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Modulation of Cytotoxicity of Chemotherapeutic Drugs by Activated H-*ras*

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ABSTRACT. Cells from a single MCF-7 clone were transfected with an isopropyl-1-thio-β-D-galactopyranoside (IPTG)-inducible construct containing activated human H-*ras* with a $Gly^{12} \rightarrow Val^{12}$ mutation. Expression of H-*ras* was induced by the presence of IPTG with low background. MCF-7-*ras* clones were examined for sensitivity to a wide variety of drugs under both induced and non-induced conditions. When expression of the activated *ras* was induced, these clones showed markedly increased resistance to cisplatin and mitomycin C, moderately increased resistance to methotrexate and trimetrexate, and no increased resistance to other drugs including taxol, doxorubicin, and etoposide. A DNA fragmentation assay revealed that DNA in MCF-7-*ras* cells treated with cisplatin under induced conditions was intact, whereas extensive degradation of DNA occurred in similarly treated cells under non-induced conditions. This result, along with the fact that MCF-7-*ras* cells, upon induction of the activated H-*ras*, showed increased resistance to drugs that bind DNA, indicates that the activated H-*ras* may play a role in the DNA repair process. BIOCHEM PHARMACOL 53;8:1203–1209, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. drug resistance; *ras*; cytotoxicity; DNA damage; MCF-7 cells; transfection

Intrinsic or acquired drug resistance is the main cause for failure in contemporary cancer chemotherapy [1–5]. Several mechanisms of drug resistance have been elucidated (reviewed in Ref. 6): (a) reduced uptake of the drug due to defective uptake [7], enhanced efflux [8, 9] or reduced retention [10]; (b) increased target enzymes due to either gene amplification or enhanced expression [11]; and (c) altered target enzymes with reduced affinity to the drug [12]. The understanding of these mechanisms of drug resistance has provided very useful information and guidance on improving the effectiveness of existing drugs and on developing new drugs. There are, however, other mechanisms of drug resistance that are either unknown or poorly understood. More recent work from this laboratory and elsewhere has linked loss of functional p53 or pRb (due to either deletions or mutations in the genes encoding for these proteins or to expression of oncoproteins such as E1A or the large T antigen that function to inactivate p53 and/or pRb) to drug and/or radiation resistance [13–16].

A number of reports showed that activation of cellular oncogenes such as *ras* and/or c-*myc* is associated with drug resistance. In 1988, Sklar showed that over-expression of the activated (i.e. mutated) H-*ras*, N-*ras*, or K-*ras* (reviewed in Ref. 17) as a result of transfection of NIH 3T3 cells with these oncogenes correlated with intrinsic resistance to ionizing radiation and CP‖ [18, 19]. This result was later confirmed independently by several other groups [20, 21] using either NIH 3T3 cells or a rat rhabdomyosarcoma cell line transfected with the H-*ras* oncogene. In addition to *ras*, over-expression of other oncogenes such as c-*myc* has been shown to be able to confer cellular resistance to MTX, CP, doxorubicin, and 4-hydroperoxycyclophosphamide [22–24]. These data suggest that there may be an underlying common cellular pathway that connects the effects of oncogene activation and expression of the drug-resistance phenotype. The exact role that *ras* or other oncogenes play in the induction of drug resistance is yet to be elucidated. Recent work by Nooter *et al.* [25] suggests that *ras* may function as an inhibitor of doxorubicin-induced apoptosis in a rat rhabdomyosarcoma cell line constitutively expressing c-H-*ras* oncogene.

All of these preliminary studies described above utilized non-inducible expression vectors and/or non-human normal or tumor cell lines as the model systems. To establish whether activated *ras* plays a role in drug resistance in human tumors and to understand this role, the current study was undertaken in which cells from a single clone of a human breast cancer cell line (MCF-7) were stably trans-

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‖ *Abbreviations*: MTX, methotrexate; DHFR, dihydrofolate reductase; BCA, bicinchoninic acid; PCR, polymerase chain reaction; IC_{50}, concentration of drug that is responsible for 50% of cell kill; IPTG, isopropyl-1-thio-β-D-galactopyranoside; ECL, enhanced chemiluminescence; and CP, cisplatin.

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fected with activated H-*ras* under the control of bacterial lactose operon regulatory elements [26]. H-*ras* expression is induced by growing cells in the presence of IPTG with minimal background. This inducible system has the advantage that the effects of activated H-*ras* on drug resistance can be studied in the same cell population (under induced or non-induced conditions), thereby eliminating (or at least minimizing) possible artifacts caused by heterogeneous (random) integration of a foreign DNA into recipient cells leading to clonal variation or other changes not necessarily related to the expression of the inserted gene.

MATERIALS AND METHODS

Chemicals

MTX was obtained from Lederle Laboratories (Pearl River, NY). Doxorubicin, taxol, vinblastine, sulforhodamine B, mitomycin C, CP, and VP-16 were obtained from the Sigma Chemical Co. (St. Louis, MO).

Cell Line

MCF-7 breast adenocarcinoma cell line was obtained from the American Type Culture Collection and was maintained as monolayer cultures at 37° in a 5%CO_2/95% air incubator in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Cloning of MCF-7 Cells

MCF-7 cells were cloned using the dilution method with a 96-well plate. Four different single clones were obtained and expanded into cell lines. One clone (No. 3) was used for transfection studies (see below). DNA sequencing analysis showed that cells from all of these clones of MCF-7 contained a normal H-*ras* gene.

Transfection

The standard calcium phosphate precipitation method (BBS Protocol) [27] was used to introduce the human activated H-*ras* gene (under the control of the *Escherichia coli* regulatory element *lac*O) into the cells from MCF-7 clone No. 3. This two-plasmid IPTG-inducible expression system has been described previously. In the standard protocol, a mixture of the plasmids pSV*lac*O*ras* (9 μg) and pHb*lac*INLS*neo* (45 μg), purified by CsCl gradient centrifugation, was used to transfect 5×10^5 cells plated in a 100-mm culture dish. Cells were exposed to the DNA for 16 hr before being washed with fresh medium. Geneticin (G418) selection at 600 μg/mL was started 48 hr later. Colonies were isolated by use of plastic cloning cylinders and then expanded into cell lines.

Identification of H-ras-Positive Clones by PCR

The protocol for specific PCR amplification of the inserted gene sequence from whole cells was adapted from Perkin-Elmer Cetus *Amplifications* (May 1989, Issue 2). About 1×10^4–1×10^5 cells (which may be grown in a 24-well plate right after colony isolation by use of cloning cylinders) were detached with trypsin digestion and washed two times with PBS. The cell pellet was resuspended in 50 μL of the following PCR-detergent buffer: 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/mL gelatin, 0.45% Nonidet P-40, 0.45% Tween-20, and 0.1 mg/mL proteinase K. After incubation at 37° for 1 hr followed by inactivation of proteinase K at 95° for 10 min, 25 μL of this reaction mixture was subjected to 37 cycles of PCR amplification by use of an SV40 promoter-specific primer and an H-*ras* exon-1-specific primer. The clones that show the expected 600 bp PCR product on the agarose gel have incorporated the inserted activated H-*ras* gene.

Western Blot Analysis

Two clones of MCF-7 cells that had incorporated the inserted activated H-*ras* as shown by PCR were grown to mid-log phase and then for an additional 24 hr in the presence or absence of 20 mM IPTG. Cells were harvested by trypsinization, washed with PBS, and solubilized with a PBS buffer containing 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS. After being centrifuged at 60,000 *g* for 30 min to remove any insoluble cellular debris, the cell extract [containing 150 μg of total protein as determined by the BCA assay according to the manufacturer's instructions (Pierce, Rockford, IL)] was precipitated with 80% acetone/20% H_2O and subjected to SDS–PAGE (15% acrylamide) [28]. The proteins were electro-transferred to nitrocellulose membrane, and the latter was probed with a monoclonal antibody against pan-ras (Cat. No. OP38, Oncogene Science, Manhasset, NY), followed by a second antibody conjugated with peroxidase. The ras protein was then visualized by treatment of the membrane with ECL reagents (Amersham, Arlington Heights, IL) and by exposure to an X-ray film.

Cytotoxicity Assays

Cytotoxicity of drugs was measured by use of a microculture technique and sulforhodamine B binding assay, adapted from Skehan *et al.* [29]. Mid-log phase MCF-7-*ras* cells (clones 3-5-5 and 3-5-11) were harvested by trypsinization and plated at a density of 3×10^3 cells/well in a 96-well plate. Each well contained a total of 180 μL fresh RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum, with or without 20 mM IPTG. After incubation at 37° overnight to allow for cell attachment, drugs in 20 μL of water or buffer were added to each well to give final concentrations ranging from 1×10^{-4} to 3×10^{-10} M (three times serial dilutions, prepared in a separate 96-well plate), and the plate was incubated at 37° for another 24 hr. Drugs were removed by washing the plate with serum-free medium, and cells were allowed to grow for 5 days at 37°. Cells were fixed to the bottom of the plate by addition of 50%

(w/v) trichloroacetic acid to a final concentration of 10% to each well and incubation at 4° for 1 hr. The plate was washed five times with water, and the fixed cells were stained with 0.4% sulforhodamine B in 1% acetic acid at room temperature for 30 min. After being washed four times with 1% acetic acid followed by solubilization of sulforhodamine B with 150 μL/well of 10 mM Tris base, the plate was read at 562 or 610 nm. IC_{50} Values were determined and the averages of duplicate or triplicate measurements were used. IC_{50}(-IPTG) indicates an IC_{50} value obtained in the absence of IPTG; IC_{50}(+IPTG) indicates an IC_{50} value obtained in the presence of IPTG.

DNA Fragmentation Assay

DNA fragmentation assay (adapted from Ref. 30) was performed for MCF-7-*ras* cells after treatment with CP under both induced and non-induced conditions. MCF-7-*ras* cells (clones 3-5-5 and 3-5-11) were grown to early log-phase and for an additional 16 hr in the presence or absence of 20 mM IPTG. Cells were exposed to different concentrations (0–30 μM) of CP for 48 hr. Cells were detached by trypsinization and combined with the dead cells in the growth medium, washed with PBS, and then resuspended in the same buffer. Cell numbers were determined by counting with a Coulter counter, and 10^6 cells were centrifuged. The cell pellet was resuspended in 15 μL of TBE buffer (89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA, pH 8.0) containing 10 mg/mL ribonuclease A, 15% Ficoll 400, and 0.01% bromophenol blue. The suspension was loaded onto a well in the gel system constructed as follows: A 150-mL vol. of 2% agarose in TBE buffer was poured into a gel support with a comb. After the gel solidified, the portion of the gel directly above the comb (*ca.* 2 cm) was removed and replaced with 0.8% agarose in TBE buffer containing 2% SDS and 1.25 mg/mL proteinase K that was added when the melted agarose had cooled to below 55°. Electrophoresis was conducted at 20 V for 1 hr and then at 90 V for 3 hr. The gel was rinsed with distilled water and incubated overnight at room temperature with gentle shaking in 200 mL TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 2 mg of ribonuclease A. After being rinsed with excess deionized water, the gel was stained with 0.5 μg/mL ethidium bromide, destained with water, and photographed under UV light.

RESULTS

Inducible Expression of H-ras in MCF-7 Cells

A common problem associated with stable transfections using non-inducible expression vectors is the likelihood of artifacts caused by clonal variation among the transfectants. To alleviate this problem, MCF-7 cells were cloned and a *single* clone (No. 3) was obtained that contained a normal H-*ras* gene as determined by sequencing analysis (data not shown). Cells from this clone of MCF-7 were used to obtain stable transfectants expressing inducible human activated

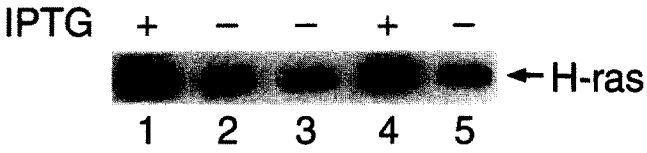

FIG. 1. Inducible expression of activated H-*ras* in MCF-7-*ras* cells. Two clones of MCF-7-*ras* cells (3-5-5 and 3-5-11) were grown to mid-log phase and then for an additional 24 hr in the presence or absence of 20 mM IPTG. Cells were harvested, and total cellular extracts were prepared. The cell extract containing 150 μg of protein was subjected to SDS-PAGE (15% acrylamide). The proteins were transferred to a nitrocellulose membrane and the ras protein was visualized by use of an anti-ras antibody followed by ECL detection as described in Materials and Methods. Lane 1, MCF-7-*ras* (3-5-5), +IPTG; Lane 2, MCF-7-*ras* (3-5-5), -IPTG; Lane 3, MCF-7-*ras* (3-5-11), -IPTG; Lane 4, MCF-7-*ras* (3-5-11), +IPTG; Lane 5, untransfected MCF-7 cells.

H-*ras* under the control of bacterial lactose operon regulatory elements. After selection with G418, two such transfectants (3-5-5 and 3-5-11) were obtained that contained the activated H-*ras* gene, as shown by PCR using a primer specific for the SV40 promoter and another primer specific for exon 1 of the H-*ras* gene (data not shown). This PCR step was necessary because the plasmid bearing the H-*ras* gene does not have a selectable marker. Western blotting analysis using a pan-ras antibody revealed that expression of the activated H-*ras* was induced by the presence of IPTG (lanes 1 and 4, Fig. 1), as compared with the level of expression in the absence of the inducer (lanes 2 and 3). The latter, which most likely represented the endogenous level of *ras* expression, was comparable to that of wild-type MCF-7 (lane 5). Growth characteristics (e.g. doubling time) as well as the morphology of the MCF-7-H-*ras* cells (clones 3-5-5 and 3-5-11) in either the presence or absence of IPTG were very similar to those of wild-type MCF-7 clones (data not shown).

Effect of Activated H-ras on Sensitivity to CP

Sensitivity (IC_{50} values) to CP with various exposure times was measured in MCF-7-H-*ras* cells (both clones 3-5-5 and 3-5-11) in the presence and absence of IPTG, and the IC_{50} ratios (+IPTG vs −IPTG) are presented in Table 1. When

TABLE 1. Effect of exposure time of drug and IPTG on the sensitivity of MCF-7-*ras* cells to CP

MCF-7-*ras* cells	IC_{50} (+IPTG)/IC_{50} (−IPTG) ratio			
	1 hr	4 hr	24 hr	48 hr
3-5-5	2.5 ± 0.5	2.2 ± 0.2	9.5 ± 1.2	12.0 ± 1.4
3-5-11	2.0 ± 0.3	3.0 ± 0.4	3.8 ± 0.5	6.3 ± 0.8

Cells were plated in the presence or absence of IPTG for 16 hr and exposed to the drug for the indicated time, washed free of the drug, and then allowed to grow for a total of 5 days after addition of the drug. Results are the means ± SEM from three experiments.

activated H-*ras* was induced by IPTG and cells were exposed to the drug for 1 or 4 hr, cells were 2 to 3-fold more resistant to the drug than cells not exposed to IPTG. When longer drug exposure times (24 and 48 hr) were used, the cells became even more resistant (3 to 12-fold) to the drug upon induction of activated H-*ras* (Table 1). A representative growth inhibition curve (that was used to calculate the IC_{50} values in Table 1) (clone 3-5-5, 48-hr exposure of CP) is shown in Fig. 2. When untransfected (control) MCF-7 cells were tested for sensitivity to CP, no significant differences in IC_{50} values were observed between cells exposed and those not exposed to IPTG (Table 2), indicating that IPTG itself does not affect CP cytotoxicity.

Effect of Activated H-ras on Sensitivity to Different Classes of Drugs

Sensitivity to different classes of drugs that have a diverse spectrum of targets and mechanisms of action was measured in MCF-7-H-*ras* cells (clones 3-5-5 and 3-5-11) in the presence and absence of IPTG (Table 2). When induced by IPTG, MCF-7-H-*ras* cells were significantly more resistant to DNA-damaging agents such as CP [with IC_{50}(+IPTG)/IC_{50}(−IPTG) ratios of 9 and 4 for clone 3-5-5 and 3-5-11, respectively] and mitomycin C [with IC_{50}(+IPTG)/IC_{50}(−IPTG) ratios of 6 and 5] than cells grown in the absence of IPTG. MCF-7-H-*ras* cells were more resistant, but to a lesser extent, to antimetabolites such as MTX and trimetrexate when induced by IPTG than were cells without IPTG induction [with IC_{50}(+IPTG)/IC_{50}(−IPTG) ratios of 1.5 to 3] (Table 2). When other classes of drugs such as vinblastine and taxol (microtubule inhibitors) or etoposide and doxorubicin (topoisomerase II inhibitors) were tested, there was no difference in the cytotoxicity of these drugs to MCF-7-H-*ras* cells in either the presence or the absence of IPTG. Interestingly, both clones of MCF-7-H-*ras* cells showed *ca.* 3-fold increases in sensitivity to cyclophosphamide when induced by IPTG as compared with cells without IPTG induction. In control experiments, untransfected MCF-7 cells showed no difference in sensitivity to CP, mitomycin C, MTX, trimetrexate and cyclophosphamide between cells exposed and cells not exposed to IPTG (Table 2).

Expression of Activated H-ras Leads to Inhibition of DNA Fragmentation Induced by CP

To understand the mechanism(s) of resistance to DNA-damaging agents induced by the activated H-*ras*, a DNA fragmentation assay was performed for MCF-7-H-*ras* cells after treatment with CP under both induced and non-induced conditions. Both clones of MCF-7-H-*ras* cells growing in the presence or absence of IPTG in log-phase were treated with various concentrations of CP. After 48 hr, cells were collected, and whole cells were subjected to electrophoresis in the presence of SDS and proteinase K. The DNA in the gel was visualized with ethidium bromide staining. As shown in Fig. 3, when MCF-7-H-*ras* cells (both clones 3-5-5 and 3-5-11) were treated with 5μM CP in the absence of IPTG, extensive DNA fragmentation occurred (lanes 3 and 10, respectively), whereas DNA fragmentation was greatly reduced in cells treated similarly but in the presence of IPTG (lanes 2 and 9, respectively). As a control, cells not treated with the drug showed no evidence of DNA fragmentation (lanes 1 and 8). Similar results were obtained when higher concentrations of the drug were used (compare lanes 4 and 5, 6 and 7, 11 and 12, and 13 and 14). Another control experiment demonstrated that when untransfected MCF-7 cells were treated similarly, no difference in CP-induced DNA fragmentation was evident between cells exposed and cells not exposed to IPTG (not

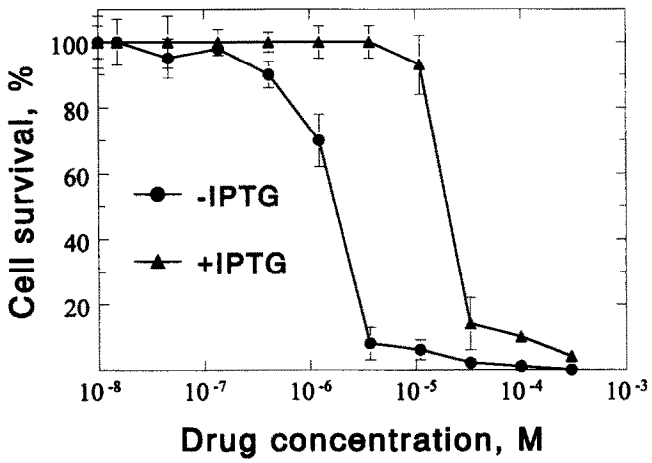

FIG. 2. Sensitivity of MCF-7-*ras* (clone 3-5-5) cells to cisplatin under induced and non-induced conditions. Cells from MCF-7-*ras* (clone 3-5-5) cells were treated with CP under both induced (+IPTG) and non-induced (−IPTG) conditions for 48 hr. After washing to remove drugs and incubation for an additional 48 hr, cell growth was measured by a standard sulforhodamine B binding assay as described in Materials and Methods. Error bars of each data point represent the SEM from three experiments.

TABLE 2. Relative resistance of MCF-7-*ras* cells to different classes of drugs

Drug	Known drug target	IC_{50} (+IPTG)/IC_{50} (-IPTG) ratio		
		MCF-7-*ras* (3-5-5)	MCF-7-*ras* (3-5-11)	Untransfected MCF-7
CP	DNA	9.5 ± 1.2	3.8 ± 0.5	1.2 ± 0.2
Mitomycin C	DNA	6.4 ± 0.6	5.3 ± 0.3	1.3 ± 0.2
MTX	DHFR	2.2 ± 0.2	1.5 ± 0.2	1.1 ± 0.1
Trimetrexate	DHFR	1.5 ± 0.1	3.1 ± 0.4	1.0 ± 0.2
Etoposide	Topo II	1.2 ± 0.2	1.1 ± 0.2	ND*
Doxorubicin	Topo II	1.0 ± 0.3†	1.1 ± 0.2†	ND
Taxol	Microtubules	1.1 ± 0.1	1.0 ± 0.2	ND
Vinblastine	Microtubules	1.2 ± 0.1	1.1 ± 0.1	ND
Cyclophosphamide	DNA	0.3 ± 0.06	0.3 ± 0.04	1.2 ± 0.3

The cytotoxicity of drugs was measured by use of a microculture technique and sulforhodamine B binding assay as described in Materials and Methods. IC_{50} (-IPTG): IC_{50} value obtained in the absence of IPTG; IC_{50} (+IPTG): IC_{50} value obtained in the presence of IPTG. Results (for all drugs except doxorubicin) are the means ± SEM from three to four experiments.

* ND, not determined.

† Average ± the difference from two experiments.

shown). These results indicated that the induced activated H-*ras* protected DNA from fragmentation when cells were treated with the DNA-damaging agent CP.

DISCUSSION

The data presented in this paper demonstrate that induced expression of an activated human H-*ras* mediated resistance to growth inhibition by DNA-damaging agents in a human breast adenocarcinoma cell line (MCF-7). Activated H-*ras* mediated this drug resistance by exerting a protective effect on DNA fragmentation induced by CP and possibly other DNA-damaging agents. Although *ras* activation has been correlated with drug and radiation resistance in a number of previous studies (see the Introduction for references), all of which, to the best of our knowledge, have used non-human cell lines, and a link between *ras* and drug resistance in human cells has not yet been established. The key features of the experimental system utilized in this study are 2-fold: (1) a human tumor cell line was used, and (2) an inducible expression system utilizing a bacterial regulatory element was used to study the effect of activated H-*ras* on drug resistance, greatly reducing if not eliminating possible artifacts often associated with clonal variations among stable transfectants obtained with conventional expression systems. Due to the highly selective nature of induction of activated H-*ras* by IPTG, the drug-resistant phenotype and the protective effect on DNA fragmentation induced by drugs were a direct consequence of expression of the activated H-*ras* transgene.

Our data indicate that induction of activated H-*ras* leads to a moderate increase in resistance to antimetabolites such as MTX whose target is DHFR. This is consistent with a previous report by Wani *et al.* [26] showing that induction of activated H-*ras* in mouse fibroblast NIH 3T3 cells resulted in increased resistance to MTX as a result of increased level of DHFR expression as well as increased frequency of gene amplification. These data suggest that *ras* may modulate the sensitivity of certain drugs by directly regulating the target enzyme levels of these drugs.

Recent work by other investigators provides evidence that *ras* may negatively regulate apoptotic pathways [31–33]. Although this could explain why induction of activated H-*ras* expression leads to resistance to DNA-damaging agents (e.g. CP and mitomycin C), it does not

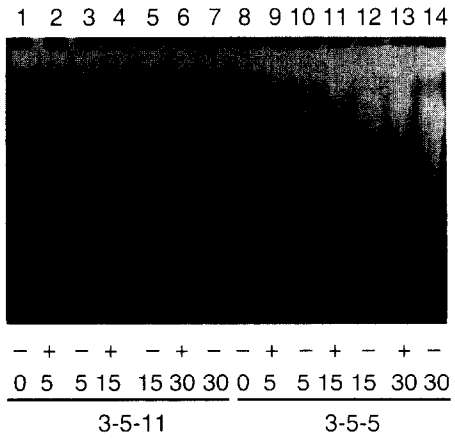

FIG. 3. DNA fragmentation assay for MCF-7-*ras* cells after treatment with CP under both induced and non-induced conditions. Cells from two clones (3-5-5 and 3-5-11) of MCF-7-*ras* cells were grown to mid-log phase in the presence or absence of 20 mM IPTG and exposed to the indicated concentrations (0–30 μM) of CP for 48 hr, with the continued presence or absence of IPTG, respectively. Cells were collected by trypsinization and processed for the *in situ* DNA fragmentation assay as described in Materials and Methods. The presence or absence of IPTG and the concentrations of CP for each treatment are indicated. Since DNA fragments smaller than 23 kbp were nearly absent, only DNA fragments larger than 23 kbp [as indicated by DNA molecular weight markers (not shown)] are shown. This is a representative of two very similar experiments.

seem to explain why induction of the activated H-*ras* expression did not result, at least in MCF-7 cells, in increased resistance to other drugs such as etoposide or doxorubicin, two agents that are also believed to kill cells by inducing apoptosis [34, 35]. In contrast to what was observed in the current study (which showed that induction of activated H-*ras* had no effect on sensitivity to doxorubicin), Nooter *et al.* [25] recently showed that expression of c-H-*ras* in a rat rhabdomyosarcoma cell line leads to increased resistance (3- to 5-fold) to doxorubicin and that constitutive expression of c-H-*ras* in the rat cell line correlated with reduced DNA strand breaks induced by the drug. This different result is most probably due, at least in part, to different cell types used in these two studies. In addition, our preliminary experiments (unpublished data; not shown) showed that induction of activated K-*ras* in the same cell line (MCF-7) up-regulated Gadd45 and p53, two proteins believed to be involved in DNA excision repair [36, 37]. This may provide a possible explanation for the observed increase in *ras*-mediated resistance to certain DNA-damaging agents such as CP and mitomycin C. Taken together, our data as well as that of others suggest that activated *ras* may play an important role in protecting DNA from drug-induced damage and, possibly, also in the DNA repair process.

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