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Carcinogen-Mediated Methotrexate Resistance and Dihydrofolate Reductase Amplification in Chinese Hamster Cells

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We have investigated different parameters characterizing carcinogen-mediated enhancement of methotrexate resistance in Chinese hamster ovary (CHO) cells and in simian virus 40-transformed Chinese hamster embryo (C060) cells. We show that this enhancement reflects dihydrofolate reductase (*dhfr*) gene amplification. The carcinogens used in this work are alkylating agents and UV irradiation. Both types of carcinogens induce a transient enhancement of methotrexate resistance which increases gradually from the time of treatment to 72 to 96 h later and decreases thereafter. Increasing doses of carcinogens decrease cell survival and increase the enhancement of methotrexate resistance. Enhancement was observed when cells were treated at different stages in the cell cycle, and it was maximal when cells were treated during the early S phase. These studies of carcinogen-mediated *dhfr* gene amplification coupled with our earlier studies on viral DNA amplification in simian virus 40-transformed cells demonstrate that the same parameters characterize the amplification of both genes. Possible cellular mechanisms responsible for the carcinogen-mediated gene amplification phenomenon are discussed.

Although environmental agents have been shown to play an important role in the initiation of most human cancers, little is known about the molecular mechanisms underlying their action. Tumorigenesis is accompanied by a variety of molecular processes including DNA rearrangements (18, 21), oncogene activation (2) and amplification (5, 24, 25), and the appearance of double-minute chromosomes and homogeneously staining regions containing amplified DNA sequences (7, 10, 16).

In recent years we studied the effect of chemical and physical carcinogens on one of these cancer-related phenomena, namely, gene amplification, in an attempt to establish its possible role in the initiation events of carcinogenesis. To study gene amplification immediately after exposure to the carcinogens rather than after the long process of establishing cell lines containing amplified genes, we have constructed an experimental model system consisting of simian virus 40 (SV40)-transformed Chinese hamster cells (C060) (12). In this system SV40 amplification can be monitored by molecular hybridization. Exposure of these cells to a variety of chemical and physical carcinogens has been shown to result in the amplification of SV40 DNA sequences. This amplification requires an active origin of replication and a functional T antigen. The amplification phenomenon is transient, reaching a maximal level on the third and fourth day after treatment with carcinogen, and disappears within a few more days (12, 14, 15). Exposure to carcinogens is followed by a prolonged S phase. Treatment of a synchronized population of cells obtained by isoleucine starvation results in increased SV40 amplification (Y. Berko and S. Lavi, manuscript in preparation).

Further studies in our laboratory revealed transient amplification of additional DNA sequences including the *dhfr* and *ras*^{H} genes in response to carcinogen treatment (15). However, due to the low level of amplification and its transient nature, it was difficult to study further details of this process by direct hybridization to DNA from carcinogen-treated cells.

Enhancement of methotrexate (MTX) resistance and *dhfr* gene amplification in mouse cells treated with carcinogens was studied by Varshavsky (29) and Tlsty et al. (27, 28). The parameters affecting *dhfr* gene amplification in the mouse system varied from those observed by us for transient SV40 amplification in Chinese hamster cells. In the present study we have investigated the carcinogen-mediated amplification of the *dhfr* gene in Chinese hamster ovary (CHO) cells and in SV40-transformed Chinese hamster C060 cells in greater detail by using the same approach used by Tlsty et al. (28). Specifically, establishment of stable cell lines containing the amplified *dhfr* gene was used as a handle to follow the different parameters affecting the initiation of *dhfr* amplification. The results of these studies reveal that carcinogen-mediated *dhfr* amplification and SV40 amplification in Chinese hamster cells share common characteristics.

MATERIALS AND METHODS

Cell culture. CHO and C060 cells were grown in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Biolabs, Jerusalem).

Plating efficiencies were determined by seeding 100, 500, and 1,000 cells in 5-cm petri dishes (Nunc, Roskilde, Denmark) in the above medium. Seven days later the colonies were fixed with methanol, stained with 10% Giemsa, and counted.

Selection of MTX-resistant colonies was done as follows. Cells were trypsinized, and 5×10^5 cells were plated in 9-cm petri dishes in medium containing 10% dialyzed fetal calf serum and 0.4 μM MTX for CHO cells or 0.3 μM MTX for C060 cells (the 50% lethal dose for CHO cells is 0.042 μM MTX, and that for C060 cells is 0.035 μM). The medium was changed three times every 3 days. Seventeen to twenty-one days after the initial seeding, colonies were either fixed, stained, and counted or picked by scratching and reseeded.

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When about 10^6 cells were obtained, half of the cells were collected, frozen in 10% dimethyl sulfoxide, and stored at −90°C. The other half was further grown, and cells were collected for determination of *dhfr* gene content.

Synchronization of CHO cell population. Synchronization was performed by a slight modification of the procedure of Mariani and Schimke (17). CHO cells were plated in 150-mm tissue culture flasks (Nunc), 30 × 10^6 cells per flask. Twelve hours later the medium was removed, the cells were washed with phosphate-buffered saline, and 10 ml of prewarmed medium was added. The flasks were then tapped three or four times, and the medium containing the mitotic cells was collected. These cells were plated in 3.5-cm petri dishes for analysis in the fluorescence-activated cell sorter or in 9-cm petri dishes for later treatment. Thirty minutes later the medium was changed again to remove dead and nonadhered cells.

Carcinogen treatment. Cells were plated in 5 ml of medium plus 10% fetal calf serum (10^6 cells per 9-cm petri dish), and the carcinogen treatment was given 24 h later. *N*-Methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG, Aldrich Chemical Co., Inc., Milwaukee, Wis.) was freshly dissolved in dimethyl sulfoxide and added to the medium at the required concentration. Ethyl methanesulfonate (EMS, Eastman Chemical Products, Inc., Kingsport, Tenn.) was added directly to the medium. The cells were then incubated in darkness for the appropriate length of time. For UV irradiation, cells were plated at 5 × 10^5 cells per 5-cm petri dish. Before irradiation the medium was removed, and 1 ml of phosphate-buffered saline was added. The cells were exposed to irradiation at 254 nm for the appropriate length of time to achieve the desired fluence. The phosphate-buffered saline was then replaced again by medium. The 50% lethal dose of UV irradiation of CHO cells is 25 J/m^2.

Determination of *dhfr* gene amplification. DNAs were prepared by a slight modification of the procedure of Brown et al. (4). Cells were grown in medium containing 0.05 μCi of [^{14}C]thymidine (Amersham, England) per ml; 2 × 10^5 cells per sample were pelleted and frozen. When all samples were collected, the pellets were thawed and lysed in 10 μl of 10 mM Tris (pH 8.0)–10 mM EDTA–100 mM NaCl–0.2% sodium dodecyl sulfate at 37°C for 15 min. A 90-μl sample of the same buffer without sodium dodecyl sulfate was then added together with RNase A (DNase free; Worthington Diagnostics, Freehold, N.J.) at a final concentration of 20 μg/ml. After 1 h of incubation at 37°C, proteinase K (E. Merck AG, Darmstadt, Federal Republic of Germany) was added to a final concentration of 100 μg/ml, and the mixture was further incubated for 2 h at 37°C. Slot blots were prepared from these samples by the NaI method of Bresser et al. (3). Slot blots of samples labeled by [^{14}C]thymidine were exposed to Kodak XAR-5 X-ray film overnight. After hybridization to ^{32}P-labeled DNA probes, those blots were autoradiographed with two sheets of paper inserted between them and the X-ray film. The paper prevents exposure of the film to the irradiation emanating from the ^{14}C but not to that from the ^{32}P label (17). The *dhfr* cDNA probe (pDHFR11) was a gift of R. T. Schimke (20). Quantitation of the exposed slots was determined with a computerized densitometer.

High-molecular-weight DNA was extracted from MTX-resistant clones as described previously (12). DNA (35 μg) digested by *Eco*RI (Biolabs) was separated on a 1% agarose gel, transferred to nitrocellulose filters, and hybridized to a nick-translated *dhfr* cDNA probe. The hybridization conditions were 35% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.2), 1× Denhardt solution (1× Denhardt solution is 0.02% [wt/vol] each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 20 mM phosphate buffer (pH 7.0), 150 μg of sonicated salmon sperm DNA per ml, and 10% dextran sulfate. Hybridization was performed at 42°C for 40 h. Filters were washed twice with 2× SSC–0.5% sodium dodecyl sulfate–20 mM phosphate buffer (pH 7.0) at 42°C for 30 min, followed by four 1-h incubations in 0.1× SSC–0.1% sodium dodecyl sulfate at 50°C.

RESULTS

Increased MTX resistance in CHO cells treated with carcinogens reflects *dhfr* gene amplification. The drug MTX inhibits DNA replication by binding to the enzyme dihydrofolate reductase, thus preventing the synthesis of purines, thymidilate, and glycine, leading to eventual cell death (6). Amplification of the *dhfr* gene has been shown to result in increased resistance to MTX. However, other mechanisms can cause enhanced resistance to the drug, including altered transport or reduced affinity of the dihydrofolate reductase enzyme to MTX (22).

To investigate whether the carcinogen-mediated enhanced MTX resistance reflects *dhfr* gene amplification, the *dhfr* gene copy number was monitored in MTX-resistant colonies originating from control and carcinogen-treated Chinese hamster cells. If a similar proportion of colonies containing amplified *dhfr* sequences occurred in the two groups of MTX-resistant cells, then an increase in the number of MTX-resistant colonies after treatment with a carcinogen would represent an increased frequency of *dhfr* gene amplification. Slot-blot hybridization was used to estimate the relative levels of *dhfr* gene amplification. Cells were prelabeled with [^{14}C]thymidine, DNA was extracted, and slot blots were prepared as described in Materials and Methods. The blots were autoradiographed to determine the ^{14}C signal, which represents the amount of DNA on the filter. They were then hybridized to a ^{32}P-labeled *dhfr* cDNA probe and exposed to a film under two sheets of paper, which eliminated the ^{14}C signal (17). The relative amount of *dhfr* sequences in various MTX-resistant clones was established by determining the ratio of ^{32}P and ^{14}C signals; we define this ratio as 1.0 for the parental MTX-sensitive CHO cells. The sensitivity of the procedure was demonstrated in reconstruction experiments (Fig. 1A). Different proportions of ^{14}C-prelabeled cells from the MTX-sensitive CHO cell line and from cell lines resistant to 0.5 μM MTX containing 15- or 5-fold amplification of *dhfr* were mixed and processed as described above. Figure 1A, lane a, shows the ^{14}C signal, and Fig. 1A, lane b, shows the hybridization of the ^{32}P-labeled *dhfr* probe. The ratios between the hybridization and ^{14}C signals (Fig. 1A, lane c) were in agreement with the expected values of the *dhfr* gene copy number in each slot (Fig. 1A, lane d). Low copy numbers of *dhfr* were underestimated by the procedure. The extent of *dhfr* amplification was monitored in MTX-resistant clones obtained after exposure of CHO cells to 1 μg of MNNG per ml (Fig. 1C) and from untreated control cells (Fig. 1B). Similar proportions of clones containing amplified *dhfr* sequences were present among the MTX-resistant colonies derived after MNNG treatment and in those obtained spontaneously (Fig. 1). The amplification varied between 2- and 10-fold in the different MTX-resistant cell lines. Thus carcinogen-induced amplification of *dhfr* sequences in treated cells can be studied by measuring the enhancement in appearance of MTX-resistant colonies in this population as compared with their appearance in untreated cells.

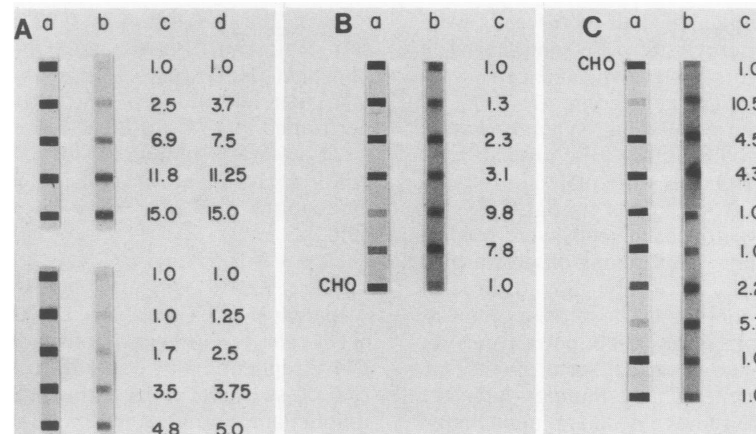

FIG. 1. *dhfr* gene amplification in MTX-resistant colonies. DNA was extracted from 2×10^5 cells prelabeled with 0.05 μCi of [^{14}C]thymidine per ml, and slot blots were prepared as described in Materials and Methods. The filters were hybridized to a ^{32}P-labeled *dhfr* cDNA probe. Lanes: a, ^{14}C signal; b, ^{32}P hybridizaton; c, *dhfr* copy number; d, expected *dhfr* copy number in the reconstruction experiments. A, DNAs from reconstruction experiments in which CHO cells and MTX-resistant cells containing 15- or 5-fold amplification of *dhfr* were mixed at ratios of 1:0, 0.75:0.25, 0.5:0.5, 0.25:0.75, and 0:1. B and C, DNAs extracted from MTX-resistant clones (grown in 0.4 μM MTX) derived from untreated control cells (B) and cells treated with 2 μg of MNNG per ml for 2 h (C).

Enhancement of MTX resistance in CHO cells treated with MNNG is dose dependent. CHO cells were treated with increasing concentrations of MNNG for 2 h and then introduced into medium containing MTX at 15 or 72 h after the treatment. Figure 2A shows the percentage of surviving cells corresponding to each dose of MNNG as measured by colony formation. Figure 2B shows the enhancement in MTX resistance as a function of decreasing cell survival. Enhancement is defined as the increase in the number of MTX-resistant colonies in the MNNG-treated culture over the number in the nontreated culture, normalized for the reduced viability of cells after the treatment. The enhancement in MTX resistance increased as cell survival decreased (Fig. 2B). To prove that we have not selected cells with increased resistance to MNNG as well as to MTX, we grew cells from a few MTX-resistant colonies and measured their plating efficiencies after treatment with carcinogens. The MTX-resistant clones obtained from carcinogen-treated cells had the same sensitivity to the carcinogens as have the parental CHO cells.

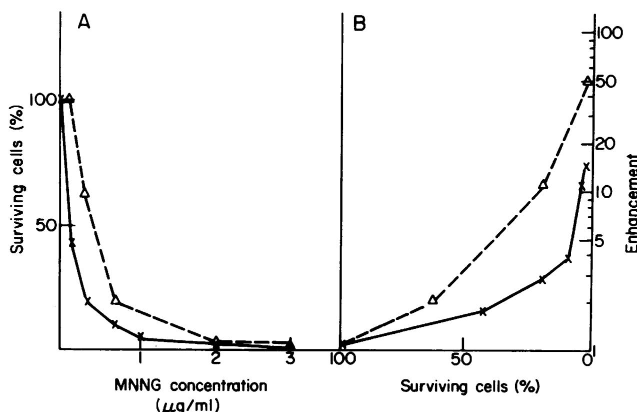

FIG. 2. Incidence of MTX resistance at different MNNG concentrations. CHO cells in 9-cm petri dishes were treated with different concentrations of MNNG for 2 h. At 15 (×) and 72 (△) h later these cells were trypsinized, and samples of 0.5×10^6 cells were plated per 9-cm dish in medium containing MTX. The selection procedure was carried out as described in Materials and Methods. Five or six plates were plated for each point.

An additional conclusion arising from Fig. 2 is that the enhancement of MTX resistance increases with time after the carcinogen treatment. The enhancement was higher when cells were placed into MTX-containing medium at 72 h posttreatment than when they were placed into selective medium at 15 h posttreatment, irrespective of the level of cell survival. For example, at 20% survival a threefold enhancement was observed when MTX selection was applied 15 h after the treatment, whereas at a similar survival level a 12-fold enhancement was observed when the selection was applied 72 h after the treatment.

Enhancement of MTX resistance in CHO and CO60 cells treated with alkylating agents is transient. To investigate in more detail the kinetics of acquisition of MTX resistance, the effect of MNNG and EMS on the enhancement was measured as a function of time posttreatment (Fig. 3). CHO cells were treated with MNNG (2 μg/ml) for 2 h or with EMS (2 μl/ml) for 30 min; cell survival in the first 3 days, as measured by colony formation, was 12 and 40% of control values, respectively. At later times the survival rates increased. At various times after treatment, control and treated cells were placed into medium containing 0.4 μM MTX. Under these selective conditions 10 ± 3 spontaneous MTX-resistant colonies were found per 5×10^5 cells. Placing the cells into MTX selection 72 h posttreatment resulted in maximal enhancement of MTX resistance for both alkylating agents used (15-fold for EMS and 10-fold for MNNG; Fig. 3). With increasing time enhancement was decreased. At 6 days after EMS treatment the enhancement of MTX resistance fell to the level of untreated cells, whereas residual enhancement was observed even 8 days after MNNG treatment.

A similar transient effect was demonstrated upon treatment of SV40-transformed Chinese hamster embryo cells (C060) with MNNG (3 μg/ml) for 2 h (Table 1). Cell survival at different intervals after the treatment was about 10% of control values. At various times after the treatment the cells were subjected to MTX selection (0.3 μM). Enhancement was already observed on the second day after the treatment (72-fold). However, on the third and fourth days the en-

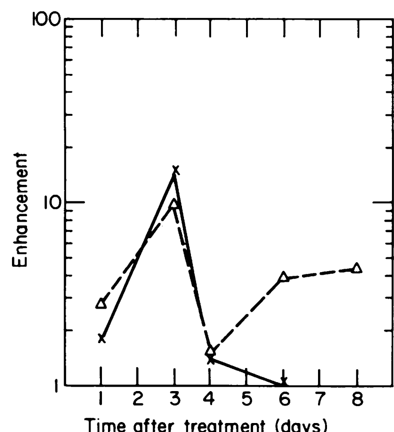

FIG. 3. Time course of enhancement in MTX resistance after treatment with alkylating agents. CHO cells were treated with 2 μg of MNNG per ml for 2 h (△) or with 2 μl of EMS per ml for 30 min (×). At various times after the treatment the cells were plated into medium containing MTX, and selection was carried out as described in Materials and Methods. Five plates were plated for each point.

hancement reached its peak of 371- and 361-fold, respectively; transient enhancement in MTX resistance was obtained in other experiments (data not shown) which also showed the decline in the enhancement after the fourth day. In parallel SV40 amplification was assayed by slot-blot hybridization (data not shown). As previously reported SV40 amplification was transient. Slight amplification was observed already on the first day after treatment. SV40 amplification reached its maximal value on the third day after treatment and decreased thereafter. As in CHO cells, the enhancement of MTX resistance in the C060 cell line reflects the amplification of *dhfr* sequences. Figure 4 presents Southern analysis of the parental MTX-sensitive C060 cells and of 15 independent MTX-resistant C060 subclones derived after MNNG treatment of C060 cells. A dramatic amplification of *dhfr* sequences was observed in eight subclones. Ethidium bromide staining of the gel (data not shown) demonstrated that the quantity of DNA chromatographed in each slot was identical. A significant increase in the hybridization to the *dhfr* probe was detected in several DNA fragments derived from MTX-resistant subclones. The pattern and intensity of hybridization to the *dhfr* probe were not identical for all the subclones with amplified *dhfr* sequences. It should be noted that SV40 amplification was observed in all 15 MTX-resistant subclones (T. Kleinberger and S. Lavi, manuscript in preparation).

Kinetics of MTX resistance in CHO cells irradiated by UV. To establish that a transient enhancement is not a phenomenon typical only of alkylating agents, CHO cells were UV irradiated. The dose of irradiation used (63 J/m^2) resulted in survival rates of 12 to 18% in the first 40 h and 30 to 45% at 60 to 135 h postirradiation. Figure 5 shows the enhancement of MTX resistance at various times after treatment. Maximal enhancement (150-fold) was found when cells were placed in MTX-containing medium at 87 h after irradiation, and it decreased thereafter. This result is similar to that found with alkylating agents.

Enhancement of MTX resistance is maximal when the carcinogenic treatment is given at the beginining of the S phase. Studies in our laboratory demonstrated that treatment of synchronized C060 cells with carcinogens resulted in increased SV40 amplification (Berko and Lavi, in preparation). To investigate the dependence of enhancement of MTX resistance on the time of treatment during the cell cycle, carcinogen-mediated amplification was studied in a synchronized population of CHO cells obtained by mitotic selection. At various times after the mitotic selection cells were treated with MNNG (2 μg/ml) for 30 min. Concomitantly, a sample was taken for analysis in the fluorescence-activated cell sorter to determine the position of the cells in the cell cycle. Seventy-two hours later MTX selection was applied to the cells. Untreated control cells gave an average of 1.8 MTX-resistant colonies per 5 × 10^5 viable cells. MNNG treatment during G1 (at 0.5 and 6 h after mitotic selection), late S, and G2 (12 and 14 hours after selection) gave approximately equal numbers of MTX-resistant colonies, i.e., an average of 11.8 per 5 × 10^5 viable cells (Fig. 6). However, treatment at the early S stage (8 and 10 h after the mitotic selection) resulted in a higher number of MTX-resistant colonies, i.e., 26.6 per 5 × 10^5 viable cells. This increase in the number of MTX-resistant colonies is statistically significant. A nonsynchronized population of cells treated at the same time yielded an average of 19.8 MTX-resistant colonies per 5 × 10^5 viable cells. These results indicate that the carcinogenic treatment induces twice as much amplification when it is given in the early S phase compared with treatment at other stages in the cell cycle.

DISCUSSION

Previous studies in our laboratory with viral amplification in SV40-transformed Chinese hamster embryo cells (C060) as a model system to investigate carcinogen-mediated gene amplification demonstrated that both chemical and physical carcinogens induce transient SV40 amplification. Cellular genes such as *dhfr* and *ras*^{H} were also transiently amplified in the C060 cells (15) but to a lesser extent, thus hindering detailed studies on their amplification.

In the experiments described in this report *dhfr* gene amplification was studied by measuring the increase in the number of MTX-resistant colonies. The finding that the frequency of MTX-resistant colonies containing amplified *dhfr* sequences is similar in spontaneously resistant colonies and in colonies obtained after treatment with carcinogens

TABLE 1. Enhancement of MTX-resistant (MTX^r) colonies in MNNG-treated, SV40-transformed C060 cells[a]

Day	Cells	MTX^r colonies/ 5 × 10^5 seeded cells	Survival rate	Avg MTX^r colonies/5 × 10^5 viable cells	Enhancement of MTX^r
0	Control	2, 3, 3	1.0	2.7	
	Treated	0, 0, 0, 0	0.06	ND	ND
1	Control	10, 11, 2, 5	1.0	7	
	Treated	6, 0, 0, 2	0.1	20	3
2	Control	2, 0, 0, 1	1.0	0.75	
	Treated	5, 10, 6	0.13	54.0	72
3	Control	2, 0, 1, 1	1.0	1.0	
	Treated	19, 42, 6, 22	0.06	370.8	371
4	Control	1, 0, 0, 3	1.0	1.0	
	Treated	14, 35, 49, 32	0.09	361.1	361

[a] C060 cells were treated with 3 μg of MNNG per ml for 2 h. At various times after the treatment the cells were plated into medium containing 0.3 μM MTX. The selection procedure was carried out as described in Materials and Methods. ND, Not determined.

justifies the employment of this strategy (Fig. 1) (28, 29). This approach to the investigation of gene amplification by following the selected expression of the *dhfr* gene permits determination of the parameters associated with the initiation of amplification by carcinogens even when it occurs in only a fraction of the population. However, amplification of part of the *dhfr* gene which is not followed by increased expression of the *dhfr* protein will not be detected by this procedure.

DHFR gene amplification was monitored by slot-blot hybridization (Fig. 1) for CHO cells and by Southern blot analysis (Fig. 4) for C060 cells. The extent of amplification was found to be 2- to 15-fold.

Although direct selection was not applied to maintain SV40 amplification in the SV40-transformed C060 cells, amplified SV40 sequences are present in most MTX-resistant clones. The SV40 persisting amplification might result from the presence of MTX, which constantly activates SV40 amplification (13), during the selection of resistant clones. In addition, SV40 T antigen might enhance *dhfr* expression, as was shown for polyoma virus T antigen (8), thus facilitating enhanced MTX resistance in cells containing amplified SV40 sequences. The presence of T antigen in C060 cells might also account for the higher level of enhancement of MTX resistance in these cells compared with that in CHO cells.

Common parameters were found to characterize carcinogen-mediated SV40 amplification and *dhfr* amplification in Chinese hamster cells. The initial *dhfr* amplification induced by the alkylating agents (MNNG in CHO and C060 cells and EMS in CHO cells) and by UV irradiation (in CHO cells) is transient and can be maintained only upon application of selective conditions. Low enhancement of MTX resistance is observed as early as 15 h after the carcinogen treatment (Fig. 2 and 5). The enhancement increases gradually and reaches maximal levels at the third and fourth days after the treatment (Fig. 3 and 5; Table 1). At later times the enhancement of amplification is reduced. Similarly temporary amplification was shown for SV40 by hybridization to DNA

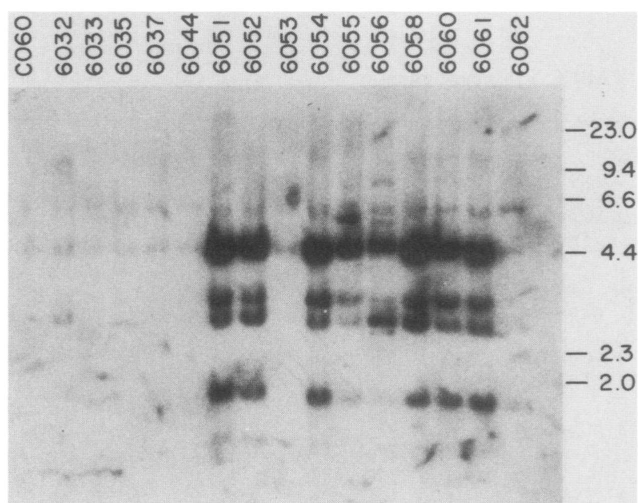

FIG. 4. *dhfr* amplification in MTX-resistant C060 subclones. DNA was extracted from MTX-resistant colonies derived from MNNG-treated C060 cells and selected on 0.3 μM MTX. DNA (35 μg) was digested by *Eco*RI, separated on 1% agarose gel, and hybridized to the ^{32}P-labeled *dhfr* cDNA probe (10^8 cpm/μg).

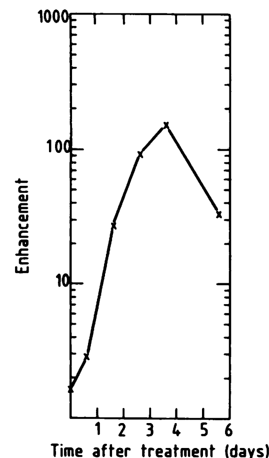

FIG. 5. Enhancement of MTX resistance after UV irradiation as a function of time. CHO cells were UV irradiated with 63 J/m^2. At various times after the treatment the cells were plated into medium containing MTX, and the selection procedure was carried out as described in Materials and Methods. Ten plates were plated for each point.

extracted from cells at different times after carcinogen treatment (12, 14). The extended period of the enhanced amplification after the treatment with carcinogens and the gradual increase in the enhancement values can be attributed to various mechanisms. These include repeated activation of the amplification machinery due to persisting adducts, accumulation of cellular factors responsible for *dhfr* and SV40 amplification, and a prolonged expression time of the functions responsible for gene amplification.

As with SV40 amplification (14), carcinogen-mediated enhancement in MTX resistance is dose dependent. Increasing doses of carcinogens decrease the survival rates and induce higher enhancement of MTX resistance. Treatment by different agents resulting in a similar survival rate yielded different degrees of enhanced MTX resistance. For example, 12% survival corresponded to enhancement of 10- to 20-fold after MNNG treatment (Fig. 2 and 3) and 150-fold after UV irradiation (Fig. 5). This can be attributed to the fact that the different agents affect the cells through different pathways or to the varied speed of repair of the different lesions.

Using a similar strategy, Tlsty et al. (28) investigated UV-induced *dhfr* gene amplification in 3T6 mouse cells. The mouse 3T6 cells and the Chinese hamster CHO cells responded differentially to UV. The time kinetics of the enhancement was different in the two systems; in the mouse cells maximal enhancement was observed between 12 and 24 h after irradiation, whereas in CHO cells the enhancement peaked between 63 and 87 h after the treatment. Moreover, in mouse cells a UV fluence, which is only slightly toxic to the cells (resulting in 70 to 80% survival), induced maximal enhancement. Higher fluences of irradiation, which decreased cell survival, induced lower levels of enhancement. The CHO cells responded in a different manner. Increasing doses of MNNG yielded higher toxic effects and induced enhanced *dhfr* amplification. The different characteristics of *dhfr* amplification in these two cell lines can be attributed to variations in the response of the cells to DNA damage. Initial experiments indicate that the CHO cells used in our experiments are repair deficient (Waldstein and Lavi, unpublished results). Such a deficiency might be associated with a prolonged persistence of DNA lesions leading to repeated

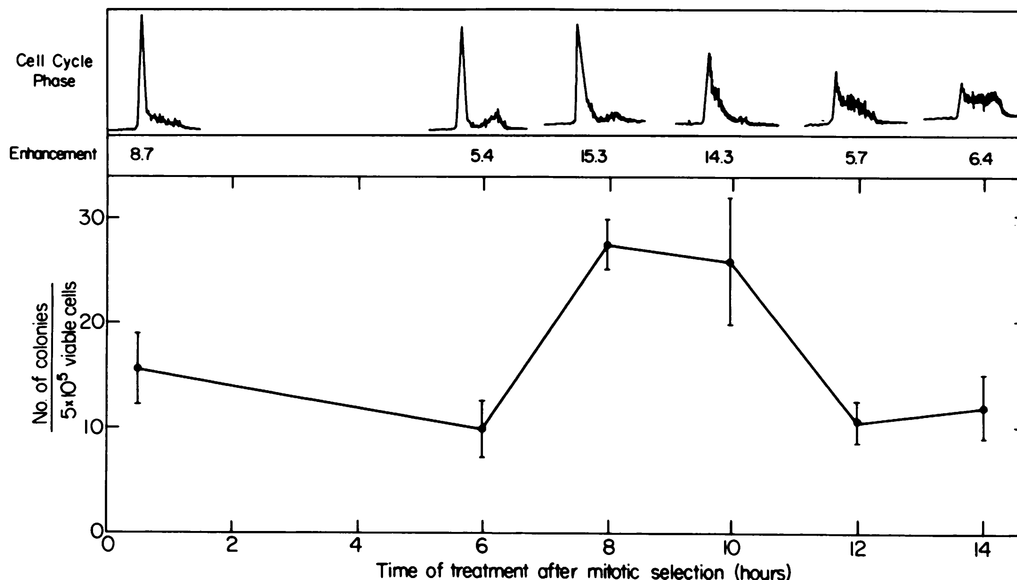

FIG. 6. Incidence of MTX resistance during different cell cycle stages. CHO cells were synchronized by mitotic selection. At various times during the cell cycle, 10^6 cells per 9-cm plate were treated with 1 μg of MNNG per ml for 30 min. A sample of cells was taken for analysis in the fluorescence-activated cell sorter; 72 h later the cells were trypsinized, and 0.5×10^6 cells per 9-cm plate were plated in medium containing 0.4 μM MTX. Four to six plates were plated for each point, and the regular selection procedure was then carried out as described in Materials and Methods. The survival rates of the cells were 13 to 16% throughout the cell cycle. Untreated control cells gave rise to 1.8 colonies per 5×10^5 cells. The number of colonies was normalized to the survival rates. The cell cycle phase of the population and the enhancement in MTX resistance are illustrated for each time point. The bars represent the standard error of the mean.

activation of the cellular machinery responsible for the amplification process. Alternatively, cellular events which are induced by the carcinogens might develop more slowly in CHO cells compared with mouse 3T6 cells. We are currently investigating the correlation between the presence of DNA adducts and DNA amplification.

Another parameter influencing the carcinogen-mediated *dhfr* amplification is its cell cycle dependence (Fig. 6). Cells treated at G1, late S, and G2 display enhanced MTX resistance (ca. 6.5-fold); however, maximal enhancement (ca. 14.8-fold) was obtained upon exposure of cells to carcinogens in the early S phase. Similarly enhanced SV40 amplification was demonstrated in synchronized cells.

The finding that enhanced *dhfr* gene amplification is not restricted to a specific stage of the cell cycle demonstrates that DNA damage throughout all phases of the cell cycle leads to enhanced gene amplification. However, cells which are in the early S phase are twice as competent for gene amplification, suggesting that the events controlling *dhfr* gene amplification are triggered at a specific time during the cell cycle, possibly at the beginning of the S phase. Studies by Mariani and Schimke (17) on the effect of hydroxyurea, an inhibitor of DNA replication, on *dhfr* amplification in synchronized cells revealed dramatic enhancement of MTX resistance in cells treated early in the S phase.

Our findings that carcinogen-induced *dhfr* and SV40 amplification share common characteristics suggest that both amplification processes are controlled by the same cellular machinery. Data have been accumulating to the effect that DNA-damaging agents and other substances which inhibit DNA synthesis induce viral and *dhfr* amplification (1, 4, 12, 14, 15, 27–29). However, the cellular mechanisms governing the induction of the amplification process are still unknown. Since this process is transient (12, 14, 15, 28) and can be induced by nonmutagenic agents, it is unlikely that mutations that permanently affect control of initiation of DNA synthesis are responsible for its induction. In bacteria, SOS repair can cause reinitiation of DNA replication at the legitimate chromosomal origin of replication (9) or at illegitimate sites (26). The involvement of an origin of replication in the amplification process mediated by carcinogens was demonstrated for SV40 amplification in Chinese hamster cells (14, 15). It is possible that DNA perturbations such as carcinogen adducts, thymidine dimers, and DNA breaks may cause premature termination of replication forks, and the arrest of DNA replication might lead to activation of events controlling the initiation of DNA replication (30). Carcinogens may induce or stabilize processes preceding DNA replication such as transcription of regulatory genes involved in the entrance to the S phase and in initiation of DNA replication. Thus, increased expression of specific genes and repeated DNA replication at specific sites might occur after carcinogen treatment. Indeed, increased synthesis of T antigen, a protein required for SV40 replication, occurs immediately after the exposure to carcinogens, in the absence of SV40 DNA replication (Berko et al., manuscript in preparation).

The induction and involvement of cellular factors in the amplification process might not require direct association between the sites of DNA perturbations and the chromosomal location of the amplified regions. Thus induction of amplification might occur in *trans*. This possibility is supported by the work of Nomura and Oishi (19), who have shown that UV irradiation of African green monkey cells induces replication of SV40 sequences in SV40-transformed Syrian hamster cells upon fusion. Similarly, Lambert et al. (11) reported indirect induction of polyomavirus DNA replication in polyomavirus-transformed rat cells. Recent studies in our laboratory indicate that in vitro replication of SV40 DNA is enhanced in cell extracts derived from carcinogen-treated cells (Berko and Lavi, in preparation). Cellular factors induced by UV irradiation were recently described

by Schorpp et al. (23). Alternatively, it is possible that unterminated DNA replication forks induce local amplification. Such amplification would be restricted only to the perturbed replicons. Amplification can occur by either one of these two mechanisms or by a combination of both.

Recently we have shown that carcinogen-induced amplification is found also in *Saccharomyces cerevisiae* (Aladjem, Koltin, and Lavi, submitted for publication). The *CUPI* gene is amplified in yeast cells after treatment with MNNG. The availability of both mammalian and *S. cerevisiae* systems to the study of carcinogen-mediated gene amplification provides us with tools to investigate the molecular and cellular mechanisms associated with this process and facilitates the characterization of amplified sequences and the determination of their role in cell regulation and tumorigenesis.

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