UV Radiation Facilitates Methotrexate Resistance and Amplification of the Dihydrofolate Reductase Gene in Cultured 3T6 Mouse Cells

THEA D. TLSTY, PETER C. BROWN, AND ROBERT T. SCHIMKE*

Department of Biological Sciences, Stanford University, Stanford, California 94305

Received 21 October 1983/Accepted 24 February 1984

Pretreatment of 3T6 murine cells with the carcinogen UV radiation or *N*-acetoxy-*N*-acetylaminofluorene increased the number of methotrexate-resistant colonies. This carcinogen-induced enhancement was seen only at low toxicities. The enhancement was transient and was observed at its maximum when cells were subjected to methotrexate selection 12 to 24 h after treatment. The addition of a tumor-promoting agent, 12-*O*-tetradecanoylphorbol-13-acetate, during or after carcinogen treatment further enhanced this effect. A large proportion of the resistant colonies had an increase in the dihydrofolate reductase gene copy number and the relative proportions of colonies with amplified genes were similar, regardless of whether selected cells were untreated, treated with carcinogen, or treated with carcinogen plus promoter. We discuss some of the variables which both enhance the generation and improve the detection of methotrexate-resistant colonies, as well as certain implications of our results for the generation and mechanism of gene amplification.

Inhibition of dihydrofolate reductase (DHFR; EC 1.5.1.3) by methotrexate (MTX) results in starvation of cells for glycine, purines, and thymidylate, resulting in inhibition of DNA synthesis, generation of chromosomal abnormalities (7), and ultimately cell death. Our laboratory (1, 41, 42) as well as others (10a, 16, 20, 31, 32, 33, 34) have shown that a common mechanism for the development of resistance to MTX is amplification of the *dhfr* gene. The karyological consequences of gene amplification include the presence of the amplified *dhfr* genes on an elongated chromosome, a so-called homogeneously staining region (8, 35), or as extra-chromosomal, self-replicating elements called double-minute chromosomes (25). Similar chromosomal abnormalities, i.e., homogeneously staining regions or double-minute chromosomes, have also been observed in cancer cells (2, 3, 21, 22, 30) where no known specific gene is known to be amplified. In the case of a mouse adrenocortical tumor with a homogeneously staining region (21), the homogeneously staining region has recently been identified as the site of amplification of the cellular oncogene *myc* (44). Amplification of *c-abl* oncogene sequences in human myelogenous leukemia (13, 14) has also been reported.

Because of the fact that chromosomal abnormalities known to occur with *dhfr* gene amplification are also observed in tumor cell lines, we asked whether agents that are known carcinogens can enhance the incidence of MTX resistance and *dhfr* gene amplification. We report here that treatment of cells with UV radiation or *N*-acetoxy-*N*-acetylaminofluorene (NA-AAF) increases the incidence of MTX resistance and *dhfr* gene amplification. This increase is further potentiated by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Similar enhancement of *dhfr* gene amplification after treatment with cellular mitogens has been previously reported by Barsoum and Varshavsky (4).

MATERIALS AND METHODS

Cells and culture conditions. Mouse 3T6 cells (clone 5) were grown in Dulbecco modified Eagle medium supple-

mented with 10% dialyzed, heat-inactivated newborn calf serum (K. C. Biological, Lenexa, Kans.), 2 mM sodium pyruvate, 2 mM additional glutamine, penicillin, and streptomycin in 10% CO₂ as described previously (10). This clone was used because of its high plating efficiency (90%), rapid growth (16-h cell cycle), and uniform colony morphology. Portions of stock cells were thawed and used within 10 passages. Cultures were tested routinely for mycoplasma.

Determination of MTX resistance. Cells were trypsinized, plated at appropriate densities, and allowed to acclimate for 6 to 12 h, and MTX (prepared from lyophilized samples) was added at the indicated concentrations. During selections, medium and MTX were replaced every 2 to 3 days as described (10). Colonies resistant to MTX were grown until visible (ca. 50 cells per colony) and were fixed with methanol: acetic acid (3:1) and stained with 2% Giemsa or were grown further to determine the *dhfr* gene copy number (see below). Colonies selected at low MTX concentrations (0 to 60 nM) required 6 to 8 days to become visible; those selected at higher concentrations (60 to 200 nM) required 2 to 3 weeks to become visible. In these studies, the incidence of MTX resistance is defined as the ratio of the number of colonies growing in MTX-containing medium to the number of cells initially plated.

Determination of *dhfr* gene copy number. To determine the *dhfr* gene copy number, individual colonies were collected by trypsinization in cloning rings (24), transferred to a 24-well cloning dish (Falcon no. 3047), and grown in MTX until a population of 10⁶ cells was achieved. The efficiency of recovering selected colonies was ca. 98%. Half of each population was frozen in 10% dimethyl sulfoxide (DMSO), and the remaining half was processed for determining the gene copy number (10). Briefly, cells were analyzed and incubated with RNase A and proteinase K. The DNA was extracted quantitatively, denatured with NaOH, neutralized, and applied to nitrocellulose for hybridization with radiolabeled cloned *dhfr* cDNA or mouse alpha-fetoprotein (*afp*) cDNA. Samples showing three times or greater hybridization intensity than seen in controls were considered to be amplified. Estimations of gene copy number were made visually and by a densitometer.

* Corresponding author.

Treatment with UV radiation, NA-AAF, or TPA. Before treatment of cells with UV radiation, cells were plated and allowed to recover for 6 to 12 h. Subsequently, excess medium was aspirated, leaving only a thin film, 1 to 2 mm thick. Cells were exposed directly to radiation at 254 nm for various times to obtain the appropriate fluence, after which the medium was replaced. After 6 to 12 h the medium was replaced with MTX-containing medium at various concentrations. NA-AAF was dissolved in DMSO and added to the medium with immediate mixing. The cells were incubated at 37°C for 20 min in the dark. The medium was changed, and 6 to 12 h later medium containing MTX was added. NA-AAF was assayed for chemical purity by high-pressure liquid chromatography before each use and was greater than 96% pure (data not shown). NA-AAF was a gift of P. Hanawalt (Stanford University, Stanford, Calif.) and J. A. Miller (University of Wisconsin, Madison). TPA (Sigma Chemical Co.) was dissolved in DMSO, frozen for storage, and used at a final concentration of 0.1 µg/ml. TPA was added to the medium with immediate mixing.

Determination of DNA synthesis. Cells were prelabeled by growth for one to three population doublings in 0.02 µCi of [*methyl*-¹⁴C]thymidine (50 Ci/mmol; Amersham Corp.) per ml. The radioactive medium was then removed, and the cells were grown for several population doublings in nonradioactive medium. DNA synthetic rate was determined by incorporation of [³H]thymidine (0.5 µCi/ml, 6.7 Ci/mmol; Amersham Corp.) at various intervals after the treatment of cells with UV radiation. Cells were labeled for 30 min, washed with ice-cold Hanks balanced salts solution supplemented with 10⁻⁵ M thymidine, and lysed on the plate by addition of lysis solution (10 mM Tris, pH 7.9, 1 mM EDTA, 0.6% sodium dodecyl sulfate, 50 to 100 µg of proteinase K per ml). The lysate was incubated at 37°C for 2 h and precipitated onto GF/C Whatman filters with 10% trichloroacetic acid, and the amount of [³H]thymidine incorporated was normalized to the [¹⁴C]thymidine content of the DNA.

RESULTS

Enhancement of MTX resistance in mouse 3T6 clone 5 cells pretreated with UV radiation. Figure 1 (closed circles) shows 3T6 clone 5 colony formation capability at a range of MTX concentrations. The 50% lethal dose is 20 nM MTX. Previous work (10, 45) has indicated that the incidence of amplification of *dhfr* is not constant along the entire range of MTX concentrations and is most readily detected when colonies are selected at 120 or 160 nM MTX. At these concentrations, 50 to 60% of emerging colonies have an increase in *dhfr* gene copy number (10).

When cells were pretreated with UV radiation (3 J/m²) the number of colonies which were resistant to MTX increased ca. 10-fold (Fig. 1., open circles). At this fluence, cell survival, as measured by colony formation in the absence of MTX, was ca. 70% of control values. The enhancement of MTX resistance as a function of the fluence of UV radiation is shown in Fig. 2. The greatest enhancement was seen at relatively low fluences (i.e., 2.5 to 4 J/m²). Fluences which reduced cell survival to 40% or less had a diminished enhancement of MTX-resistance, even after correction for the increased cell death produced by UV radiation (data not shown).

To determine whether the increased resistance to MTX had its basis in an increase in *dhfr* amplification frequency, mouse cells were irradiated at 0 or 3 J/m² and selected at 160 nM MTX for 2 to 3 weeks. The colonies which arose were analyzed for *dhfr* gene copy number as described above.

FIG. 1. Effects of UV radiation on resistance to MTX as measured by colony formation. Exponentially growing cultures of 3T6 clone 5 cells were trypsinized, plated at various densities, and allowed to acclimate for 12 h. Medium was removed, cells were exposed to 254-nm light, and fresh medium was added to each plate. Twelve hours after the UV pretreatment, MTX-containing medium was added to the plates. Colonies were fixed, stained, and counted after 2 to 3 weeks. Each point was measured in quadruplicate, and bars represent sample standard deviation. Plating densities for the various MTX concentrations were: 40 nM, 1×10^4 ; 60 nM, 1×10^5 ; 80 nM, 2×10^5 ; 120 nM, 5×10^5 ; and 160 nM, 1×10^6 in 10-cm-diameter plates. Symbols: ●, control; ○ pretreatment with UV radiation (3 J/m²). Cell viability in this experiment was 74% of control values. Data are corrected for reduced viability for the UV-irradiated samples.

Figure 3 shows representative data from these experiments. DNA from several colonies exhibited increased hybridization to radiolabeled *dhfr* cDNA probe. DNA samples from these colonies, hybridized in parallel to *apf* cDNA, showed no increase in intensity of hybridization. Of 20 control colonies (resistant to 160 nM MTX but not exposed to UV), 60% showed a detectable increase in intensity of hybridization with the *dhfr* probe when compared with the parental level of hybridization. Of the 22 colonies selected after treatment with UV radiation, 15 (68%) showed an increase in *dhfr* copy number.

Transient enhancement of MTX resistance by UV radiation. The enhancement of MTX resistance after UV radiation was not detected if the UV-irradiated cells were put into MTX-containing medium immediately after the UV treatment. Figure 4 shows the degree of enhancement as the time between UV treatment and placement in MTX-containing medium was varied. Maximum enhancement was detected when the UV-irradiated cells were placed in MTX-containing medium 12 to 24 h after treatment and decreased as that time was increased beyond 24 h.

FIG. 2. Incidence of MTX resistance at different UV fluences. Exponentially growing 3T6 clone 5 cells were plated at 2×10^5 cells per 10-cm plate and allowed to acclimate for 12 h. Medium was removed and cells were exposed to 254-nm light for increasing times. Fluence was calculated from dosimeter values (data not shown). Cell viability after 0, 1, 2, 2.5, 3, 4, and 5 J/m² was 100, 93, 81, 75, 70, 55, and 40%, respectively. After 12 h the cells were placed in drug-containing medium which was changed every 3 days until discrete colonies were visible 3 weeks later. Colonies were fixed, stained, and counted. Relative plating efficiency was calculated by using the plating efficiency of the cells grown without UV pretreatment in 160 nM MTX as the 100% value. The graph has not been corrected for UV toxicity to the cells.

Two variables which affect the incidence of MTX resistance include trypsinization of cells and the cell density at the time of the initial placement in MTX (data not shown). Newly trypsinized cells show a lower relative colony-forming capability when placed in MTX than do cells which have been allowed to recover for 6 to 12 h. On the other hand, recovery times greater than a full cell cycle (18 to 24 h) result in variable cell growth which leads to inaccuracy in the

FIG. 3. *dhfr* gene amplification in MTX-resistant colonies. Randomly picked colonies resistant to 160 nM MTX were grown in 160 nM MTX for a minimum of 15 generations. Each subclone was tested for amplification of the *dhfr* gene as described in the text and by Brown et al. (10). Equal portions of DNA were placed in adjacent slots and hybridized. The left half of each pair was hybridized with *afp*-radiolabeled probe, and the right half was hybridized with the *dhfr*-radiolabeled probe. Parental 3T6 clone 5 cells are marked (S) to the left of each sample. Those samples showing amplification of *dhfr* are marked (*) to the right of each sample. Control, 3T6 clone 5 colonies resistant to 160 nM MTX without UV pretreatment; UV radiation, 3T6 clone 5 colonies resistant to 160 nM MTX after pretreatment with 3 J of UV radiation per m².

FIG. 4. MTX resistance at various times after UV irradiation. Exponentially growing 3T6 clone 5 cells were trypsinized and plated at 2×10^5 cells per 10-cm plate and allowed to acclimate for 12 h. A 4-J/m² dose of UV radiation was given, and drug-free medium was replaced on the plates. At 0, 3, 6, 9, and 12 h after UV pretreatment, medium containing 160 nM MTX was added to each set of plates and changed every 3 days until MTX-resistant colonies were evident (ca. 3 weeks). Samples for the 12-, 24-, 48-, 72-, and 96-h points were held in drug-free medium, trypsinized, and plated at 2×10^5 cells per 10-cm plate 12 h before MTX-containing medium was put on the plates. The medium was changed every 3 days until MTX-resistant colonies were detected. Relative plating efficiency was calculated by using the plating efficiency of control cells in 160 nM MTX as the base value. Each point was normalized to its respective control value (i.e., without UV pretreatment) and corrected for the reduced viability after UV pretreatment. Plating efficiencies of control cells in drug-free medium were taken at 0, 6, 24, 48, and 96 h and found to be constant. Relative plating efficiency is shown on a log scale.

estimation of the number of MTX-resistant colonies. To avoid these added variables, the cells were always irradiated, incubated for various times, and then split to a constant cell density 12 h before they were placed in MTX-containing medium. This was done for each sample taken after the 12-h point of the experiment shown in Fig. 4. For all samples taken before that point (0, 3, 6, and 9 h), cells were not trypsinized. Cell number in both control and UV-treated samples in these instances increased no more than twofold, a difference in cell density that has minimal effect on colony formation in MTX.

Enhancement of MTX resistance after pretreatment with NA-AAF. 3T6 cells were also treated with 5 μ M NA-AAF, a concentration which reduced plating efficiency by 50% relative to untreated controls (data not shown). The incidence of resistance to several concentrations of MTX was increased after pretreatment with NA-AAF (Fig. 5). Concentrations of NA-AAF which resulted in greater than 50% kill showed diminished enhancement of generating MTX resistance, even when increased toxicity was taken into account (data not shown).

Effect of TPA on the generation of MTX resistance and *dhfr* gene amplification. The oncogenic activity of carcinogens is facilitated by tumor-promoting agents. We therefore wished to determine whether TPA would further enhance the effect of UV radiation on the incidence of MTX resistance. Cells were exposed to various combinations of UV radiation, TPA, and MTX (Fig. 6). The cells were grown in 160 nM MTX until MTX-resistant colonies were apparent (2 to 3 weeks). A striking effect was seen when the cells were incubated with TPA for a brief period of time after treatment

FIG. 5. Effects of NA-AAF on MTX resistance as measured by colony formation. Exponentially growing cultures of 3T6 clone 5 cells were treated with 5 μ M NA-AAF as described in the text. MTX-containing medium was replaced every 3 days with fresh medium, and after 2 to 3 weeks colonies were fixed, stained, and counted. Each point was done in quadruplicate, and bars represent sample standard deviation. Plating densities and symbols are as in Fig. 1. NA-AAF treatment reduced cell viability to 50% relative to untreated controls.

with UV radiation (treatment c, Fig. 6). An approximate 100-fold increase in the number of MTX-resistant colonies was observed. Since MTX is known to be a weak carcinogen (39a), we determined the effect of preincubation of cells with TPA and MTX on the generation of MTX-resistant colonies. A 6-h pretreatment with MTX followed by TPA stimulated MTX resistance, but to a lesser extent (treatment d, Fig. 6). When TPA was present throughout the selection with MTX, it produced an approximate 80-fold increase in the generation of MTX-resistant colonies. At the concentrations used, the effects of TPA and DMSO on plating efficiency were negligible (data not shown). Pretreatment with either TPA or DMSO alone had no effect on the incidence of MTX-resistant colonies.

Representative colonies from each type of treatment were analyzed for their *dhfr* copy number as described above. The same proportion of colonies which had amplified *dhfr* as a mechanism of resistance in single-step selections (with or without UV pretreatment) was also seen after pretreatment with UV plus TPA (30 to 60%). The extent of amplification in the various colonies was similar to that seen with the UV pretreatment alone, i.e., maximum of 5- to 10-fold increase in the *dhfr* gene copy number.

Effect of UV radiation on DNA synthesis. Carcinogens are known to damage cell constituents and inhibit DNA, RNA, and protein synthesis. Thus, we determined the effect of resistance-enhancing fluences of UV radiation on the syn-

thesis of DNA. Cells were prelabeled with [¹⁴C]thymidine and pulse-labeled with [³H]thymidine at various intervals after UV radiation. DNA synthesis decreased immediately after UV radiation and reached a minimum value (60% of control values) within 1 h (Fig. 7). Over the next 5 to 7 h the rate increased until it approached that of the control value. The extent and duration of inhibition of DNA synthesis was found to be dose dependent (data not shown, see below).

DISCUSSION

In this study we have shown for the first time that treatment of cells with agents, i.e., UV and NA-AAF, that are known mutagens and carcinogens can also increase the incidence of *dhfr* gene amplification. The demonstration of this enhancement requires attention to a number of parameters. The first parameter is the dose employed. The enhancement was greatest when UV and NA-AAF were employed in doses that produced intermediate toxicity, i.e., 30 to 40% kill of the cell population. Higher doses with increased toxicity did not further increase the incidence of MTX resistance. In addition, our selections were done within a range of MTX concentrations which allows for optimal detection of *dhfr* gene amplification (10). We did not detect either spontaneous or induced single-step resistance to MTX at concentrations greater than 300 nM MTX.

Carcinogens such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and ethyl methanesulfonate, when used at toxicities comparable to those used in our studies, increase the incidence of MTX resistance (19, 36). Flintoff et al. (19) reported that the increases in MTX resistance resulted from alterations in MTX transport or altered binding affinity of MTX for DHFR. Although they reported no increases in the incidence of DHFR overproducers, they noted that 12 out of 31 spontaneously MTX-resistant isolates had unstable phenotypes. Ethyl methanesulfonate treatment increased the frequency of MTX-resistant colonies 5- to 10-fold, some of

FIG. 6. Effects of UV irradiation and TPA on the incidence of MTX resistance. Exponentially growing 3T6 clone 5 cells were plated as described in the legend to Fig. 1. When UV irradiation was given it was at 3 J/m². TPA was present in the medium for 6 h at a final concentration of 0.1 μ g/ml. Plates were rinsed with fresh medium after TPA treatment and grown in fresh (drug-free) medium for 6 h before 160 nM MTX-containing medium was put on all plates. At the end of 3 weeks, colonies were fixed, stained, and counted. Open bars indicate TPA present in medium. The dashed line indicates 160 nM MTX present in medium. Numbers represent the average value from three plates.

FIG. 7. Effect of UV irradiation on [^3H]thymidine incorporation. Exponentially growing 3T6 clone 5 cells were trypsinized and plated at 2×10^5 cells per 10-cm plate. The cells were allowed to acclimate for 12 h and then some were treated with 3 J of UV per m^2 . [^3H]thymidine was added to each culture (control and UV treated) 30 min before they were harvested. Open circles represent values of UV pretreatment samples which have been normalized to the control values (represented by dashed line).

which were unstably resistant. Inasmuch as the amplified genotype is often accompanied by instability, in particular when cells are first selected (26, 27), it is likely that some of the unstable variants contained amplified *dhfr* genes.

A second parameter is the time which elapses between treatment of the cells and selection for MTX resistance. If cells were placed in selection medium immediately after UV treatment, only a minor increase in the incidence of resistance was seen; maximal enhancement occurred when cells recovered for 12 to 24 h before addition of MTX, and declined after 24 h (see Fig. 3).

A third parameter is the growth state of cells. The increase in incidence of MTX-resistant colonies was observed only in cell populations in logarithmic growth. Confluent cultures showed negligible enhancement of MTX resistance (data not shown). Mariani and Schimke (30a) have reported that the effect of hydroxyurea in enhancing the frequency of *dhfr* gene amplification occurs only when the hydroxyurea block is instituted after the initiation of the S-phase in a synchronized cell population. The position of cells within the cell cycle was also critical to the effect of UV radiation in increasing gene amplification (T. D. Tlsty and R. T. Schimke, manuscript in preparation). We conclude that to detect enhancement of the incidence of gene amplification, cells actively synthesizing DNA must be employed.

A fourth parameter is the cell line employed. Brown et al. (10) showed that different clones of a population of 3T6 cells have different propensities for generating colonies which are resistant to MTX. These observations emphasize the necessity for careful characterization of various parameters to allow for optimum detection of gene amplification.

In these studies, we have shown that the tumor promoter TPA increased the incidence of UV-induced MTX resistance, but only when TPA was present during or after UV treatment. Pretreatment with MTX plus TPA also enhanced the incidence of MTX resistance. Pretreatment of cells with TPA alone did not increase the incidence of MTX-resistant colonies. This observation is important when relating this work to studies presented previously by Varshavsky (47) and Barsoum and Varshavsky (4). In their studies, TPA (or another mitogen) was included during the entire MTX selec-

tion. Since MTX is a known carcinogen, this protocol is equivalent to the carcinogen and promoter being present simultaneously. Under these conditions, an approximate 100-fold increase in the incidence of MTX-resistant colonies was obtained. The great majority of the MTX-resistant colonies had amplified *dhfr* sequences. In our own studies, when TPA was added to the medium after UV irradiation and before MTX selection, a similar (100-fold) increase in the incidence of resistance was observed. To be effective in enhancing the incidence of amplification, TPA must be preceded by treatment with a carcinogen or be present simultaneously with that carcinogen.

We recognize that only a proportion of the MTX-resistant colonies (ca. 50%) were resistant by virtue of *dhfr* gene amplification, irrespective of whether colonies were selected without prior treatment (10), after treatment with hydroxyurea, UV, or NA-AAF, or after combined MTX-TPA treatment. What is the genotype of the MTX-resistant colonies which did not exhibit *dhfr* gene amplification? Two possibilities come immediately to mind. One possibility is that amplification of non-*dhfr* sequences may lead to MTX resistance. Recently, several groups (5–7, 28) have provided evidence that resistance to vinca alkaloids is associated with overproduction of one or more proteins, and the karyotypes of such cells show the classical consequences of gene amplification. That amplification of two different DNA sequences can lead to the generation of resistance to the same drug is shown by the studies of Coderre et al. (12) and Beverly et al. (submitted for publication) in *Leishmania tropica*. Thus, although the mechanism(s) of resistance of the other 50% of 3T6 cells has not been examined, amplification of other genes resulting in MTX resistance cannot be excluded. The second possibility for the increase in MTX-resistant colonies which did not exhibit amplified *dhfr* sequences is that they arose by alternative mechanisms which are well documented in this system. Among such mechanisms are altered affinity of MTX for the DHFR protein (19, 23) and altered transport (40). Our present data do not distinguish between these or other possible explanations.

All of the agents we have employed to increase the incidence of MTX resistance and *dhfr* gene amplification produce a myriad of effects. One effect they have in common is the transient inhibition of DNA synthesis. The pattern of inhibition of DNA synthesis in 3T6 cells after UV radiation (Fig. 6) was similar to that described previously for mouse L cells (17) and HeLa cells (18) with equivalent UV fluences. A similar result was seen with NA-AAF treatment (data not shown). Aberrant replication is known to take place after transient inhibition of DNA synthesis in both procaryotes (9, 38, 39) and eucaryotes (29, 48, 49). Lavi has shown that treatment of simian virus 40-transformed Chinese hamster embryo cells with various chemical and physical carcinogens induces amplification of the simian virus 40 sequences. This amplification does not produce infective virus and is dependent on the viral A gene (29). The effect of TPA on DNA synthesis is complex (15). Peterson et al. have reported a mitogenic effect of TPA and suggest that it results from partial synchronization of cells after an initial inhibition of DNA synthesis (37). We propose that inhibition of DNA synthesis can trigger a cellular response which results in aberrant replication, the consequence of which is gene amplification (also see reference 46). Indeed, Mariani and Schimke (30a) have shown that after transient inhibition with hydroxyurea (conditions which increase the incidence of *dhfr* amplification) rereplication of a portion of the genome occurs. Thus, we favor the explanation that these agents

influence the incidence of gene amplification by transiently inhibiting DNA replication. Of course, other effects caused by the complex action of these agents may also influence these processes. We are currently attempting to determine the specific effect(s) exerted by these agents.

Several recent papers have reported oncogene amplification in tumor cell lines (11, 13, 14, 43, 44). Many questions remain to be answered. Is amplification causally related to the development or progression or both of the malignant state? Does our demonstration as well as that of Lavi (29), showing enhancement of gene amplification by carcinogenic agents, have direct bearing on the mechanism of carcinogenesis?

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