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X-Ray Induction of Methotrexate Resistance Due to *dhfr* Gene Amplification

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Abstract—*The effect of ionizing radiation on methotrexate (MTX) resistance and gene amplification in cultured mammalian cells was investigated. X-irradiation of mouse EMT-6 cells induced cell killing and MTX resistance due to amplification of the dihydrofolate reductase (*dhfr*) gene in a dose-dependent manner. The highest yields of mutant cells were obtained at approximately D_{37} (the dose at which 37% of the cells survive), where the frequency of MTX-resistant cells was four- to eightfold over that of the unirradiated population. The proportion of MTX-resistant cells among the survivors increased logarithmically with dose, up to a 1000-fold increase over unirradiated cells at 1000 cGy, the highest dose tested. The induced frequency of MTX resistance after X-irradiation was greater than the induced frequency of 8-azaguanine resistance, which indicates deletion of the hypoxanthine phosphoribosyltransferase gene. Inhibition of poly(ADP-ribose) polymerase by the addition of 3-aminobenzamide before irradiation increased both cell killing and MTX resistance. Metaphase spreads of chromosomes from EMT-6 cells that had been irradiated and subjected to stepwise increases in MTX concentration showed numerous double minutes. Pulsed-field gel electrophoresis of the DNA from cells containing radiation-induced double minutes showed that many copies of the dhfr gene were present on circular DNA molecules of 10^6, 2×10^6, and 3×10^6 base pairs. These results suggest a relationship between the induction of chromosome aberrations and the induction of gene amplification.*

INTRODUCTION

Gene amplification is a genetic rearrangement that results in an increased number of copies of a given gene and much of the flanking chromosomal regions. It is associated with both drug resistance and tumor progression in mammalian cells (1–3). Mammalian cell resistance to methotrexate (MTX) in culture due to amplification of the dihydrofolate reductase (*dhfr*) gene has received the most attention. Although the molecular events responsible for this type of rearrangement are not known, there is substantial genetic evidence for a complex process that involves sequences associated with the gene being amplified (4–7) as well as totally unrelated genes (8).

Cytogenetic observations of metaphase chromosomes from cells resistant to very high levels of drug have revealed two forms of gene amplification. Amplified genes have been located both in expanded chromosomal domains, referred to as homogeneously staining regions (9, 10), and on extrachromosomal elements, referred to as double minutes (11).

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Recent evidence suggests that submicroscopic circular DNA molecules are associated with some cell lines that contain double minutes (12), and at least the smaller double minutes themselves may be circular (13). Indeed, Carroll et al. (14) had shown that in one case the submicroscopic circular DNA molecules had a structure compatible with having arisen by recombinational excision events, leading those investigators to propose that double minutes arise by multiple dimerizations of small circular excision products.

Many DNA-damaging agents have been shown to increase the frequency of amplified genes in a cell population (15–18), but this phenomenon has been considered to be a consequence of inhibition of DNA synthesis and only indirectly related to the damage induced. It has been suggested that perturbations in the replicative process induced by repair activity lead to multiple rounds of DNA synthesis in a single cell cycle (19–21) and then to multiple copies of a particular gene in a single cell. However, Morgan et al. (22) and Hahn et al. (23, 24) have suggested that early events in gene amplification are a direct result of misrepair of the damaged DNA in the classic sense of mutation. Chromosome aberrations resulting from misrejoined or unrejoined double-strand breaks are hypothesized to lead to gene amplification or deletion, depending on whether or not the aberrations are retained at mitosis. Thus, gene amplification or deletion would result from unequal segregation at mitosis rather than from multiple rounds of initiation at certain origins of replication.

To test this hypothesis, we examined the effect of ionizing radiation on the induction of gene amplification. X-radiation induces high frequencies of chromosome aberrations, but it does not cause many single base change mutations and does not increase sister chromatid exchanges (25–28). Although X-radiation causes high levels of both single- and double-strand breaks in chromosomal DNA (29), most genetic effects are associated with the double-strand-breaking activity. Consistent with its chromosome-breaking activity, X-radiation produces primarily gene deletions and rearrangements, most of which are lethal. If gene amplification results from the same type of mutation that gives rise to deletions, then X-radiation would be expected to dramatically increase the frequency of gene amplification events. Furthermore, if the deletion products are circular precursors to double minutes, then it should be possible to detect these products in X-ray-induced MTX-resistant cell lines that harbor double minutes.

Since the DNA strand breakage induced by X-rays can be potentiated by the poly(ADP-ribose) polymerase inhibitor 3-aminobenzamide (3AB) (30), we also examined the effect of 3AB on X-ray-induced gene amplification. Poly(ADP-ribose) polymerase is activated by the presence of strand breaks in chromosomal DNA, resulting in extensive ribosylation of nuclear proteins and a concomitant depletion of nuclear NAD^+ (for review see reference 30). Although the molecular role of poly(ADP-ribosylation) remains unknown, the net effect of its inhibition is to increase the levels of DNA damage over the amount that occurs when the polymerase is active. The potentiation of X-ray-induced gene amplification by 3AB would suggest that gene amplification is related to DNA double-strand breakage and repair.

MATERIALS AND METHODS

Cell Culture. EMT-6/SY mouse cells were obtained from R. Kallman (Stanford University) in 1978 and have been maintained since then in continuous culture in our laboratory. These cells are capable of growing as tumors in mice, and at every 10 passages in culture they were injected back into mice and regrown as tumors. After reisolation of cells, approximately 10 passages were required to "adapt" these cells to the dialyzed serum. A large number of cells then were grown and

frozen to be used as needed. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 15% dialyzed fetal calf serum or CPSR 3, a serum replacement (Sigma), 10 mM HEPES, and 1% penicillin and streptomycin.

MTX Sensitivity. To test the sensitivity of normal EMT-6 cells to MTX, 200 cells per plate (in triplicate) were grown in Dulbecco's modified Eagle's medium supplemented with dialyzed fetal calf serum with or without MTX at concentrations of 25, 50, 75, or 100 nM. After 14 days, colonies containing greater than 50 cells were counted. Because no cells survived treatment with 100 nM MTX, we selected 150 nM MTX for the assay for *dhfr* gene amplification. This concentration is sufficient to kill MTX-sensitive cells in which the *dhfr* gene is not amplified.

X-Ray-Induced MTX Resistance and 8-Azaguanine Resistance. EMT-6 cells were irradiated with 125, 250, 500, 750, or 1000 cGy from a 320-kV Philips irradiator (300 kVp; 15 mA; nominal half-value layer, 0.35 mm Cu), at a rate of 3.18 Gy/min. One day after irradiation, 10^4 cells per plate (in triplicate) were plated into 150 nM MTX to determine MTX resistance frequency. The remaining cells were cultured for an additional nine days and passaged every two or three days. Ten days after irradiation, an additional 10^4 cells per plate (in triplicate) were plated into 150 nM MTX, and 10^5 cells per plate (in triplicate) were plated into medium with 1 μM 8-azaguanine (8AG) to determine the frequency of hypoxanthine phosphoribosyltransferase (*HPRT*) deletion. 8AG kills all cells that have a functional *HPRT* gene and is used to quantify *HPRT* gene inactivation; in the case of X-irradiation this is primarily due to *HPRT* gene deletion. Ten days has been established as the optimal phenotype expression time for 8AG resistance (unpublished observation).

Quantification of MTX Resistance and dhfr *Gene Amplification.* To test whether surviving colonies were genuinely resistant to MTX, a population of EMT-6 cells was plated into 150 nM MTX, and 30 single colonies were isolated—15 into medium supplemented with 150 nM MTX to monitor their ability to grow in the presence of MTX and, as a control, 15 into medium without MTX to determine their ability to survive the isolation procedure. An additional 30 colonies were isolated from a population of cells that had been irradiated with 750 cGy of X-rays before plating into 150 nM MTX. Again, 15 MTX-resistant colonies were plated directly into medium containing MTX and 15 into medium without MTX.

To determine whether the surviving MTX-resistant colonies were resistant owing to amplification of the *dhfr* gene, DNA from MTX-resistant isolates was subjected to Southern blot analysis to measure *dhfr* gene hybridization relative to total cellular DNA. Five MTX-resistant single-colony isolates from the unirradiated population (plated in MTX) and five MTX-resistant isolates from the irradiated population (plated in MTX) were grown in the presence of 150 nM MTX and expanded to approximately 5×10^7 cells each. Since we were simultaneously looking for chromosome fragments with *dhfr* genes by pulsed-field gel electrophoresis, the cells were then trypsinized, washed in saline solution, and adjusted to 10^7 cells per 200 μl in 0.75% low-melt agarose, placed in 200-μl molds to form "plugs," and digested in 0.5 mg/ml proteinase K, 1% sarcosine, and 0.5 M EDTA, pH 8.4, overnight at 55°C. Representative plugs from each isolate were quartered, washed in Tris EDTA, digested to completion with HindIII, electrophoresed through a 0.75% agarose gel, and blotted onto nylon membranes. The membranes were probed first with pSV2 (a plasmid that contains *dhfr*) labeled with ^{32}P by using a BRL random primer kit in a Hoeffer "hybrid-ease" chamber according to the Church and Gilbert (31) procedure and then autoradiographed for 10 days at −70°C with two intensifying screens and Kodak X-Omat AR X-ray film. To quantify DNA

transferred to the membrane, the membranes were subsequently stripped and reprobed with ^{32}P-labeled EMT-6 DNA, followed by autoradiography for 20 h at ambient temperature with intensifying screens and Kodak X-Omat AR film.

To quantify the strength of the hybridization signals, the region corresponding to the 3-kb HindIII band in the autoradiographs was scanned with a densitometer (Automatic Isodose Plotter; ATC Medical Technology, Sunnyvale, California). The bands from the pSV2 *dhfr* probe were scanned in the direction of the migration of the lane, whereas the autoradiographs of the whole DNA probe were scanned across the lanes (perpendicular to the scan of the band intensities) of the same region, as determined by overlaying the autoradiographs. Autoradiographs were compared at similar levels of exposure (low-grain density). Values were obtained by averaging two scans of each "band" and dividing the intensity of the *dhfr* band by the intensity of the total DNA in that region (integration was by excising and weighing scan tracings). The several control (nonresistant) values on each gel were further averaged, and the intensities of all *dhfr* 3-kb bands were expressed as normalized intensity relative to the average normalized intensity of control DNA on that gel.

Interaction of 3AB with X-Irradiation. To test the effect of 3AB on the frequency of induced gene amplification, 2 mM 3AB was added to cultures 30 min before X-irradiation. Control cultures received no 3AB. A population was divided into ten 25-cm^2 tissue culture flasks, five of which were treated with 2 mM 3AB before 200, 400, 600, or 800 cGy of X-rays. Irradiated cells, with or without 3AB, were given fresh medium after irradiation and plated the following day in 150 nM MTX. Colonies were counted 14–21 days later.

Cytogenetics. Metaphase chromosome preparations from highly MTX-resistant EMT-6 cells were prepared essentially as described (22). A population of cells that had originally received 750 cGy was subjected to a stepwise increase in MTX concentration to 100 μM, at which time single colonies were isolated, expanded, raised to 160 μM MTX, and maintained in that concentration. Control (unirradiated) EMT-6 cells were prepared in parallel.

Pulsed-Field Gel Electrophoresis. To examine the double minutes by pulsed-field gel electrophoresis, we used a modification of the method of van der Bliek et al. (13), who used ionizing radiation to break the circular *Escherichia coli* genome and double-minute chromosomes in mouse cells to facilitate entry into agarose gels. Single-colony isolates were prepared as described for Southern blot analysis and irradiated with 4000 cGy of X-rays. Paired samples with and without irradiation were subjected to pulsed-field gel electrophoresis with a CHEF apparatus (32) from Owl Scientific (Boston, Massachusetts). Two electrophoretic conditions were used. One set of conditions separates chromosomal markers of both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe,* resulting in separation of DNAs ranging in size from 2×10^5 to 1×10^7 base pairs (bp). These conditions require a 50-V setting with a series of pulse times in each direction of 6, 12, 24, 42, 66, and 96 min, repeated for 7–10 days at 8°C. The pulse controller is a PP200 from MJ Research. The second set of conditions separates just the *S. cerevisiae* chromosomal markers, with a maximum separation around 10^6 bp. These conditions require 125 V with pulse times increasing from 60 sec to 300 sec by 4-sec increments for three days.

RESULTS

MTX Resistance Due to dhfr *Gene Amplification.* After determining the survival of normal EMT-6 mouse cells in MTX (LD_{50}, 35 nM), we selected 150 nM MTX for the assay for *dhfr* gene amplification. We wished to determine the relative gene copy number in the cells in the resistant colonies,

and we also wished to ensure that most of the entities we were counting as colonies were composed of living cells. Therefore, one half (15 of 30) of each set of colonies were plated into medium without MTX to determine viability, and the other half were plated into medium supplemented with 150 nM MTX to determine whether they were both viable and resistant to MTX. Approximately half of the 60 colonies selected survived as populations, but there was little difference between the subgroups. Therefore, most of the entities we call colonies probably contained bona fide MTX-resistant cells. Ten of the surviving MTX-resistant single-colony isolates—five from the unirradiated population and five from the irradiated population—then were expanded in the presence of 150 nM MTX to measure *dhfr* gene hybridization intensity. DNA from all 10 colonies exhibited at least 1.5-fold increased signal intensity after normalization for total DNA attached to the nylon membrane in the region of the *dhfr* gene (Fig. 1).

X-Ray Induction of MTX and 8AG Resistance. HPRT gene inactivation is a well-established assay for mutagenesis in mammalian cells and is frequently used to assess X-ray mutagenesis (25, 26, 34, 35), since it is one of the few assays that yield a measurable response. We wished to compare *dhfr* gene amplification as measured by MTX resistance frequency with *HPRT* gene deletion as measured by 8AG resistance to assess the relative importance of gene amplification and gene deletion in EMT-6 cells.

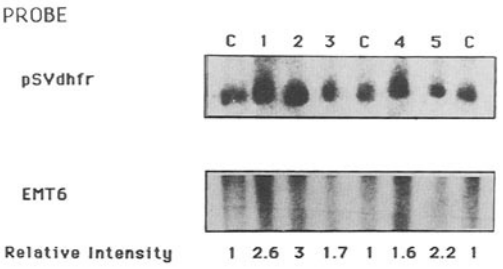

Fig. 1. Southern blot analysis of *dhfr* gene hybridization. HindIII-digested DNA in agarose plugs from the five single-colony isolates (labeled 1–5) recovered in 150 nM MTX after 750 cGy of X-rays. X-irradiated DNA was compared with wild-type DNA (C) for hybridization intensity. The DNA was probed with a *dhfr*-specific probe (pSVdhfr) followed by a probe for total DNA. The relative intensity of the isolates is expressed as an average of two separate blots. Each sample on each blot was normalized for total DNA attached to the membrane at the position of the HindIII band and then expressed relative to the average of the controls on that membrane. Only one set of hybridizations is shown.

After irradiation with 125–1000 cGy of X-rays, cells were allowed to recover overnight in fresh medium and either plated in 150 nM MTX (the normal MTX resistance protocol) or subcultured for 10 days before plating in 150 nM MTX or 1 μM 8AG (the normal *HPRT* gene inactivation assay).

MTX resistance was increased at all X-ray doses tested, whether it was measured one day after irradiation (Fig. 2B,C) or 10 days later (Fig. 2D), and the response was dose dependent (Fig. 2B). At 1000 cGy, MTX resistance among the surviving cells was 1000-fold over that of unirradiated cells. After 10 days, at all X-ray doses tested, MTX resistance was more common than 8AG resistance. Cell killing by X-irradiation also was dose dependent (Fig. 2A). Although the shapes of the survival curves differed in separate experiments (data not shown), MTX resistance achieved maximum levels (four- to eightfold higher than that of the unirradiated population) at approximately D_{37} (Fig. 2C).

3AB Potentiation of X-Rays. To determine whether 3AB would increase X-ray-induced MTX resistance, we treated EMT-6 cells with 3AB alone or in combination with X-rays. When we irradiated cells in the presence of 3AB, both cell killing and MTX resistance were increased over levels achieved with X-rays alone (Fig. 3). However, when X-ray-induced MTX resistance is expressed as a function of cell killing, either in the presence or absence of 3AB, the "isoeffect" curves are indistinguishable (Fig. 3A). This suggests that the same DNA damage that causes cell death also causes MTX resistance.

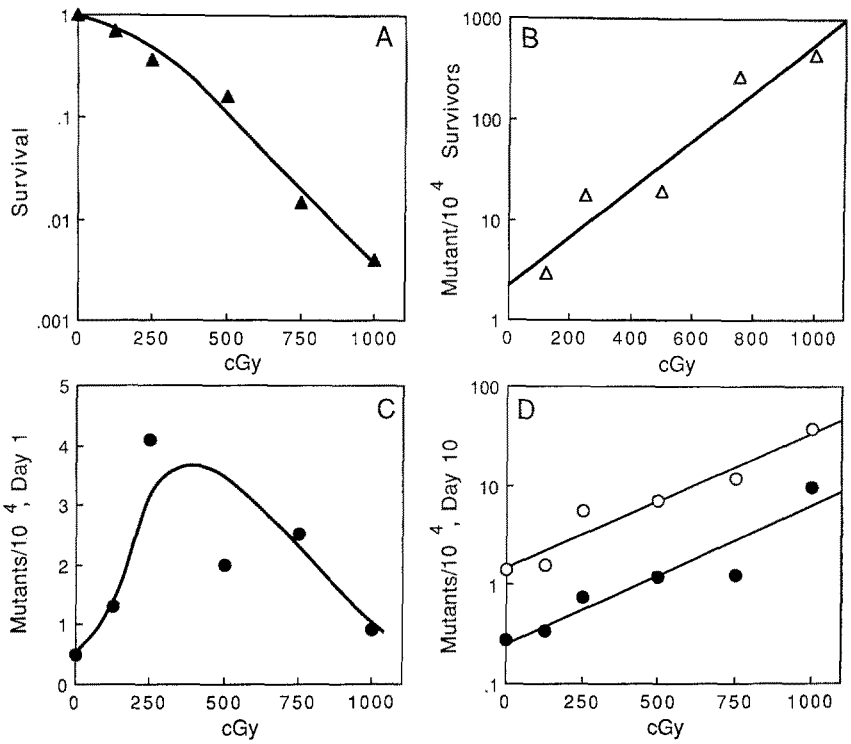

Fig. 2. Effect of radiation on survival, *dhfr* gene amplification, and *HPRT* gene deletion in EMT-6 cells. (A) Survival of cells one day after X-irradiation as a function of X-ray dose. (B) Frequency of MTX-resistant cells among the survivors. (C) Live cells with mutations to MTX resistance one day after irradiation, as a function of cells plated without regard to survival. (D) Frequency of MTX-resistant variants (O) and 8AG-resistant variants (●) after 10 days of subculture after irradiation to allow time for the expression of the $HPRT^-$ phenotype.

Double Minutes. Naturally MTX-resistant cells and MTX-resistant cells isolated after X-irradiation with 750 cGy were subjected to stepwise increases in MTX concentration up to 100 μM, at which time single colonies were isolated, raised to 160 μM MTX, and prepared for cytogenetic analysis. In all highly resistant populations, numerous double minutes were present in virtually every metaphase spread (Fig. 4).

Pulsed-Field Gel Electrophoresis. Because we were testing whether radiation-induced excision events could lead to gene amplification, we examined the MTX-resistant cells for evidence of amplified chromosomal fragments. We were looking for low numbers of small circular molecules in the single-colony isolates resistant to 150 nM MTX and many copies of larger molecules accompanying the double minutes in the highly resistant multicolony isolates resistant to 160 μM MTX. It is important to note that all of the isolates, although related, are quite different. They were all derived from the same original population of cells but through different routes. The isolates resistant to a low concentration of MTX (150 nM) were isolated as single-colony, single-step mutants, and they fall into two classes, irradiated and unirradiated. Presumably, most of the unirradiated MTX-resistant cells were in the population before selection, and most of the irradiated MTX-resistant cells were generated by the X-radiation itself (based on the induction frequency at 750 cGy). The isolates resistant to a high concentration of MTX (160 μM) were derived from the same original population but were subjected to several rounds of

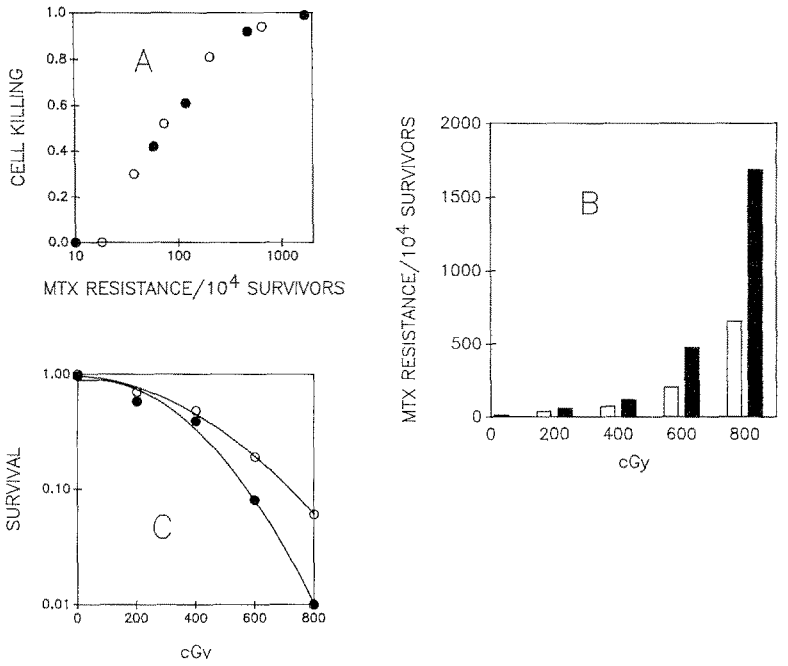

Fig. 3. Effect of interaction of 3AB and X-radiation on survival and MTX resistance in mouse EMT-6 cells. (A) Induction of MTX resistance as a function of cell killing by X-radiation in the presence (●) or absence (○) of 2 mM 3AB. (B) Induction of MTX resistance as a function of X-ray dose in the presence (■) or absence (□) of 2 mM 3AB. (C) Survival as a function of X-ray dose in the presence (●) or absence (○) of 2 mM 3AB.

selection, or to X-radiation before several rounds of selection, before single-colony isolation. These isolates were probably derived from the fastest growing or most resistant cells to survive 150 nM MTX (the first selection). We were able to locate DNA with large circular *dhfr* genes only in the isolates from the highly resistant irradiated population.

A large fraction of the *dhfr* genes were located on molecules that migrated as three "bands" in this size range. These were observed only after the isolated DNA had received further irradiation, indicating that they were probably circular molecules (Fig. 4). Both highly resistant single-colony isolates from the irradiated population showed similar size classes of molecules containing the *dhfr* gene, although in different proportions. They both contained species of approximately 1, 2, and 3 × 10^6 bp (Fig. 5). Figure 6 shows a blot of a pulsed-field gel run under conditions that would give maximum resolution around the size of the smallest "band." The highly resistant isolates from the unirradiated population contained double minute chromosomes (observed cytogenetically), but they were evidently too large to enter the gels under the conditions we used, which separated molecules between 2 × 10^2 and 7 × 10^6 bp.

We were unable to locate any circular double minutes in any of the 10 samples from the isolates resistant to 150 nM MTX. This does not mean that they were not there, only that we were unable to detect them, presumably because of their size or low copy number. We can detect only circular molecules with a single double-strand break, since unbroken circular molecules do not enter the gel and circles with more than one break migrate as random lengths. The maximum number of "single-hit" molecules will be determined by the size of the circle and will always be less than 100%. However, without knowing the

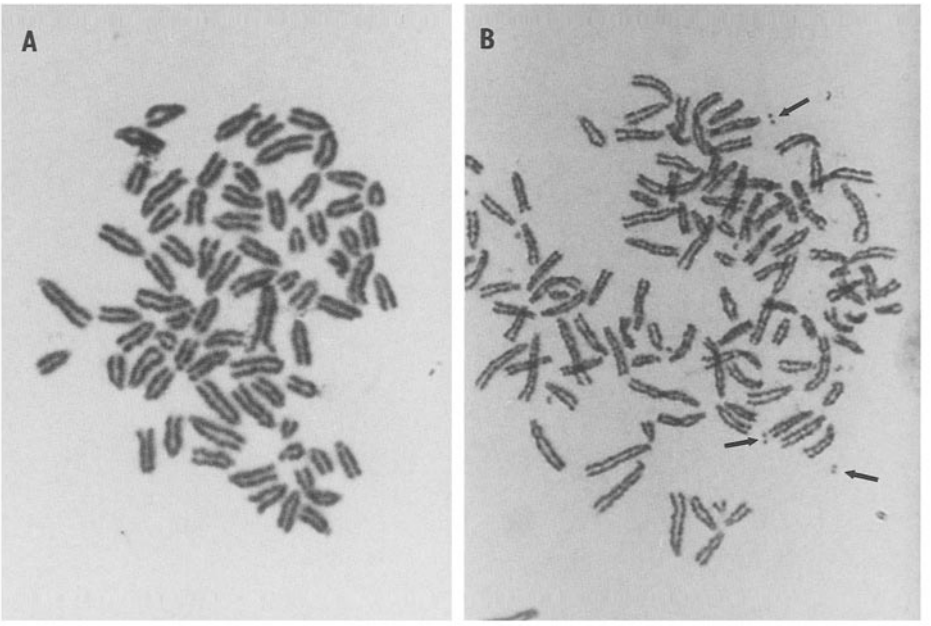

Fig. 4. Metaphase chromosome preparations from (A) normal EMT-6 cells and (B) EMT-6 cells resistant to 150 μM MTX. Arrows denote double minutes.

size in advance, one can optimize neither the amount of DNA-breaking X-radiation nor the electrophoretic separation conditions.

DISCUSSION

Evidence presented here suggests that chromosome aberrations, cell death, and MTX resistance are all due to the same X-ray-induced DNA damage. Both cell killing and MTX resistance were increased by X-rays in a dose-dependent manner, and both were potentiated by 3AB. This result has implications for the mechanism of gene amplification. Although the exact chemical nature of the lesions produced by X-rays remains obscure, DNA strand breakage is generally the result. Unrepaired double-strand breaks lead to chromosome aberrations, which generally lead to cell death. 3AB potentiates the cytogenetic damage (27). Stable rearrangements leading to mutations, principally translocations and large deletions, have been observed after X-irradiation, and X-ray-induced base substitutions have been seen in some systems (33, 34). Because most of the measurable genetic effects (including cell killing) of X-radiation are related to misrejoining or nonrejoining of DNA double-strand breaks, it seems reasonable to infer that the gene amplification that follows X-irradiation is also due to misrejoining or nonrejoining of double-strand breaks. In agreement with this hypothesis are the observations of Cavolina et al. (35), who found that the restriction endonucleases PvuII, BamHI, and EcoRI, which make only DNA double-strand breaks, induced *N*-phosphonacetyl-L-aspartate resistance in Chinese hamster cell clones due to amplification of the *CAD* gene.

Amplification of the *dhfr* gene appears to behave like a simple mutation. The relationship between X-ray dose and mutant yield followed the simple model of Haynes and Eckardt (36) for a linear-quadratic cell survival curve and a linear mutation induction

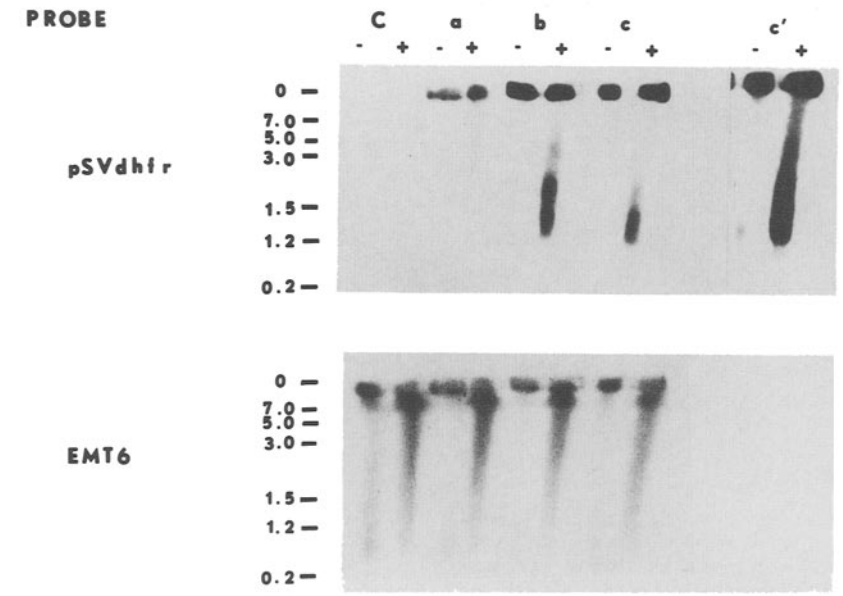

Fig. 5. Pulsed-field gel electrophoresis of DNA from cells containing double minutes. Wild-type DNA (C) and DNA from naturally highly resistant (a) and radiation-induced highly resistant (b, c) isolates of EMT-6 cells were electrophoresed without irradiation (−) or immediately after irradiation with 4000 cGy (+) to break circular molecules. Some DNA (c') was exposed for a longer time to show small numbers of double minutes in the absence of irradiation. The gel was probed first with a *dhfr*-specific probe, then stripped and reprobed with a nonspecific DNA probe. The positions of molecular weight standards (in millions of base pairs) are indicated at the left; they represent the three chromosomes of *S. pombe* and the two largest plus the smallest chromosomes of *S. cerevisiae*. O = origin of plug.

curve. This strongly suggests that X-ray-induced DNA damage directly caused the amplification of the *dhfr* gene.

Our results are consistent with the observations of Carroll et al. (4), who demonstrated that deletions can lead to the formation of double minutes. A simple explanation for the presence of the radiation-induced double minutes in our experiments is that the radiation caused an $\sim 10^6$ bp deletion that included the *dhfr* gene, which circularized. Selection for retention of the acentric circular fragment, coupled with unequal segregation at mitosis, then led to amplification. The 2×10^6 and 3×10^6 bp species observed by pulsed-field gel electrophoresis could have arisen by dimerization. There is ample precedence for this possibility. Rings are commonly observed chromosome aberrations that result from radiation and other DNA-damaging agents. Morgan et al. (37) have shown that hydroxyurea-induced ring chromosomes persist in the population even in the presence of agents that increase sister chromatid exchanges, which would be expected to lead to interlocked and unresolvable structures. At the other end of the size scale, Carroll et al. (4, 14) and Ruiz et al. (12) have observed numerous persistent circular molecules of $<10^6$ bp in cells containing double minutes.

This study was designed to establish a role for chromosome fragmentation in gene amplification. What we have shown is a high correlation between X-irradiation, cell killing, and induction of *dhfr* gene amplification. Although there is little doubt that X-radiation-induced cell killing is mediated primarily by chromosome fragmentation, some caution must be observed in concluding that the same activity leads directly to gene amplification in the survivors. Several months elapsed between the X-irradiation/MTX plating and the anal-

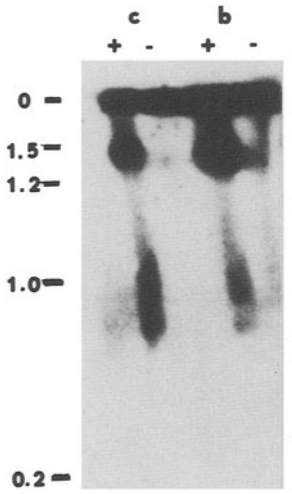

Fig. 6. Pulsed-field gel electrophoresis of DNA containing the smallest double minutes from Fig. 5 (b and c). Markers at the left of the gel indicate the three largest chromosomes of *S. cerevisiae*. O = origin of plug.

ysis of DNA from the populations derived from the surviving cells. It is possible, for instance, that other X-ray effects such as membrane transport alterations (38) could lead to very slow growing survivors, and a subsequent amplification event (perhaps induced by MTX) could lead to a rapidly growing subclone whose DNA is analyzed in the Southern blots. On the other hand, double-minute chromosomes must become involved at some point, and X-radiation is known to readily induce chromosome aberrations that are cytogenetically indistinguishable from double minutes containing amplified genes. The simplest explanation is that the X-radiation leads directly to double-minute formation.

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