not significantly alter cell cycle profiles or doubling times (shown for 75 μM) (Table 1), indicating that suppression of micronucleus formation or chromosome instability was not attributable to changes in growth rates.

Instability Induced by Constitutive Activation of MAPK Involves Chromosome Missegregation and Binucleation—Micronucleus formation reflects damage induced by agents that disrupt the mitotic spindle or by agents that induce double-strand DNA breaks (45–52). In the former case, the micronuclei represent whole chromosomes that have not been incorporated into the daughter nucleus. In the latter case, the micronuclei are comprised of acentric chromosome fragments. These alternatives can be distinguished by staining micronuclei with the anti-centromeric antibody CREST. An example of a micronucleus stained with CREST is presented in Fig. 4D. Staining with CREST antibody revealed that at the high levels of activated MAPK induced by v-ras and v-mos, the percentage of CREST-positive micronuclei increased dramatically (Fig. 7A). The approximate increase was 14-fold in cells infected with v-ras and 7-fold in cells infected with v-mos. CREST-negative micronuclei increased by approximately 6- and 2-fold in v-ras and v-mos-infected cells, respectively (Fig. 7B). The fact that both v-ras and v-mos induce a disproportionate increase in the number of CREST-positive micronuclei is suggestive of mitotic instability, since most of the missegregated chromosomes are lost as whole chromosomes. To confirm that v-ras was, indeed, inducing mitotic instability, we estimated the frequency with which v-ras and v-mos induced the formation of binucleated cells, as an independent measure of mitotic instability (43, 55). Expression of Ras and Mos resulted in elevation of the frequency of binucleated cells to 10 and 12%, respectively (Fig. 8). Binucleation by RAS also seems to be mediated by MAPK,
since treatment of v-ras cells with 75 μM PD98059 suppressed the formation of binucleated cells by 43% (Fig. 8).

Fig. 4. Expression of ras and mos induces genomic instability. NIH 3T3 cells infected with MuLV (A), v-ras (B and D), or v-mos (C) were stained with CREST antibodies directed against kinetochore proteins and an ALEXA-488-labeled secondary antibody (in green) and counterstained with propidium iodide. Images were obtained with a Bio-Rad confocal microscope using a ×60 objective. Blue and white arrows indicate micronuclei, and the yellow arrow indicates a binucleated cell. Most of the nuclei shown in this figure are in interphase. Panel B shows a cell in metaphase (left) with 2 micronuclei and a cell in interphase with a micronucleus (right). Panel D shows an interphase cell containing two CREST-positive micronuclei and a CREST-negative micronucleus.

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resulted in an increase in centrosome amplification, leading to the formation of multiple mitotic spindles (31) and in mitotic bridges, which are the result of the formation of dicentric chromosomes (38). Both of these processes were reduced by treatment with MAPK inhibitors, but not with an inhibitor of the PI3K signal transduction pathway (Figs. 9 and 10).

DISCUSSION

The Ras signal transduction pathways control normal cellular functions such as growth (12, 13), differentiation (13, 14), and cell morphology (15–18, 28). The Ras signal transduction pathways can also mediate responses to stress such as hypoxia (19), as well as contribute to the induction of apoptosis in hematopoietic cells in response to FAS (24, 25) or TNF (26).

Mutations within the cellular Ras genes render Ras and its signal transduction pathways constitutively active and lead to potentially deleterious cellular consequences including cellular transformation and uncontrolled growth rates (4, 12, 13, 15, 17, 32). Overexpression of constitutively active Ras also induces programmed cell death (reviewed in Ref. 56) in primary mouse embryo fibroblasts (43), NIH 3T3 cells (57), in COS-7 cells expressing BAD (58), and in Jurkat human lymphoblastoid T-cells (59). Overproduction of oncogene products related to proteins within the Ras signal transduction pathway, such as Mos, induce apoptosis in Swiss 3T3 (55) and p53+/− and p53−/− mouse embryonic fibroblasts (45).

Expression of the Ras oncogene also leads to genomic instability. In NIH 3T3 cells, selective induction of the human EJ Ha-Ras oncogene expression leads to several phenotypes associated with genomic instability, such as gene amplification (34, 35), generation of aberrant chromosomes (33, 36) within a single cell cycle (33), and chromosome missegregation (37, 38). Induction of genomic instability by oncogenic Ras and oncogenes within the Ras signal transduction pathway seems to be a general phenomenon, since it occurs in cell lines such as rat
mammary carcinoma cells (39), rat prostatic tumor cells (40), human colon carcinoma cells (39), Swiss 3T3 fibroblasts, and p53−/− mouse primary fibroblasts (43, 55).

Since the Ras signal transduction pathway is not strictly linear, but is complex with multiple intersections and bifurcations (9–11), we asked whether activation of a single branch, namely the MAPK pathway, was sufficient to induce chromosome instability. We have chosen to dissect this pathway by using the MAPKK inhibitors PD98059 and U0126 and by over-expressing the v-mos oncogene, which leads to direct activation of the MAPK pathway (60, 61). As a control, we inhibited the Ras/PI3K signal transduction pathway with the chemical inhibitor wortmannin. We studied Ras/PI3K signal transduction pathway with the chemical in-

Our results indicate that selective expression of oncogenic ras results in whole chromosome and chromosome fragment loss, as measured by the micronucleus assay. Micronuclei are a confocal microscope and a ×100 objective. The number of cell populations analyzed and the p values resulting from an unequal variance t test is as follows: MuLV (n = 14); v-ras (n = 12, compared with MuLV, p ≤ 7.63E-8); v-ras + 75 μM PD98059 (n = 7, compared with v-ras p = 1.58E-6); v-ras + 50 μM U0126 (n = 6, p ≤ 2.35E-6); v-ras + 80 μM U0126 (n = 5, p = 6.79E-7); v-ras + 100 μM U0126 (n = 3, p ≤ 8.67E-7); v-ras + 1 μM wortmannin (n = 5, p ≤ 0.23).

Table I

| Transduced population | Doubling time | G1* | S* | G2/M* |
|-----------------------|--------------|-----|----|-------|
| MuLV                  | 24 ± 1       | 75.2 ± 3 | 14.35 ± 3.7 | 10.6 ± 1.8 |
| v-ras (75 μM PD98059) | 43 ± 0       | 60.7 ± 3.3 | 15.8 ± 1.8 | 23.3 ± 2.8 |
| v-ras (50 μM U0126)   | 44 ± 6       | 59.2 ± 4.6 | 22.12 ± 4.4 | 17.7 ± 1.12 |
| v-ras (50 μM U0126)   | 57.25 ± 0.07 | 23.75 ± 2.06 | 19 ± 2.12 |
| v-ras (80 μM U0126)   | 61.2 ± 1.3   | 24.45 ± 0.8 | 14.4 ± 2.12 |
| v-ras (1 μM wortmannin) | 55.6 ± 0.57 | 19.7 ± 0.99 | 24.75 ± 1.6 |

* Percentage of cells in G1, S, or G2/M as determined by flow cytometry.

There is little available evidence to indicate which of the Ras-mediated pathways most actively promotes genomic instability. The MAPK pathway has been implicated since ras induces genomic instability via MAPK, and since there are no known bifurcations of the MAPK pathway downstream of Mos (43, 55). To directly test whether ras induces genomic instability via MAPK, we treated ras-overexpressing cells with the specific MAPKK inhibitors PD98059 and U0126. We also infected cells with the mos oncogene. We have shown that micro-

formation of ras-induced micronuclei, and direct induction of MAPK activation by mos also resulted in micronuclei formation. In contrast, total inhibition of the PI3K signal transduction pathway did not inhibit micronucleus formation. Formation of micronuclei cannot be attributable to higher growth rates, since the ras cells showed a similar replication index that vector control cells, and slowed down during mitosis, as has been described for mos-transformed cells (63). To ascertain the predominant mechanism by which ras and mos induce micronuclei, we stained micronuclei with the anti-kinetochore antibody CREST, which recognizes the centromeric proteins CENP-A, -B, and -C (64). Increases in CREST-negative micronuclei have been associated with DNA-damaging agents such as γ-irradiation, and correlates directly with loss of centromeric α-satellite DNA sequences, whereas increases in CREST-positive micronuclei results from agents that disrupt the mitotic spindle (45, 62). At high levels of activated MAPK, ras and mos disproportionately increased the frequency of CREST-positive micronuclei, suggesting that most of the micronuclei induced by ras and mos are missegregated whole chromosomes. The fact that ras and mos also increased the frequency of CREST-negative micronuclei is consistent with previous observations that ras induces chromosome breaks leading to increases in the frequency of acentric fragments (33), and other chromosome anomalies (33, 36–41), and to gene amplification (34, 35) resulting from the induction of multiple bridge-break fusion cycles (reviewed in Ref. 65). The fact that both CREST-positive and negative micronuclei are generated suggests that at least two mechanisms may be operative in the MAPK-mediated induction of genomic instability. One is the generation of acentric fragments, possibly as a result of endonucleolytic activity (66). The second is the improper segregation of whole chromosomes that contain the necessary centromeric machinery to be segregated properly. To confirm that ras and mos induced mitotic instability, we quantitated the increase in binucleated cells, which serves as an independent measure of mitotic instability.
due to abnormalities of the mitotic spindle (43, 55). Expression of ras and mos increased the frequency of binucleated cells, which was suppressed by treatment of the cells with the MAPK inhibitor PD98059. Formation of micronuclei correlates with an increased number of mitotic defects induced by high activation of the MAPK pathway. The predominant defect induced by v-ras is the formation of mitotic bridges, which are the result of the acquisition of one or more centromeres due to breakage and fusions of chromosomes and the consequent stretching of chromosomes when the daughter cells separate during mitosis (38). Another defect induced by v-ras is the induction of centrosome amplification, which results in the formation of multiple mitotic spindles and the consequent mis-segregation of chromosomes. Both of these phenotypes are strongly inhibited by MAPK inhibitors.

The observation that mitotic instability induced by oncogenic ras involves the MAPK pathway makes it possible to dissect the mechanism(s) that produce this instability in mammalian cells. The initial evidence implicating MAPK in the regulation of mitosis came from studies of Xenopus meiosis where MAPK promotes oocyte maturation (67–73). In contrast, during embryogenesis, MAPK plays a negative regulatory role, since activation of MAPK by Mos or injection of active MAPK into Xenopus embryos leads to mitotic arrest (74, 75). MAPK also plays a role in the Xenopus spindle assembly checkpoint, an evolutionarily conserved mechanism that monitors defects in the mitotic spindle or improper alignment of chromosomes during mitosis (reviewed in Refs. 76 and 77). MAPK activation establishes a mitotic spindle checkpoint; active MAPK increases dramatically in nocodazole-treated Xenopus egg extracts, and the cells could be released from the arrest by addition of the MAPK phosphatase MKP-1 (78). In somatic cells, evidence involving MAPK in the spindle assembly checkpoint stems from recent observations that MAPK is activated in response to nocodazole and that the arrest is overridden by the MAPK phosphatase XCL100 (79). Similarly, immunodepletion of MAPK in cycling Xenopus extracts or treatment with PD98059 causes precocious termination of mitosis and interferes with production of normal mitotic microtubules (80).

The relationship between MAPK activation and its involvement in mitosis in mammalian cells is less clear. Microinjection of fibroblasts with antibodies against c-src blocks entry into mitosis, suggesting a link between upstream components of signal transduction pathways and mitosis (81). Recent reports suggest a possible role for MAPK in regulation of mammalian mitosis. Active MAPK localizes to the kinetochores during mitosis and phosphorylates proteins such as CENP-E, a motor protein involved in chromosome movement (54). MAPK also phosphorylates proteins containing the 3F3/2 phosphoantigen (82), which are involved in the mammalian mitotic checkpoint (83). The dephosphorylation of this antigen is required for progression to anaphase (84). Interestingly, one of the proteins sharing the 3F3/2 antigens is topoisomerase II, an enzyme that generates regulated strand breaks to ensure proper condensation of chromosomes during mitosis (85). Topoisomerase II has been postulated to be the enzyme that may be involved in the chromosomal breaks and recombination induced by the ras oncogene (41), is modulated by the ras oncogene (86), and is activated by the MAPK pathway (87). Whether topoisomerase II is the enzyme which actually leads to RAS-induced chromo-

FIG. 7. Constitutive activation of MAPK results in loss of whole chromosomes and chromosome fragments. NIH 3T3 cells were infected with MuLV, v-ras, or v-mos. Eight days after infection, immunocytochemistry was performed on infected cells using CREST antibody, which detects kinetochore proteins. Cells were counterstained with propidium iodide. Each cell containing a micronucleus was optically sectioned three to five times on a Kalman/slow mode by confocal microscopy under a ×100 objective. A, percent CREST-positive micronuclei. The number of analyzed populations and the p values resulting from an unequal variance t test is as follows: MuLV (n = 5); v-ras (n = 6; compared with MuLV, p = 5.6E-5); v-mos (n = 5; compared with MuLV, p = 0.03). B, percent CREST-negative micronuclei. The number of analyzed populations and the p values resulting from an unequal variance t test is as follows: MuLV (n = 5); v-ras (n = 6; compared with MuLV, p = 0.0006); v-mos (n = 5; compared with MuLV, p = 0.11).

FIG. 8. Ras and mos induce binucleation. Cells infected with MuLV, v-ras cultured in the presence or absence of 75 μM PD98059, and v-mos were stained with CREST anti-kinetochore antibody and counterstained with propidium iodide. The percentage of binucleated cells was obtained by confocal microscopy from the analysis of at least 500 nuclei. Percentages represent the average of three to four independently infected populations. The number of analyzed populations and the p values resulting from an unequal variance t test is as follows: MuLV (n = 4); v-ras (n = 3, compared with MuLV p = 0.02); v-ras + 75 μM PD98059 (n = 3, compared with v-ras p = 0.05), v-mos (n = 4; p = 0.02).
some breaks that produce the breakage-fusion cycle leading to the formation of CREST-negative micronuclei (acentric chromosomes), dicentric chromosomes, and mitotic bridges remains to be elucidated.

Acknowledgments—We thank Drs. Yolanda Sanchez, Jeffrey Knauf, and Anthony Capobianco for helpful discussions. We also thank Dr. George Babcock and Jim Cornelius for assistance in the flow cytometry.

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