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Genetics

The human Ha-*ras* oncogene induces genomic instability in murine fibroblasts within one cell cycle

(NIH 3T3 cells/inducible Ha-*ras*/chromosomes)

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ABSTRACT **Many human tumors contain an activating mutation in one of the *ras* protooncogenes. Additionally, these tumor cells are often heteroploid and characterized by chromosome breaks and rearrangements that are consequences of the genomic instability that is thought to contribute to tumor progression. The concurrence of *ras* mutations and genomic instability in tumors prompted us to ask whether selective induction of an activated Ha-*ras* gene could render a genome unstable. The NIH 3T3 cells used in this study contained mutant p53 genes and carried a selectively inducible activated (EJ) Ha-*ras* transgene under the control of bacterial lactose regulatory elements. When stably transfected cells were induced to express activated Ha-*ras* by isopropyl β-D-thiogalactoside administration, there was a marked increase in the number of gross chromosomal aberrations including acentric fragments, multicentric chromosomes, and double minutes, which occurred within the time frame of a single cell cycle from the time of induction. To confirm that these aberrations occurred within the first cell cycle after mutant Ha-*ras* induction, the cells were arrested in G_1 phase by serum depletion and, subsequently, released by administration of isopropyl β-D-thiogalactoside or serum. The mitoses from cells released with isopropyl β-D-thiogalactoside contained a 3-fold elevation in the fraction of chromosomes containing aberrations compared to mitoses from parallel cell cultures that were released with serum. Thus, the induction of activated Ha-*ras* gene expression in these cells results in genomic instability that can be detected as aberrant chromosomes at the next mitosis.**

The *ras* gene family encodes small (20–22 kDa) proteins that bind and hydrolyze GTP and participate in the transduction of signals from plasma membrane receptors to nuclear effectors (1). Specific missense mutations in the genes that encode Ras proteins decrease the ability of the proteins to hydrolyze GTP and result in constitutive Ras activity (1). The *ras* gene family is made up of Ha-*ras*, K-*ras*, and N-*ras*, which collectively are among the most commonly mutated genes in human cancers (2). Recently, several groups have implicated oncogenic *ras* as an initiator of abnormal karyotypes in cells that were selected as tumorigenic in nude mice (3, 4) and in spontaneous and UV-induced genetic changes *in vitro* (5). However, the mechanisms by which the mutant proteins contribute to deregulated cell growth, malignancy, and/or genomic instability remain unclear.

We have used an NIH 3T3 cell line in which expression of an activated Ha-*ras* gene (EJ) is under the control of an inducible promoter SV*lac*O (6), to study early consequences of mutation in Ha-*ras*. In addition to the mutant Ha-*ras* transgene, these cells harbor the plasmid pHβ*lac*INLS*neo* that encodes the *lac* repressor to sustain repression of the transgene. The transgene can be derepressed selectively and rapidly by administration of isopropyl β-D-thiogalactoside (IPTG), a nonmetabolizable lactose analogue. The mutant Ha-*ras* transcript level increases 20-fold within 4 h of IPTG administration, and the cells subsequently acquire a transformed phenotype (6). Using this system, we have addressed a genetic consequence of this conditionally transformed state, genomic instability, by utilizing damage to mitotic chromosomes as an indicator.

Genomic instability, manifested in part by an abnormal karyotype, is a distinctive feature of many tumors and is thought to contribute to tumor progression and metastasis (7–9). The genetic and biochemical pathways that lead to genomic instability are poorly understood. One pathway involves the loss and/or mutation of the p53 gene, a gene that encodes a DNA binding protein that has tumor suppressor function (10, 11). Absence of a functional p53 gene leads to an inability of cells to arrest properly in the G_1 phase of the cell cycle in response to DNA damaging agents (12). This lack of G_1 arrest is thought to impair timely repair of damaged DNA before replication, thereby, contributing to genomic instability (13), as measured by the capacity to amplify drug-resistance markers (14, 15) and by loss of a normal karyotype (16). However, loss of a functional p53 gene is not the sole determinant of genomic instability since some cell lines with wild-type p53 display an amplification permissive phenotype (14). In this report, we demonstrate that in NIH 3T3 cells, selective induction of a mutant Ha-*ras* transgene also can contribute to genomic instability that results in damaged chromosomes and heteroploidy within one cell cycle.

MATERIALS AND METHODS

Cell Lines. NIH 3T3 cells and derivative cell clones 2-42 (constitutive expression of EJ Ha-*ras*) and 2-12 (inducible expression of EJ Ha-*ras*) have been described (6). NIH 3T3 cell clone derivatives G5 (also with constitutive expression of EJ Ha-*ras*) and D5 (also with inducible expression of EJ Ha-*ras*) were generated similarly (6) by transfection with pSVlacO*ras* in the former case and by cotransfection of plasmids pHβ*lac*INLS*neo* and pSVlacO*ras* in the latter. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with penicillin (100 units/ml); streptomycin (100 μg/ml); 10% (vol/vol) fetal bovine serum (FBS), 10% (vol/vol) newborn calf serum, or a mixture of 5% FBS and 5% newborn calf serum; and G418 (300 μg/ml) in the transfected lines. For experiments involving G_1 arrest and release, 2-12 cells were plated at a density of 1.3×10^3 cells per cm^2 in a series of 100-mm dishes for 24 h in 10% FBS.

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Abbreviations: IPTG, isopropyl β-D-thiogalactoside; FBS, fetal bovine serum.

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Cells in one-quarter of the plates were maintained in this medium with 10% FBS for the duration of the experiment. Cells in the remaining plates were deprived of serum by washing the dishes twice in phosphate-buffered saline (PBS) and were refed with medium containing 0.1% FBS. After 24 h, one-third of the serum-deprived plates received FBS to a final concentration of 10%, one-third received IPTG to a final concentration of 20 mM, and one-third received no addition. At increasing time intervals after addition of IPTG or serum, dishes were treated with Colcemid (0.1 μg/ml) for 30 min, cells were trypsinized and collected, and chromosomes were prepared as described below. A total of 500–1000 cells were counted under phase-contrast microscopy and scored for mitotic figures. The values are averages of two experiments with the average difference in the two experiments of 0.5% for IPTG and 0.6% for FBS.

Chromosome Analysis. Chromosomes were prepared as described (17). The DNA probe was PCR-amplified from mouse γ-satellite (18) and was labeled directly by substitution of Biotin-11-dUTP for dTTP. Hybridizations were carried out for 2–4 days in 50% (vol/vol) formamide/6× standard saline citrate (SSC)/10% (wt/vol) dextran sulfate/carrier DNA (500 μg/ml). The slides were washed three times in 50% formamide/2× SSC at 42°C, stained for 20 min in fluoresceinated avidin, and counterstained with propidium iodide. The photographs were taken at ×400–1000 on either a Nikon Microphot or a Zeiss Axiovert.

p53 Sequence Determination. p53 transcript sequence was determined by dideoxynucleotide sequencing (Sequenase, United States Biochemical) of PCR product amplified from reverse-transcribed mRNA isolated from 2-12 cells and cloned into pBSSK$^+$ (Stratagene). First-strand cDNA was synthesized from 10 μg of total mRNA by standard protocols (19). This cDNA was then used as template for a PCR (20 cycles of 94°C, 60 sec; 57°C, 30 sec; 72°C, 30 sec) using primers homologous to sense-strand nucleotides 559–579 of the p53 cDNA (GGTGAAGCGTGCCCTGTGC) and complementary to sense-strand nucleotides 1048–1068 (CACTTTGCTCTCCCTGGG).

Analysis of γ-Ray-Induced Cell Cycle Block in 2-12 Cells. Cell cycle distributions were determined by two-dimensional flow cytometry as described by Kastan *et al.* (12). Briefly, cells were irradiated with 500 cGy by using a ^{60}Co source and were allowed to cycle for 16 h. They were pulse-labeled with 10 μM bromodeoxyuridine (BrdUrd) for 60 min, trypsinized, and fixed in 70% ethanol. Cells were treated with 2 M HCl and 0.5% Triton X-100 for 60 min, followed by 10 min in 0.1 M $Na_2B_4O_7$ (pH 8.5) to produce single-stranded DNA and then 30 min with fluoresceinated anti-BrdUrd antibody, as suggested by the manufacturer (Becton Dickinson). The cells were counterstained with propidium iodide (5 μg/ml) in PBS and scanned in a Becton Dickinson FACScanner.

RESULTS

Activated *ras* Expression Induces Chromosome Damage. Structural changes in chromosomes were monitored by examining each chromosome in every scored metaphase before or after EJ Ha-*ras* gene induction. In these experiments, changes in chromosome number and morphology were determined by fluorescence *in situ* hybridization analysis using a pan-centromeric probe (18). Acentric fragments and multicentric chromosomes are a good measure of genomic instability since they occur rarely under normal circumstances and will result in improper segregation at mitosis and in additional deleterious genetic events (20). Two classes of chromosome anomalies were observed after IPTG administration: gross structural changes and alterations in ploidy. Examples of some of these chromosome aberrations are depicted in Fig. 1. Fig. 1 *A* and *B* shows mitotic figures from

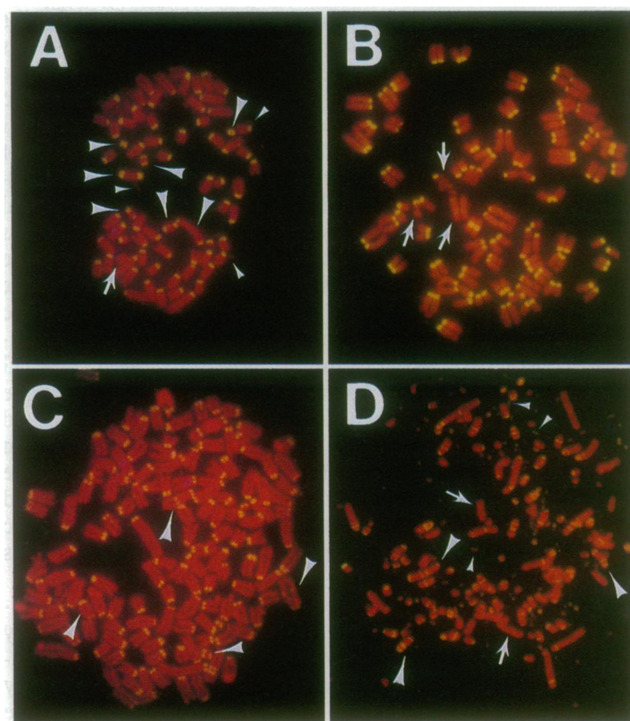

FIG. 1. Four representative metaphase spreads displaying aberrant chromosomes. (*A* and *B*) Mitotic figures from inducible Ha-*ras* 2-12 cells after exposure to IPTG for 120 h. (*C*) Mitotic figure from 2-12 cells after release from a serum-deprivation block by IPTG and the resultant one cell cycle in the presence of Ha-*ras* (see Fig. 3). (*D*) One of the extremely heavily damaged mitotic figures from clone G5 that constitutively expresses Ha-*ras* (at 8 weeks after transfection). Control NIH 3T3 cells contain a modal number of 72 monocentric chromosomes (ref. 18 and see Fig. 4). Acentric fragments (arrows), polycentric chromosomes (large arrowheads), and double minutes (small arrowheads) are marked. *B* contains a rare cell that has undergone endoreduplication and, therefore, contains four copies of each chromatid.

inducible clone 2-12 cells 120 h after IPTG treatment, Fig. 1*C* is a mitotic figure from clone 2-12 cells obtained 29 h after IPTG release from G_1 arrest due to serum deprivation (see Fig. 3), and Fig. 1*D* depicts an extensively damaged mitotic figure from early-passage clone G5 cells, which constitutively express the mutant Ha-*ras* transgene. The types of chromosome aberrations that appeared after Ha-*ras* induction included acentric chromosomes (arrow), dicentric and other multicentric chromosomes (large arrowhead), double minute chromosomes (small arrowhead), and endoreduplicated chromosomes (Fig. 1*B*). In the majority of aberrant chromosomes (95%), both chromatids were affected, suggesting that the lesion was incurred prior to DNA replication. The metaphase in Fig. 1*B* contained chromosomes with four paired chromatids for each homolog, indicating that this cell had undergone two rounds of DNA synthesis without a nuclear division (endoreduplication). In this metaphase, there are three examples of acentric chromosomes. One chromosome set had lost its centromere before the first of the two replication cycles, producing four acentric chromatids. The second chromosome set had lost a centromere between the two rounds of DNA synthesis, producing one chromatid pair containing a centromere and a second pair in which the centomere is missing. The third chromosome set retained all its centromeres but contained a DNA double-strand break that was replicated, producing a pair of acentric chromatid fragments.

The impact of activated Ha-*ras* on chromosome stability over time is shown in graphic form in Fig. 2. Fig. 2*A* shows

that there was a decrease in the fraction of metaphase figures that had no detectable chromosome damage (i.e., metaphases that have neither an acentric chromosome fragment nor a multicentric chromosome) as a function of exposure time to IPTG. Two separate cell clones with IPTG-inducible Ha-*ras* transgenes manifested a significant increase in the fraction of metaphases with altered chromosomes after IPTG administration. The rapidity with which the frequency of normal metaphases decreased suggested that the impact of Ha-*ras* expression on chromosome stability occurs within the time frame of a single cell cycle (see below). Fig. 2*B* shows the increase in the aggregate number of acentric and multicentric chromosomes as a function of IPTG exposure time. Multicentric chromosomes were found only after >48 h of IPTG induction of mutant Ha-*ras* (four tricentrics at 120 h). The fraction of mitotic cells with multiple aberrations increased with the length of time after Ha-*ras* transgene induction from 0.53% at 0 h to 24.3% at 120 h (data not shown). The background of aberrant chromosomes in the noninduced SV*lacO**ras* cells (0 h of IPTG) was due primarily to dicentric chromosomes (one acentric and nine dicentric chromosomes per 50 metaphases).

Neither the parental NIH 3T3 cells nor cells with constitutive expression of the Ha-*ras* transgene were affected by the IPTG (Fig. 2*A*). However, the two clones that constitutively express an activated Ha-*ras* transgene displayed very different basal levels of chromosomal damage. One cell clone (2-42), described previously (6), has been in culture for >2 years. This clone had a relatively low (19–26%) frequency of aberrant metaphases with no heavily damaged metaphases. The second cell clone (G5), which had been in culture for 8–12 weeks at the time of analysis, displayed a relatively high (32–38%) frequency of aberrant metaphases. In ≈5% of metaphases prepared from cells of this latter clone, damage was sufficiently severe that few chromosomes were recognizable as normal (see Fig. 1*D*).

Induction of Chromosome Damage Occurs Within One Cell Cycle. To confirm that the effect of mutant Ha-*ras* induction on genomic instability seen in Fig. 2 occurred within a single cell cycle, 2-12 cells were arrested in G_1 phase by serum deprivation and released from G_1 arrest by IPTG administration. Fig. 3*A* shows that the frequency of mitotic figures reached a maximum 29 h after IPTG administration, which was roughly the same time period required for reaching a peak mitotic index after addition of FBS (ref. 21 and Fig. 3*A*). Consistent with previous observations regarding the relative levels of [^{3}H]thymidine incorporation (6), there were about three times more mitotic cells after release by serum than by IPTG administration. Examination of metaphase chromosomes showed that cells treated with IPTG produced an ≈3-fold higher frequency of aberrant metaphases than cells treated with 10% serum (Fig. 3*B*). In addition, 18% of the aberrant mitotic figures from cells collected after release with IPTG contained multiple chromosome lesions, while only 6% of the aberrant mitotic figures in the cells collected after release with serum contained multiple aberrant chromosomes. The observation that chromosome damage is evident in cells in mitosis after release from G_1 arrest by IPTG indicates that induction of mutant Ha-*ras* expression produces genomic instability within one cell cycle. Most of the aberrations (>90%) involved both chromatids of a chromosome, indicating that DNA was damaged before it had replicated.

Expression of Activated *ras* Increases Heteroploidy. Fig. 4 depicts the distribution of chromosome number within individual metaphases after EJ Ha-*ras* induction and demonstrates that induction of oncogenic Ha-*ras* produced cells that were highly heteroploid. By 120 h after induction of activated Ha-*ras*, ≈25% of the 2-12 cells in mitosis had significantly increased their chromosome number (Fig. 4 *B* and *C*). In contrast, the parental NIH 3T3 cells and the G5 cell line constitutively expressing Ha-*ras* showed no change in the modal chromosome number with culture in medium containing IPTG (data not shown). However, there was no clear

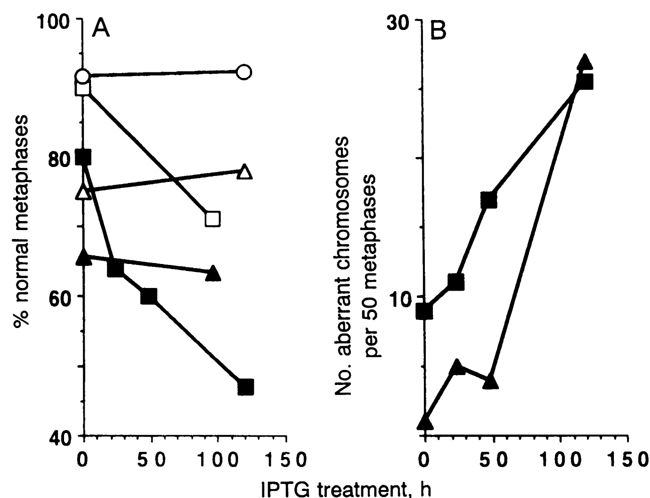

FIG. 2. Chromosome aberrations as a function of time after induction of activated Ha-*ras*. (*A*) The decrease in percentage of metaphases that were normal (i.e., those that had neither an acentric nor polycentric chromosome) as a function of time grown in medium with IPTG. ○, NIH 3T3 (parental); □, D5 (IPTG-inducible Ha-*ras*); ■, 2-12 (IPTG-inducible Ha-*ras*); △, G5 (constitutive Ha-*ras*); ▲, 2-42 (constitutive Ha-*ras*). Forty to 60 metaphases were scored for each point in duplicate experiments (with duplicate values ± 5%), 0- and 24-h points were scored blind by three people. (*B*) The same metaphase figures were scored to illustrate the increase in each of the major types of aberrant chromosomes in 2-12 cells as a function of time in IPTG. ▲, Acentric fragments; ■, multicentric chromosomes. The 48-h point was from a single experiment.

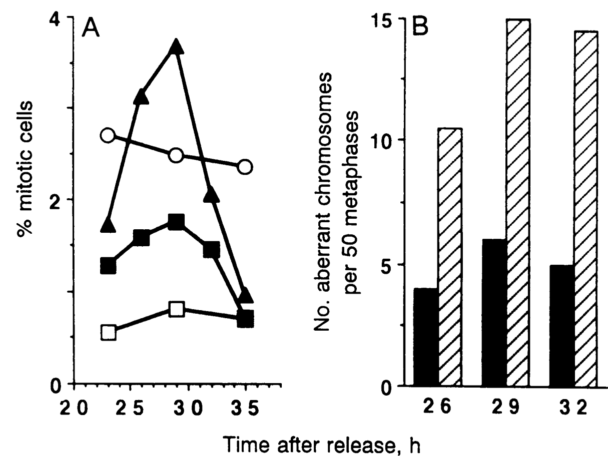

FIG. 3. Mitotic index (*A*) and frequency of aberrant chromosomes (*B*) in (EJ) Ha-*ras*-inducible 2-12 cells after release from serum starvation by addition of fresh serum or IPTG. (*A*) ○, 2-12 cells maintained in medium with 10% FBS for the duration of the experiment; □, cells arrested in G_1 and not released; ▲, G_1-arrested 2-12 cells released with 10% FBS; ■, cells released with IPTG. (*B*) Fifty metaphases were examined from the cells collected at increasing times after release from a serum-deprivation-induced block (*A*). The total number of chromosomes with aberrations were scored. From all metaphases examined that were released with IPTG, there were 13.2 ± 2.7 aberrations per 50 metaphases; from those metaphases released with FBS, there were 5.2 ± 1.0 aberrations per 50 metaphases. In the cells released with IPTG (hatched bars), 19% (12/63) of the aberrant metaphases had multiple aberrations; in the cells released with FBS (solid bars), 6% (2/31) of the aberrant metaphases had multiple aberrations. Metaphases scored from the unreleased cells contained 3 aberrant chromosomes per 50 metaphases.

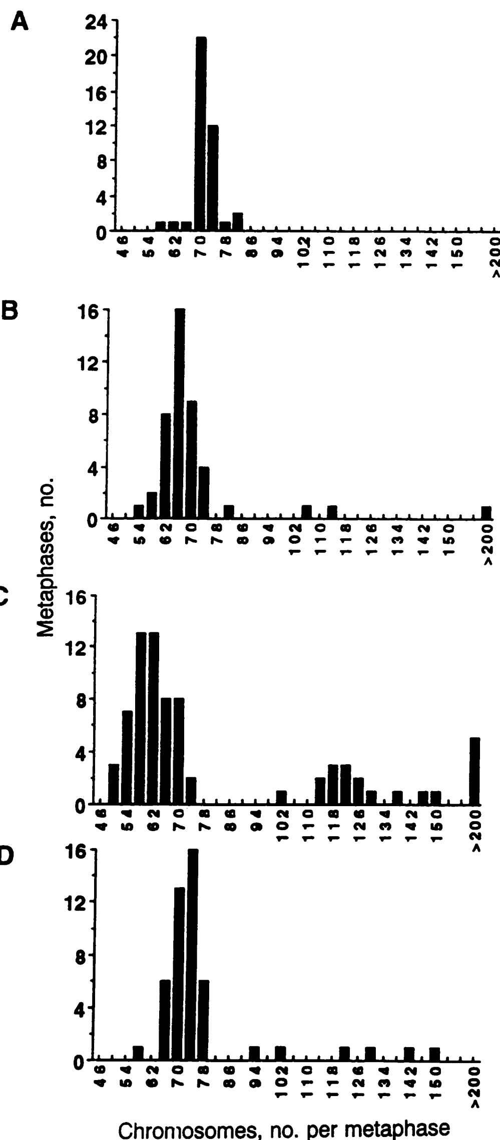

FIG. 4. Effect of activated Ha-*ras* induction on the distribution of the number of chromosomes in individual metaphases. (*A*) Parental NIH 3T3 cells; (*B*) 2-12 cells without IPTG; (*C*) 2-12 cells 120 h after activated Ha-*ras* induction by IPTG; (*D*) G5 cells, a clone that constitutively expresses an activated Ha-*ras*. Each panel contains counts from 40 to 50 metaphases, totaled from duplicate (20–25 metaphase) experiments.

relationship between the induced 2-12 cells that were heteroploid and those that had damaged chromosomes. The metaphases that contained >90 chromosomes did not have a higher frequency of aberrant chromosomes than the metaphases that contained <90 chromosomes when normalized for total chromosomes (>90, 0.0151 ± 0.009 aberration per chromosome; <90, 0.0131 ± 0.003 aberration per chromosome). Interestingly, G5 cells that had been in culture for 8 weeks at the time of analysis also had an elevated frequency of heteroploid cells.

2-12 Cells Harbor Mutant p53 Genes. Because loss of wild-type p53 function has been implicated in impaired cell cycle control (12) and reduced genomic stability (13–16), it was important to establish the status of the p53 genes in 2-12 cells. The p53 mRNA was reverse-transcribed, PCR-amplified, cloned, and sequenced. Two mutant and no wild-type alleles were found. An A → G transition at bp 804 resulted in a Asn → Ser change at amino acid 236 in one allele, and a G → A transition at bp 886 resulted in a Met → Ile substitution at amino acid 243 in the other. Both of these missense mutations reside in conserved region 4, which commonly is mutated in human cancers (22). To assess whether a p53 function was compromised in these cells, they were examined for radiation-induced cell cycle block. The cells were exposed to ionizing irradiation (500 cGy) and the cell cycle distribution was examined by two-dimensional flow cytometry (12). There was no evidence of cell accumulation in either G_1 or G_2 phase (data not shown), consistent with loss of p53 function (12).

DISCUSSION

Our data indicate that induction of an activated Ha-*ras* gene in NIH 3T3 cells leads to rapid genomic instability. Although the mechanism by which activated Ha-*ras* produces chromosomal abnormalities is unknown, several hypotheses can accommodate the data. Direct action by Ras upon the cell's chromosomes seems unlikely because Ha-Ras is localized at the plasma membrane (1). However, Ras is known to affect multiple cellular pathways that could impinge upon the integrity of chromosomal DNA. *ras*-responsive promoter elements have been described (23), and it is conceivable that activated Ha-*ras* may alter transcription of genes whose products are involved in DNA metabolism. Alternatively, signal transduction initiated by activated Ha-Ras may activate a nucleolytic activity, resulting in sufficient damage to saturate the DNA repair capacity prior to replication. Another possibility is that activated Ha-*ras* may alter the interactions between regulatory accessory factors such as Ras–GAP and Ras–GEF, thereby leading to deregulation of one or more members of the GTP-binding *ras* superfamily (1), such as *ran*/TC4 (24). The product of *ran*/TC4 is necessary for the function of RCC1, which is a gene product involved in coordinating the end of the S phase and chromosome condensation (25). Finally, activated Ras may perturb cell cycle regulation, thereby interfering with maintenance of DNA integrity.

There are considerable data indicating that inappropriate expression of *ras* can interfere with normal cell cycle progression. Microinjection of recombinant EJ Ha-Ras into quiescent fibroblasts causes them to synthesize DNA (26). Conversely, microinjection of anti-Ras antibodies can block the progression of serum-deprived cells from G_0 to S phase after addition of growth factors (27). In this study, we have shown that selective induction of an activated Ha-*ras* transgene with IPTG is sufficient to disrupt at least one cell cycle control point by stimulating serum-deprived cells to progress from G_1 arrest into S phase. Consistent with this observation, disruption of an activated K-*ras* gene *in vitro* by gene targeting can lead to a decrease in the rate of cell proliferation (28), whereas the introduction of an activated Ha-*ras* in a different cell type can lead to an increase in the rate of cellular proliferation (29). In aggregate, these data argue that in some cells activated *ras* can facilitate the transition from G_1 to S phase of the cell cycle.

Disruption of the cell cycle by several mechanisms can result in DNA and chromosome damage (14–16, 30). Therefore, it should not be surprising that perturbation of the cell cycle by mutant Ha-*ras* can induce genomic instability. Induction of activated Ha-*ras* may stimulate cells to enter the S phase precociously, resulting in genomic instability and chromosome damage. Under normal circumstances, proliferating cells have a G_1 phase of defined duration during which cells are prepared for DNA synthesis before entering S phase. In response to stress, wild-type p53 protein normally is elevated and can function as a G_1 check point by blocking cells from entering S phase. In 2-12 cells where p53 is mutant, the G_1/S restriction point may be compromised by the induction of oncogenic Ha-*ras*, which we have shown in-

duces DNA synthesis and mitosis in G_1-arrested cells (ref. 1 and Fig. 3). Thus, activated Ha-*ras* may accelerate the cells' traverse through G_1, resulting in premature entry into S phase before DNA damage arising from endogenous sources, such as free radicals or nucleases, can be repaired. The predominance of chromosome rather than chromatid aberrations indicates that the unrepaired damage observed in mitosis was incurred before replication of the affected DNAs. Replication of DNA with double-strand breaks would produce broken chromosomes and acentric fragments with "sticky ends." The chromosome fragments can fuse with one another to produce acentric or multicentric chromosomes that are incapable of proper segregation at mitosis.

Double-strand DNA breaks and the resultant broken chromosomes also have been invoked in models of gene amplification (31, 32). In support of these models, we have found that 2-12 cells have a 25-fold enhanced capacity to amplify genes under selection when cultured in IPTG (33). The collaboration between activated Ha-*ras* and mutant p53 with respect to transformation and amplification competence recently has been demonstrated in the REF52 cell line (34, 35). We draw on this analogy to hypothesize that the impact of oncogenic Ha-*ras* on genome instability in 2-12 cells is due to the cumulative effect of defects in at least two genetic elements, p53 and Ha-*ras*, that jointly can participate in controlling the passage of cells through a G_1-phase check point.

The G5 cells, which constitutively express activated Ha-*ras*, have a significantly higher background of damaged chromosomes than either 2-42 cells or uninduced 2-12 cells. The primary difference between G5 and 2-42 cells is that the former had been in continuous culture for 8 weeks at the time of analysis, whereas the latter had been grown for >2 years. This time difference in continuous culture might account, in part, for the high background of chromosome instability seen in G5 cells. The occurrence of acentric and dicentric chromosomes leads to unequal chromosome segregation and to cells with karyotypes incompatible with viability. An alternative mechanism by which cells might become increasingly heteroploid is by endoreduplication, such as the example shown in Fig. 1*B* where the chromosomes were replicated twice without nuclear division. Endoreduplicated cells were seen at low frequency (1–3%), but only in the inducible Ha-*ras* cells after exposure to IPTG. It has been noted that tumor cells with divergent karyotypes eventually achieve a modal but aneuploid chromosome or DNA content (36), suggesting that there may be a selective pressure for cells to revert to genetic stability. In contrast to G5 cells, 2-42 cells had been in extended culture for sufficient time to select for mutations that suppress genomic instability but not the transformed phenotype. This would result in a lower background of damaged chromosomes in 2-42 cells than in the G5 cell line. Such a mechanism is plausible since genetic instability, as measured by the capacity to undergo gene amplification, is separable from the transformed phenotype (37).

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