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The Effects of Bifunctional Alkylating Anti-Tumor Agents on Oncogene Structure and Function in the Human Tumor Cell Line Colo3320 HSR

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THE EFFECTS OF BIFUNCTIONAL ALKYLATING ANTI-TUMOR AGENTS
ON ONCOGENE STRUCTURE AND FUNCTION IN THE
HUMAN TUMOR CELL LINE Colo320 HSR

by

Bernard Walter Futscher

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

April

1990

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To my love, Karin, her encouragement and forbearance made the tough times tolerable.

Finally, to my parents, Erich and Ruth Futscher, their contribution to this achievement is immeasurable. Words can never express adequately my appreciation.

VITA

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Mr. Futscher has accepted a position as a Research Associate at the Arizona Cancer Center, University of Arizona in Tucson.

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LIST OF ABBREVIATIONS

μCi	microcurie
μg	microgram
μl	microliter
μM	micromolar
4-HC	4-hydroperoxy cyclophosphamide
bp	base pair
C2	4-S-(propionic acid)-sulfidocyclophosphamide
CAT	chloramphenicol acetyl transferase
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
dGTP	deoxyguanosine triphosphate
DHFR	dihydrofolate reductase
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetraacetic acid
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HN2	mechlorethamine
HSR	homogeneously staining region
kb	kilobases
kd	kilodalton
L-Pam	L-phenylalanine mustard
M	molar

LIST OF ABBREVIATIONS (Cont.)

mCi	millicurie
mg	milligram
ml	milliliter
mmol	millimolar
mRNA	messenger RNA
nm	nanometer
PBS	phosphate buffered saline
PUVA	4-hydroxymethyl-4,5',8 trimethylpsoralen plus ultraviolet A irradiation
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
t 1/2	half life
TBE	Tris-borate-EDTA gel electrophoresis buffer
TCA	trichloroacetic acid
TE	Tris-EDTA buffer
Tris	(Tris-[hydroxymethyl]-aminomethane)
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
x g	times gravity
°C	degrees celsius

INTRODUCTION

Evidence indicates activated proto-oncogenes (oncogenes) play a causal role in the induction of human cancer. Thus, it follows that delineating the effects of anti-tumor agents on oncogene structure and function ultimately may be of chemotherapeutic significance. Although the mechanisms by which oncogenes induce malignant growth continue to be defined, the effects of DNA-damaging anti-tumor agents on oncogene structure and function are not understood. The goal of this dissertation project was to characterize the effects of clinically-used bifunctional alkylating agents on oncogene structure and function, and was predicated on the hypothesis that these compounds may affect differentially the function of these genes.

The anti-tumor agents examined in this study were the bifunctional alkylating agents mechlorethamine (HN2, nitrogen mustard), L-phenylalanine mustard (L-PAM), and forms of cyclophosphamide which are active in vitro - 4-S-(propionic acid)-sulfidocyclophosphamide (C2) and 4-hydroperoxy-cyclophosphamide (4-HC). These agents are cytotoxic, and experimental evidence suggests that the DNA interstrand crosslink is a critical cytotoxic cellular lesion. This lesion is formed by the covalent binding of the bis-chloroethyl group of the alkylating agent to the N7 position

of guanines on opposite strands of the DNA, and may alter the transcription, and ultimately the replication, of oncogenes. To help characterize the actions of DNA-damaging anti-tumor agents on oncogenes, the effects of the aforementioned bifunctional alkylating agents on a number of parameters regarding gene structure and function were explored. Specifically, studies presented in this dissertation attempted to address the following questions:

1) Do differences exist between the bifunctional alkylating agents, HN2, L-PAM, and C-2, in their ability to react with the N7 position of guanine in the primary sequence of the c-myc oncogene in vitro?

2) Do HN2, L-PAM, and 4-HC differentially affect the steady state expression of specific oncogenes within a given human tumor cell line?

3) Are gene-specific DNA interstrand crosslinks responsible for any changes seen in the steady state expression of these genes?

To perform these studies a human tumor cell line, Colo320 HSR, was used as a model system. This cell line was chosen because it was of human origin, it was obtained from the patient prior to the onset of chemotherapy, and it expressed an activated oncogene (c-myc). Exposures of HN2, L-PAM, or 4-HC used for these studies were chosen for their ability to produce one or three logs of cell kill in Colo320 HSR cells, as determined by soft agar colony formation assays. Using a

modification of the Maxam and Gilbert DNA sequencing technique, it was possible to determine the extent of N7 guanine modification produced by these anti-tumor agents in vitro, using an exon 2 fragment of the c-myc oncogene as the probe. The c-myc fragment used in these studies originally was isolated and cloned from the Colo320 HSR cell line. Effects of drug exposure on the steady state levels of gene expression in Colo320 HSR were determined by Northern blot analysis. Gene-specific crosslinks were measured by the combined use of DNA denaturation-renaturation gel electrophoresis and Southern blot analysis.

REVIEW OF RELATED LITERATURE

oncogenes

The genetic basis of cancer has been a tenet of cancer biology for almost a century. It was not until recently however, that it was proven that specific genes play a causal role in oncogenesis. In 1969, work by Huebner and Todaro led them to propose that cancer genes, or oncogenes, existed in the genome of RNA tumor viruses, and were transmitted from parent to progeny in a covert form. It was further hypothesized that damage to these genes would result in their activation (Huebner and Todaro, 1969). This suggestion proved to be incorrect. In 1976, experiments by Bishop and Varmus demonstrated unequivocally that the oncogene of the Rous Sarcoma virus is found in the genome of higher organisms, suggesting that the virus had transduced the gene from eukaryotic organisms (Stehlin et al., 1976).

The cellular homologs of viral oncogenes have been termed proto-oncogenes (Bishop, 1983). These proto-oncogenes are not inherently cancerous, but instead their conservation throughout evolution suggests that proto-oncogenes play a fundamental role in normal cellular function and evolutionary development (Bishop, 1987). The proteins encoded by proto-oncogenes include growth factors and their receptors, plasma membrane proteins involved in signal transduction, and nuclear

proteins involved in transcriptional regulation and perhaps DNA replication (Chiu et al., 1988, Studzinski et al., 1986, Fleming et al., 1989, Bos, 1988, Coussens et al. 1986).

The activation of a proto-oncogene to an oncogene can occur by a wide variety of mechanisms, but all involve the disruption of the integrity of the gene. For instance, a single point mutation in the protein coding region of the ras family of proto-oncogenes gives rise to a non-conservative amino acid substitution, and hence an altered protein with oncogenic potential (Capon et al., 1983). Another mechanism is chromosomal translocation involving proto-oncogenes, which may lead to the disruption of normal gene regulation (e.g. c-myc) or the production of a modified protein (e.g. c-abl); either event is capable of oncogenic transformation (Battey et al. 1983, Heisterkamp et al. 1983, Shtivelman et al. 1985). Finally, the amplification of proto-oncogenes, resulting in increased copies of the gene and increased levels of the gene product within the cell is also a method of inducing transformation (e.g. c-myc and N-myc)(Collins and Groudine, 1982, Schwab et al. 1983).

A number of studies have shown activated oncogenes to play a pivotal role in human neoplasia. Furthermore, it has been hypothesized that two cooperating, activated oncogenes are necessary to transform a normal cell to a malignant cell (Land et al. 1983). Although evidence supporting this hypothesis has been achieved in vitro, it has been difficult

to demonstrate this hypothesis in vivo. The activation of a single proto-oncogene in vivo however, has been readily demonstrated, and more importantly the activation of specific oncogenes have been shown to occur consistently in some tumor types. For example, a somatic mutation in the K-ras proto-oncogene has been implicated in playing a role in virtually all tumors of the exocrine pancreas (Almoguera et al. 1988). The Philadelphia chromosome involves a reciprocal chromosome 9 to chromosome 22 translocation, is indicative of a disrupted c-abl proto-oncogene, and is observed in 90% of patients with chronic myelogenous leukemia (Teich 1986). Finally, amplification of the N-myc proto-oncogene is frequently observed in neuroblastoma, and more importantly the level of amplification of N-myc (> 10 copies) is strongly correlated with a poor clinical prognosis (Schwab et al. 1984, Brodeur et al. 1984).

Although much is known of the role oncogenes play in carcinogenesis, a paucity of data exists regarding the effects of DNA-damaging anti-tumor agents on oncogene structure and function. It is conceivable, however, that different anti-tumor agents may inhibit selectively the expression of specific oncogenes. For example, a preliminary report indicated that exposure of the Burkitt lymphoma cell line Daudi to HN2 caused suppression of c-myc expression, but did not affect ras expression (Watt et al. 1986). Another report examined the effect of a variety of clinical anti-leukemic

chemotherapeutic regimens on c-myc expression in patients with leukemia (Venturelli et al., 1988). Although no alkylating agents were included in the chemotherapeutic regimens employed, this study demonstrated that depression of c-myc expression 24 hours after treatment correlated with tumor response in patients with acute myelogenous leukemia or acute lymphocytic leukemia. These data suggest that drug effects on oncogene expression may ultimately serve as indicators of prognosis in specific types of cancer.

Colo320 HSR

The cell line, Colo320 HSR, is a human colon carcinoma of neuroendocrine origin, isolated from a patient prior to the onset of chemotherapy (Quinn et al., 1979). Consistent with this cell line being derived from the neuroectoderm are the products it synthesizes and secretes. These include epinephrine, norepinephrine, serotonin, parathyroid hormone, and adrenocorticotrophic hormone. Furthermore, this cell line does not produce carcinoembryonic antigen, nor does it demonstrate morphologic characteristics consistent with it being of epithelial origin (Quinn et al. 1979). Colo320 HSR contains a homogeneously staining region (HSR) indicative of gene amplification, and in fact, contains approximately 30 to 40 copies of the c-myc oncogene (Alitalo et al., 1983). In addition to the amplification and high expression of the c-myc oncogene, we also have detected low levels of expression

of two other oncogenes; N-ras and c-fos. The salient features of each gene are discussed below.

c-myc

The c-myc gene has been intensely studied, however, the gene and its functional role remain enigmatic. c-myc is a member of an oncogenic multigene family which also includes the genes N-myc and L-myc (Nau et al., 1985, Kohl et al. 1986).

The activation of c-myc from proto-oncogene to oncogene is attributed to either the amplification or translocation of the gene, or retroviral integration near the gene locus (Hayward et al., 1981, Little et al. 1983, Taub et al. 1982). It appears that over production of a c-myc mRNA and protein are responsible for oncogenic activation. c-myc activation has been implicated in the induction of tumors of the colon, lung, breast, thyroid, and prostate, as well as glioblastomas and hematological malignancies (Trent et al. 1986, Escot et al. 1986, Yamashita et al. 1986, Dalla Favera et al. 1982, Calabretta et al. 1985, Fleming et al. 1986, Slamon et al. 1984, Yokota et al. 1986). The widespread role that c-myc appears to play in human oncogenesis makes the delineation of c-myc responses to chemotherapeutic agents a worthwhile venture.

The c-myc gene is mapped to chromosome 8q24, and is composed of three exons and two introns (Taub et al. 1982).

exon 1 is an unusually long untranslated leader sequence while exons 2 and 3 contain the protein coding information (Kohl et al. 1986).

The mature messenger RNA (mRNA) transcript coded for by c-myc has been reported to be 2.1-2.7 kb in both normal and malignant cells, and has a half life ($t_{1/2}$) of 20-30 minutes (Kohl et al. 1986). The gene is expressed in proliferating cells and can be induced in resting cells by a variety of stimuli including serum, platelet-derived growth factor (PDGF), and interleukin-1 alpha (Armelin et al. 1984, Kovacs and Oppenheim 1986, Cole 1986, Kelly and Siebenblast 1986).

The predicted molecular weight for the c-myc protein derived from nucleic acid sequences is 49 kd, however, SDS polyacrylamide gel electrophoresis (PAGE) measurement yields two bands migrating at approximately 66 and 62 kd (Persson and Leder, 1984). The reason for this anomaly is not known, but it is not due to multiple RNA species. A recent report suggests that the larger form originates from a non-AUG initiation codon in the mRNA near the 3' end of exon 1 (Hann et al., 1988). The c-myc protein, a phosphoprotein localized in the nucleus of normal and tumor cells, is capable of binding single-stranded and double-stranded DNA, and has a $t_{1/2}$ of about 30 minutes (Persson and Leder, 1984). The function of the c-myc protein is unknown, but may be involved in DNA replication or transcriptional regulation (Studzinski et al., 1986, Lech et al. 1988).

N-Ras

Like c-myc, N-ras is a member of an oncogenic multigene family which includes the H-ras and K-ras genes and two pseudogenes. The activation of the N-ras proto-oncogene to its oncogenic form can occur by a single point mutation in codons 12, 13, or 61. These mutations lead to a single amino acid substitution in the protein, which leads to altered protein function. Enhanced expression of the N-ras gene also leads to oncogenic activation, as in the case of gene amplification. Thus, it appears that overproduction of a normal ras protein, or the normal production of a mutated protein are both mechanisms for oncogenic activation of ras genes. Activated N-ras has been implicated as a transforming gene in an assortment of tumors including neuroblastoma, mammary carcinoma, fibrosarcoma melanoma, leukemia, and rhabdomyosarcoma (Taparowsky et al., 1983, Shimzu et al. 1983, Bos, 1988).

The N-ras gene is located on chromosome 1 and is composed of four exons with exon 1 being an untranslated leader sequence (Hall et al., 1983). The protein product is located on the cytoplasmic surface of the cell membrane, can bind GTP and GDP, and has GTPase activity. The above facts suggest that this protein is a G-protein involved in signal transduction from membrane bound receptors to adenylate cyclase (Bos, 1988).

c-fos

The c-fos gene has been implicated in normal cell growth, differentiation, and development. The gene has been mapped to chromosome 14q21-31, and is composed of four protein coding exons. It gives rise to a mature mRNA transcript of approximately 2.2 kb, which can be detected at high levels in some normal tissues, notably a wide variety of prenatal tissues and postnatal hemopoietic cells. It also is found at extremely low levels in a number of other tissues (van Straaten et al., 1983, Verma and Graham, 1987).

To activate the oncogenic potential of the c-fos gene two perturbations to the gene must occur. First, a strong promoter (such as a retroviral long terminal repeat) must be inserted near the gene's 5' end, and secondly, a 67 base pair stretch located in the gene's 3' non-coding region must be removed. The strong promoter serves to amplify normal transcription, and the 67 bp deletion is presumed to stabilize the c-fos mRNA transcript (Miller et al., 1984). From these observations it appears oncogenic activation of c-fos involves the aberrant expression of a normal protein.

Interestingly, the c-fos gene can be induced rapidly and transiently following various stimuli in a number of different tissues and cell lines. The induction can be observed within a few minutes following stimulation, and can disappear within one hour (Greenberg and Ziff, 1986). For example, pheochromocytoma cell lines exhibit c-fos expression following

stimulation by cholinergic agonists (e.g. nicotine) and nerve growth factor. In vivo, cells of the CNS can be induced to express c-fos by sensory stimulation, electrically-induced seizures, or chemically-induced seizures (e.g. with metrazole) (Greenberg et al., 1986, Hunt et al., 1987, Dragunow and Robertson, 1987 Morgan, 1987). Non-neuronal cells also can be stimulated to induce c-fos expression by a wide variety of agents, including platelet-derived growth factor, epidermal growth factor, phorbol esters, and the mitogens concanavalin A and lipopolysaccharides (Kruiger et al., 1984, Greenberg and Ziff, 1984, Muller et al., 1984).

The protein product of the c-fos gene is a nuclear phosphoprotein with an apparent molecular weight of 62 kd. This protein functions as a transcriptional regulator, is associated with other transcriptional proteins, and the protein appears to regulate its own expression (Rauscher et al., 1988, Sassone-Corsi et al., 1988, Lech et al., 1988). From these observations it has been speculated that c-fos acts as a third messenger - coupling short term stimuli to long term responses through the alteration of gene expression (Sagar et al., 1988).

α -Satellite DNA

α -satellite DNA is composed of a family of DNA sequences found in the genome of many vertebrates. The majority of α -satellite DNA exists as a series of tandemly repeated

monomers. In the human genome the monomer is approximately 171 bp in length. In turn, the tandemly repeated monomer is part of a higher order array. The higher order array can continue, uninterrupted by non-alpha sequences, for as much as several million base pairs, and can account for several percent of the entire human genome. These sequences are localized to the heterochromatic centromeric regions of the chromosome. The α -satellite DNA is not a gene and produces no protein product. α -satellite sequences are not transcribed into RNA; thus the function of α -satellite DNA is not well understood (Willard, 1987).

Although unclear functionally, α -satellite DNA organization is becoming clearly defined. Studies indicate that these α -sequences are organized in a highly chromosome-specific fashion in the human genome. These chromosomal subsets display different higher-order organization based on different multimers of the approximately 171 bp monomer. It is this organization which is chromosome-specific. The unique chromosome-specific, higher-order repeats can often be revealed by restriction enzyme digestion and Southern blot analysis (Willard, 1985, Waye et al., 1988).

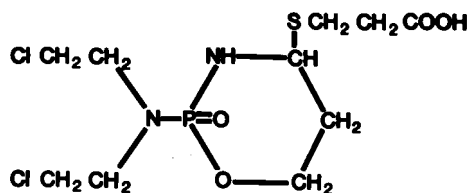
The chromosomal α -satellite DNA examined in this project is specific for human chromosome 20. Upon digestion of genomic DNA with the restriction enzyme, EcoRI, an approximately 1.7 kb fragment is revealed by Southern blot analysis. This fragment consists of eleven 171 bp monomers,

is present in 100-1000 copies per genome, and hence covers a stretch of approximately 200-2000 kb (Oncor Inc., unpublished results).

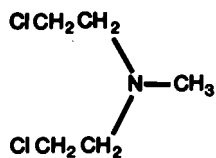
Bifunctional Alkylating Agents

Nitrogen mustard was one of the first non-hormonal compounds to show clinical efficacy in the treatment of human cancer, and still is clinically used today (Figure 1)(Goodman et al. 1946). The biological activity of this compound is due to the presence of the bis-(2-chloroethyl) group. A number of derivatives of this initial compound have been synthesized in which the methyl group has been replaced by a wide variety of carrier molecules, and many of these have found a niche in cancer chemotherapy. Some of these compounds have been examined in this dissertation, namely HN2, L-PAM, and two forms of cyclophosphamide which do not require metabolic activation: 4-S-(propionic acid)-sulfidocyclophosphamide (C2) and 4-hydroperoxy-cyclophosphamide (4H-C) (Hirano et al., 1979, Takamizawa et al., 1975).

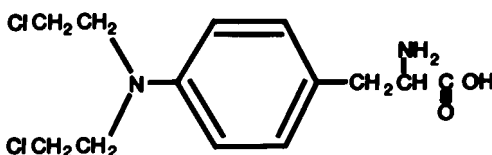
These agents are extremely reactive electrophilic compounds, which alkylate a number of cellular macromolecules including protein and RNA, but it appears that they exert their cytotoxic effects by producing lesions in the DNA (Johnson and Ruddon, 1967, Ruddon and Johnson, 1968, Lawley and Brooks, 1963, Erickson and Zlotogorski, 1984, Brooks and Lawley, 1965, Kohn et al., 1966). Alkylation of the DNA



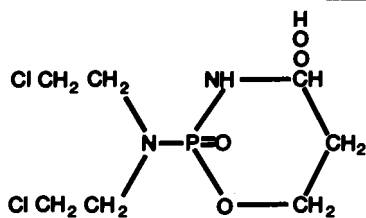
4-S(Propionic Acid)-Sulfidocyclophosphamide
(C-2)



Nitrogen mustard
(HN2)



Melphalan
(L-Pam)



4-Hydroperoxycyclophosphamide
(4-HC)

Figure 1. The chemical structures of HN2, L-PAM, 4-HC, and C-2.

occurs primarily at the N7 position of guanine, and results in the formation of monoadducts, as well as DNA intrastrand and interstrand crosslinks. Studies using derivatives of the nitrogen mustards which were only capable of forming monofunctional adducts in the DNA demonstrated that while these compounds were mutagenic, they were not effective anti-tumor agents compared to those compounds capable of forming interstrand crosslinks (Colvin, 1982). These observations have stimulated considerable interest in understanding the chemical nature of the bifunctional adducts and the subsequent biological consequences of this DNA damage.

Brooks and Lawley first demonstrated mustard compounds could form crosslinks in DNA by isolating di(guanin-7-yl) derivatives from cells treated with sulfur mustard (Brooks and Lawley, 1961). The bifunctional alkylating agents also form DNA interstrand crosslinks through covalent bonding at the N7 position of guanines on opposite strands of the DNA. The DNA interstrand crosslink is an infrequently occurring lesion in cells treated with nitrogen mustards. Only 100-1000 interstrand crosslinks are thought to be formed in cells following cytotoxic exposures to nitrogen mustards (Kohn et al., 1981). Nevertheless, the DNA interstrand crosslink has been strongly correlated with nitrogen mustard-induced cytotoxicity. Mechanistically, it has been hypothesized that the DNA interstrand crosslink inhibits DNA replication, by preventing strand separation, which ultimately leads to cell

death (Brooks and Lawley, 1965). Additionally, preventing strand separation may theoretically inhibit RNA transcription. Recent work supporting this possibility demonstrated that chloramphenicol acetyltransferase (CAT) expression vectors exposed to HN2 displayed a concentration-dependent increase in DNA interstrand crosslinks and a concomitant decrease in CAT expression (Dean et al., 1988). These experiments did not rule out the possibility that the decreased CAT expression was not the result of some other lesion. The molecular mechanisms of cellular repair of DNA interstrand crosslinks is not understood, but is probably a multi-step, multi-enzymatic process, similar to other DNA repair systems.

Heterogeneous DNA Damage and Repair

Many lines of evidence indicate that chemotherapeutic and carcinogenic DNA damaging agents interact with DNA in a non-random fashion. Damage studies of carcinogens reacted with DNA in vitro indicate that differences exist at the primary sequence level (Hatley et al., 1986, D'Andrea and Haseltine, 1978, Mattes et al., 1986, Bole and Hogan, 1984, Hurley et al., 1985). Moreover, other reports demonstrate that DNA-damaging chemotherapeutic agents and carcinogens preferentially react with actively-transcribed genes, as well as with DNA-associated with the nuclear matrix where transcription and replication are thought to occur (Tew et al., 1978, Irwin and Wogan, 1984, Obi et al., 1986).

Although the production and disappearance of nitrogen mustard-induced DNA interstrand crosslinks in the entire genome has been well documented, the production and disappearance of DNA interstrand crosslinks within specific genes is unknown. Employing the rapid renaturation kinetics of crosslinked DNA, size fractionation of DNA by gel electrophoresis, and Southern blot analysis, it has been possible to detect DNA interstrand crosslinks in defined genomic restriction fragments (Vos and Hanawalt 1987).

Using this type of experimental approach, gene-specific DNA interstrand crosslinks produced by the chemotherapeutic agents, Mitomycin C, and 4-hydroxymethyl-4,5',8-trimethylpsoralen plus ultraviolet A irradiation (PUVA) have been assayed. Using Mitomycin C to produce DNA interstrand crosslinks, human cell lines derived from persons with Fanconi's anemia removed interstrand crosslinks from their ribosomal RNA (rRNA) genes more slowly than cell lines derived from control individuals (Matsumoto et al., 1989). PUVA-induced DNA interstrand crosslinks and monoadducts also were assayed in the dihydrofolate reductase (DHFR) gene of a methotrexate-resistant human cell line (VA2-6A3). The DNA interstrand crosslinks disappeared more rapidly than the monoadducts, suggesting different types of DNA lesions are processed with different repair kinetics (Vos and Hanawalt, 1987).

Using similar experimental approaches, UV-induced DNA

damage and repair of specific genes has been studied (Bohr et al.). Results from studies examining UV-induced pyrimidine dimers have indicated that these lesions are preferentially removed from certain regions of the genome, or in other words, DNA repair is heterogeneous. Using UV repair-deficient Chinese Hamster Ovary (CHO) cell lines, it was demonstrated that 24 hours after exposure to moderate doses of UV (<15% cytotoxicity), 85% of the UV-induced lesions were removed from the actively-transcribed DHFR gene, while the overall repair of the entire genome was only 15% (Bohr et al., 1985). On the basis of these results, it was hypothesized that the relative resistance of rodent cell lines to UV irradiation was due to their ability to repair essential genomic regions. Using a rodent fibroblast cell line (Swiss mouse 3T3), studies also have shown that the active c-abl proto-oncogene is repaired faster than the silent c-mos proto-oncogene, suggesting that repair may be coupled to RNA transcription (Madhani et al., 1986). In support of the possibility that DNA repair is coupled to RNA transcription, it has been recently reported that, UV-damage in the lac operon of E. Coli was preferentially removed from the transcribed strand of these genes when RNA transcription of these genes was induced, but repair of the two strands was about equal when the genes were not being actively transcribed. (Mellon and Hanawalt, 1989)

From this body of evidence, it is possible to suggest that DNA reactive drugs may preferentially modify specific

regions of the genome, and the subsequent repair of the resultant lesions may be removed preferentially from specific portions of the genome. By delineating the heterogeneity, it may be possible to find specific genomic regions that are susceptible to drug attack and/or repair bypass. Ultimately, this may lead to the elucidation of genomic regions critical for DNA damage-induced cytotoxicity.

MATERIALS AND METHODS

Cell Culture

Colo320 HSR was the cell line used in this study, and was purchased from American Type Culture Collection. The cell line was cultured in RPMI 1630 (Gibco, Grand Island, N.Y.) supplemented with 15% heat inactivated bovine calf serum (Hyclone Laboratories, Logan, Utah) 1 mM glutamine (Gibco), penicillin (50 U/ml), plus streptomycin (50 μ g/ml, Gibco) at 37° C, 95% O₂, 5% CO₂. These cells have a doubling time of approximately 24 hours and were passaged twice a week to maintain logarithmic growth. Cells were counted on a Coulter Counter Model ZBI to determine cell number.

Bifunctional Alkylating Agents

HN2 and L-PAM were obtained from the Drug Development Branch of the National Cancer Institute. HN2 and L-PAM were dissolved in sterile filtered 0.1 N HCl and maintained as a frozen stock solution. C-2 was kindly supplied by Prof. Dr. Helmut Ringsdorf, University of Mainz, Federal Republic of Germany. C-2 was dissolved in sterile phosphate buffer immediately prior to use. 4H-C was the generous gift of Dr. Michael Colvin, of The Johns Hopkins School of Medicine. 4-HC was dissolved in sterile RPMI 1630 immediately prior to use. 4-HC and C-2 were used instead of cyclophosphamide

because of the need for cyclophosphamide to undergo microsomal activation. C-2 was used for all DNA sequencing studies because it could be dissolved in phosphate buffer and did not detrimentally effect PAGE. 4-HC was used for all experiments involving cells because it was more readily obtained, and because it is in clinical use. Both 4-HC and C-2 spontaneously decompose in vitro to 4-OH-cyclophosphamide; the same intermediate resulting from the microsomal activation of cyclophosphamide.

Survival Assays

To determine doses of the antineoplastic agents used in this study, soft agar colony survival assays were utilized. Exponentially growing Colo320 HSR cells were exposed to drug for one hour at 37° C. The cells were washed free of drug by centrifugal pelleting, resuspending the cells in an equal volume of warm media, pelleting the cells again, and finally resuspending in one volume of warm media. The cells then were counted, and serially diluted into tubes to give ranges in cell concentration from 10^2 to 10^5 per ml. One ml of control and drug treated cells were seeded into culture tubes containing 3 mls of media and noble agar (Difco, Detroit, Mi.) to produce a semi-solid support in which the cells could form colonies. The final concentration of noble agar in the media was 0.1% (Chu and Fisher, 1968). Cells were allowed to grow undisturbed for two weeks, at which time the number of

colonies was scored. Colony forming efficiency of untreated control cells was typically 75%. Colony forming efficiency was defined as the [number of countable colonies formed per tube/number of cells seeded per tube] x 100. Percent survival was defined as the [number of colonies formed from drug treated cells/number of colonies expected from untreated cells] x 100.

General Methods

A number of general procedures, techniques, and solutions used in this dissertation were employed in the experimental protocols. These are fundamental to the general laboratory work, and merit description for the sake of completeness. These basic procedures are described below.

A. Reagent Preparation

Organic solvents were often used in the isolation of nucleic acids, but need to be prepared properly before use. Phenol was purchased as a solid, and was liquefied by heating at 68°C. The anti-oxidant 8-hydroxyquinilone was added to the liquefied phenol to a final concentration of 0.1% (w/v), and the reducing agent 2-mercaptoethanol was added to a final concentration 0.2% (v/v). The phenol solution then was extracted sequentially with 1.0 M Tris, pH 8.0; 0.1 M Tris, pH 8.0; and finally 0.01 M Tris, 0.001 M ethylenediamine tetraacetic acid (EDTA), pH 8.0 (TE, 8.0). This procedure neutralizes the acidic phenol, and adequate neutralization was

ensured by checking the pH of the final aqueous extraction (TE). The pH of the aqueous phase was typically ~7.5. Another organic solvent used in nucleic acid preparation was sevag, which is a 24:1 (v/v) mixture of chloroform:isoamyl alcohol.

Some compounds were deionized before use. Compounds such as those used in this dissertation project included 100% formamide and 7 M glyoxal. These compounds were deionized by mixing with RG 501-X8 mixed bed ion exchange resin (Bio-Rad Laboratories, Richmond, Ca.) for one hour at room temperature. The sample:resin mixture ratio is 1:1. The mixed bed resin then was removed by filtering the solution through a 0.45 μm pore size filter.

B. Plasmid Isolation

DNA fragments utilized in the DNA sequencing studies and used as gene-specific probes in hybridization studies often were purified from a number of bacterial plasmids. Large scale plasmid preparations were employed to obtain large quantities of these fragments. Bacteria harboring these plasmids were grown overnight in Luria-Bertani broth (per liter: 10 grams Bacto-tryptone, 5 grams Bacto-yeast extract, and 10 grams NaCl) at 37° C in an orbital shaking bacterial incubator.

The bacteria were lysed, and the nucleic acids were recovered by the SDS-lysozyme procedure (Maniatis et al., 1982). Five hundred mls of bacterial culture were pelleted

at 3750 rpm (4000 x g) in the Beckman Model J-6M centrifuge at 4°C for 10 minutes. The pellet was washed once in 1x PBS, pelleted, and resuspended in 10 ml of ice cold 10% sucrose, 0.05 M Tris, pH 7.5. To this, 0.5 ml lysozyme (10 mg/ml) and 2 ml of 0.25 M EDTA were added, the tubes were inverted and then incubated for 10 minutes on ice. Ten mls of 10% sodium dodecyl sulfate (SDS), and 1.5 ml of 5 M NaCl was added, and the lysate was incubated on ice for 1 hour. The bacterial debris was pelleted in thick-walled polycarbonate tubes by ultracentrifugation (Beckman Model L8-70M) in a Beckman Ti70.1 fixed angle rotor at 30,000 rpm (83,000 x g) and 4°C for 30 minutes. The aqueous layer was saved and extracted twice with an equal volume of phenol/sevag (1:1), and once with sevag alone. Phases were separated by centrifugation in the Beckman Model J-6M at 2370 rpm (1600 x g) at room temperature for 10 minutes. Nucleic acids were precipitated by the addition of 2.5 volumes of ice-cold non-denatured 95% ethanol. Nucleic acids were recovered by centrifugation in a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont) using the ss-34 rotor. Centrifugation runs are at 4°C for 30 minutes at 10,000 rpm (10,000 x g). Nucleic acids were dried in the lyophilizer, resuspended in TE 8.0, and treated with RNase (10 µg/ml) for 1 hour at 37°C. Proteins were removed by extraction with organic solvents as described above.

To separate supercoiled plasmid DNA from the linear bacterial chromosomal DNA, total DNA was centrifuged in 6 M

CsCl, 0.8 mg/ml ethidium bromide gradients in the type 70.1 rotor, for 48-60 hours at 45,000 rpm, 20° C. After centrifugation, the plasmid band was detected by brief irradiation with a hand-held 254 nm source, the tube was punctured with an 18 g syringe needle, and the plasmid DNA withdrawn. Ethidium bromide was removed by 5 extractions with isoamyl alcohol. The DNA was dialyzed against TE, pH 8.0, and precipitated with 0.1 volume 3 M sodium acetate and 2 volumes of ice-cold, non-denatured 95% ethanol.

C. Nucleic Acid Quantitation

Nucleic acids were quantitated by UV spectrophotometry. Absorbance measurements of an aliquot of the nucleic acid samples were taken at 260 nm and 280 nm. The absorbance of the solution at 260 nm is directly proportional to the amount of nucleic acid, and the 260/280 nm absorbance ratio is an approximation of the purity of the nucleic acid (Maniatis et al., 1982). Ideally, a ratio of 1.8 to 2.0 is desired.

D. Agarose Gel Electrophoresis

Agarose gels were used to assess the molecular weights of isolated DNA, the completion of restriction enzyme digests, separation of genomic restriction enzyme digests for Southern blot analysis, and for separating, sizing and checking the integrity of RNA molecules. Agarose concentrations (0.4-1.2%, w/v) were selected depending upon the size and conformation of the DNA or RNA to be analyzed. Electrophoresis grade agarose (Seakem ME, FMC Marine Colloids) was dissolved in a

given electrophoresis running buffer by heating to boiling in a microwave oven. Unless otherwise noted, the running buffer for DNA gels was 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH ~8.3 (1x TBE buffer), and the running buffer for RNA gels was 0.01 M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 6.8. The molten agarose solution was cooled to 50 degrees, and cast in either a BRL "Babygel" or "H4" apparatus or the FMC "Resolute" apparatus. The gel was allowed to solidify for 30 minutes, and then submerged in electrophoresis running buffer.

Aqueous DNA samples were mixed with a 10X loading buffer (50% glycerol, 0.25% bromphenol blue) and loaded into preformed wells, and electrophoresed at 1-15 volts/cm (distance between the electrodes). Molecular weight standards utilized included intact lambda phage DNA, lambda phage DNA digested with HindIII, 123 bp ladder (BRL), or the supercoiled DNA ladder (BRL). To stain DNA in the gel, ethidium bromide was either included in the buffer at a concentration of 0.5 $\mu\text{g}/\text{ml}$, or the gel was stained with this solution following electrophoresis. DNA was then visualized and photographic records of the gel were made using a Fotodyne UV-440/Polaroid MP-4 photographic transilluminator system.

The secondary structure of RNA must be removed to accurately size RNA molecules (Lehrach et al., 1977). Total cellular RNA samples were denatured by heating at 50°C for 1 hour in a mixture of 50% dimethyl sulfoxide (DMSO), 1M deionized glyoxal, 0.01 M sodium phosphate, pH 6.8. The

samples were chilled on ice, 1/6 volume of a 40% sucrose, 0.25% bromphenol blue loading dye was added, and the sample then was loaded onto the gel. The RNA was electrophoresed for 20 minutes to allow the RNA to enter the gel, at which time a Manostat ministaltic pump was turned on to recirculate the buffer during electrophoresis which prevents the formation of pH gradients in the gel. Electrophoresis was carried out at 3-5 volts/cm. Molecular weight markers were the 18 and 28 S rRNA bands. After electrophoresis, the marker lanes were cut off the gel and were de-glyoxalated by soaking the gel marker lanes in 0.05 M NaOH for 30 minutes at room temperature. The gel marker lanes then were rinsed with water, and stained with 0.5 $\mu\text{g/ml}$ ethidium bromide in 0.012 M Tris, 0.006 M sodium acetate, 0.0003 M EDTA (1x TAE). Photographic records were made as described above.

It is possible to calculate the size of any nucleic acid fragment, whether detected by hybridization to radioactive labelled gene-specific probes used in blot hybridizations, or detected by ethidium bromide staining the nucleic acids directly in the agarose gel. Linear, double-stranded DNA molecules, or denatured single stranded RNA molecules migrate through the gel matrix at rates that are inversely proportional to the \log_{10} of their size (Helling et al., 1974). By plotting the \log_{10} of the nucleic acid size markers in base pairs (bp) versus the distance migrated a linear relationship is observed. Thus, by knowing the distance migrated of any

nucleic acid fragment, and comparing it to the relative migration of a known set of nucleic acid size markers, it is possible to calculate the size of any sample in bp.

E. Gel Purification of DNA Fragments

Target DNA fragments were separated on agarose gels as described. Lanes included a single wide preparative lane, and two narrow marker lanes to the outside of the gel. After electrophoresis for appropriate separation of the target fragment(s) away from other DNA fragments, the target DNA was recovered using a combination of trough electroelution and electrophoresis onto a dialysis membrane. This procedure involved cutting a rectangular trough ahead of the band of interest, and inserting a piece of dialysis membrane over the forward surface of the gel, down the front wall of the trough, along the bottom of the trough, and under the back of the gel. The trough is filled with buffer and the DNA fragment electrophoresed through the trough and onto the dialysis membrane. The polarity of the gel was reversed for 1 minute to electroelute the DNA off the membrane. The buffer containing the DNA was removed, transferred to a 1.5 ml microfuge tube and extracted (1:1) sequentially with phenol, phenol/sevag, and sevag. The DNA in the recovered aqueous phase was then precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ice cold, non-denatured 95% ethanol. The recovered DNA was collected by centrifugation at top speed at 4°C for 30 minutes in a Beckman Model 11 microfuge. The

DNA was resuspended in deionized water and purified of any contaminants using a Elutip nucleic acid purification cartridge (Schleicher and Schuell, Keene, N.H.) following the manufacturer's instructions.

F. Nucleic Acid Blotting

Following separation by agarose gel electrophoresis, nucleic acids were transferred to a filter support membrane by a process known as nucleic acid blotting. The filter membrane then can be probed for specific nucleic acid sequences by using radiolabelled nucleic acid probes complementary to a specific sequence of interest. Many procedures are available, and the one used in this dissertation project is described below. The transfer of both DNA and RNA to filter membranes was performed essentially as described (Southern, 1975). The nucleic acids were transferred to Genescreen plus nylon membranes (NEN Research Products, Boston, Ma.) by capillary action using 10X standard saline citrate (SSC) as the transfer solvent (1x SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0). Briefly, a piece of Whatman 3MM paper was laid across a smooth solid support, which was placed above a tray containing 10X SSC. The piece of 3MM paper had both of its ends in the 10X SSC, and served as a wick to allow for the 10x SSC to act as a transfer solvent. The gel was laid upside down on the Whatmann 3MM paper and care was taken to remove any air bubbles between the 3MM paper and the gel. Next, the nylon membrane, cut to the

size of the gel, was carefully laid on the gel such that the nucleic acid binding side (side "B") of the filter was in direct contact with the gel. Again, care was taken to remove any air bubbles between the gel and the nylon membrane. Sequentially added on to the top of the membrane were: five pieces of 3MM paper cut to equal size, a stack of gel blot paper (GB004, Schleicher and Schuell, Keane, N.H.), a glass plate, and a weight of ~500 to 700 g. Before transfer of DNA or RNA, Genescreen plus nylon membranes were prepared for blotting. First, the membranes are wetted with deionized water for one minute and then 10x SSC for fifteen minutes. Immediately following nucleic acid transfer, membranes with RNA were immersed first in 0.05 M NaOH for 15 seconds and then 0.2 M Tris, pH 7.5, 1X SSC for 35 seconds. Membranes with transferred DNA were immersed first in 0.4 N NaOH for 45 seconds, and then 0.2 M Tris, pH 7.5, 2X SSC for one minute. Transfer of the nucleic acids to the membrane was visualized, and therefore assured, by briefly irradiating the filter with 254 nm light.

G. Hybridization Probes

A number of gene-specific probes were used in the analysis of northern and Southern blots. These probes were as follows: the 420 bp PstI exon 2 fragment of the human c-myc oncogene (Figure 2), the 1 kb PstI fragment of the viral fos oncogene (v-fos)(Figure 2), a 350 bp HindIII fragment containing exon 1 of the human N-ras oncogene (Figure 2), and

a 770 bp fragment of a chicken cDNA encoding β -actin. Additionally, a 1.7 kb probe for α -satellite DNA was used in Southern blot analysis. The β -actin and α -satellite probes were purchased as aqueous DNA solutions from Oncor, Inc. (Gaithersburg, Md.). The oncogene probes were isolated from plasmids originally purchased from ATCC (Colby et al., 1983, Curran et al., 1982, Taparowsky et al., 1983, Cleveland et al., 1980, Willard, 1987).

H. ^{32}P -Labelling of Hybridization Probes

Radiolabelling of the DNA probes from these gene-specific probes was done by utilizing the random primer protocol. (Feinberg and Vogelstein, 1983, 1984). In this protocol, the fragment to be used as a probe was released from the vector in which it was contained, by restriction enzyme digestion. Using a low gelling temperature agarose (Sea Plaque LE, FMC) known amounts of the DNA fragments were separated by electrophoresis, using 0.04 M Tris-acetate, 0.005 M sodium acetate as the electrophoresis buffer. The buffer contains 0.2 $\mu\text{g}/\text{ml}$ of ethidium bromide to visualize the DNA. The desired band was cut out of the gel and placed into a preweighed 1.5 ml microfuge tube. Deionized water was added to a ratio of 3 ml water to 1 gram of gel. The tube

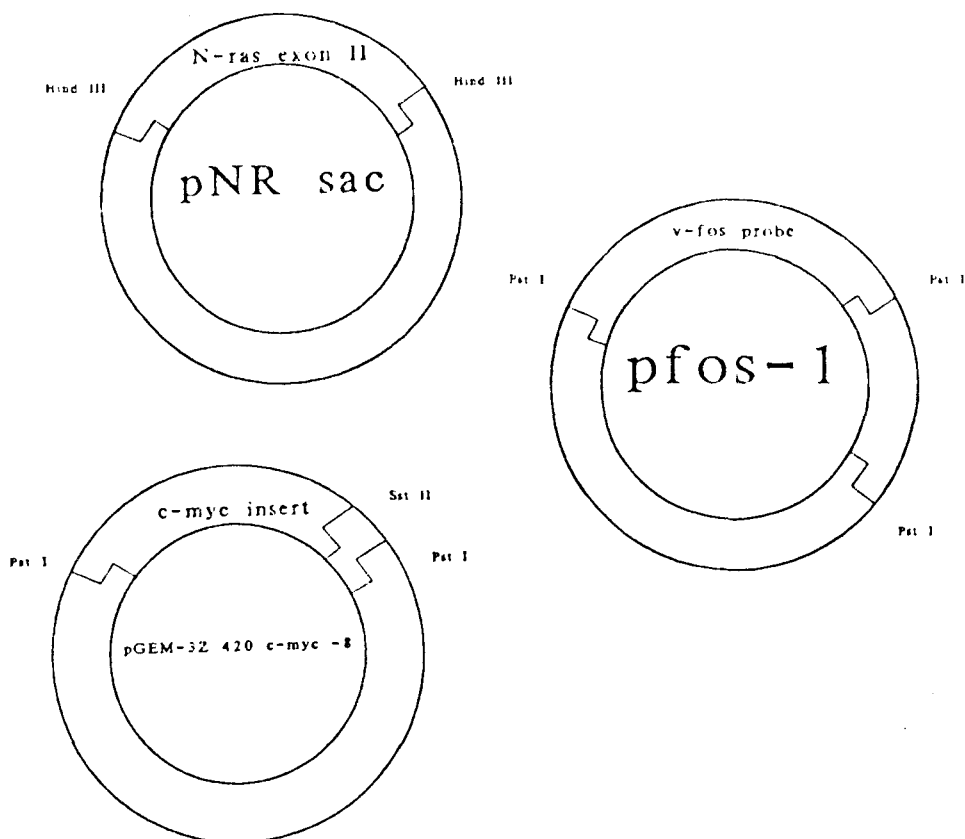


Figure 2. Maps of plasmids containing the restriction fragments used as probes for nucleic acid hybridization assays. The relevant restriction sites and the regions used as hybridization probes are noted.

containing the DNA was boiled for 7 minutes to melt the gel and denature the DNA, and then stored for subsequent use at -20°C.

The labelling reactions are carried out using the Prime-a-Gene labelling kit (Promega, Madison, Wi.) and α -³²P dCTP (NEN specific activity 3000 ci/mmol). Briefly, target DNA was boiled for 3 minutes to remelt the agarose and denature the DNA, and 25 to 40 ng of the DNA was added to the labelling reaction mixture. This mixture consisted of random sequence hexadeoxyribonucleotides, acetylated bovine serum albumin, nonradioactive dTTP, dGTP, dATP, and 50 μ ci of α -³²P dCTP. After the addition of the denatured target DNA, 4.5 units of the Klenow fragment of E. Coli DNA polymerase I was added. Radiolabelled probe DNA was synthesized because the random hexamers hybridize to the single-strand target DNA, and serve as primers for the synthesis of new DNA by the enzymic action of Klenow fragment.

Following a reaction time of 2 to 4 hours at room temperature, the polymerization reaction was stopped by the removal of unincorporated nucleotides by Sephadex G-50 gel filtration. G-50 quick spin columns were purchased from Boehringer Mannheim (Indianapolis, In.), and used following the manufacturer's instructions. Levels of incorporation were monitored by trichloroacetic acid (TCA) precipitation. Specific activity of the probes generated was typically 0.7 to 1.0×10^9 cpm/ μ g DNA.

I. Quantitation of Incorporation of ^{32}P into DNA Probes

The level of radioactivity incorporated into DNA probes was measured by trichloroacetic acid precipitation. One percent of the labelled probe was added to 25 μg of yeast transfer RNA and the nucleic acids were precipitated by the addition of 1 ml of cold 10% TCA and placed on ice for 10 minutes. The nucleic acid precipitate then was layered onto a Whatman GF/C glass fiber filter wetted with 10% TCA and positioned in a vacuum manifold (Millipore Bedford, Ma.). The tube was rinsed with cold 2% TCA, vortexed, and also layered onto the filter. Finally, the filter was washed with 95% ethanol, and placed in a scintillation vial. Eight ml of scintillation fluid (Ready Gel, Beckman) were added to the vial containing the filter, and the sample was counted in a Beckman Model LS 5800 liquid scintillation counter.

J. Nucleic Acid Filter Hybridization

Prehybridization of the membrane was done in an effort to minimize non-specific binding by the radiolabelled nucleic acid probe. For all probes, except the α -satellite DNA probe, the membrane was pre-hybridized by incubating in 50% formamide, 10% dextran sulfate, 5X SSPE (1X SSPE is 0.15 M NaCl, 0.01 M dibasic sodium phosphate, and 0.001 M EDTA, pH 7.4), 250 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, 1% sodium dodecyl sulfate (SDS), and 0.1X Denhardt's solution (0.002% Ficoll 400, 0.002% polyvinylpyrrolidone, 0.002% bovine serum albumin, Pentax Fraction V) at 42°C for 4-24 hours in a shaking water

bath. The filter pre-hybridization for the α -satellite probe was exactly the same as described above, except the salt concentration was reduced from 5X SSPE to 3X SSPE. Hybridization was carried out in the same pre-hybridization buffer to which approximately 1 million cpm/ml of ^{32}P labeled single stranded DNA probe had been added, for 18-24 hours at 42°C in a shaking water bath. After hybridization the membrane was washed once in 250 ml of 2X SSPE containing 0.1% SDS for 45 minutes at room temperature on a rocker platform. Except for filters hybridized with the v-fos probe, the membrane was then washed in a Disk Wisk membrane washer (Schleicher and Schuell, Keene, N.H.) in 0.1X SSPE at 60 to 65°C for 6.5 minutes. Filters probed for c-fos sequences received a final wash as described above, except 0.2X SSPE was substituted for 0.1X SSPE. After washing in the Disk Wisk, the membrane was blotted to dampness with Whatman 3 MM paper, heat sealed in plastic bags, and autoradiographed at -70°C for appropriate times.

K. Autoradiography

Detection of ^{32}P labelled nucleic acids on filter membranes, agarose gels, or DNA sequencing gels was achieved by autoradiography. Gels were wrapped in Reynold's plastic wrap and filter membranes were heat-sealed in plastic bags before exposure to X-ray film. In the darkroom, the sample was placed in an X-ray film holder and covered with one or two pieces of x-ray film. The X-ray film used was either Kodak

x-Omat AR or DuPont Cronex 10. The Kodak film was approximately four to five times faster than the DuPont film. Further increases in the sensitivity or speed of the film was accomplished by the use of either one or two calcium-tungstate-phosphor intensifying screens (DuPont Cronex Lightning-Plus). Films were exposed to x-ray film with or without screens at -70°C for various periods of time (hours to days). Films were developed in earlier experiments by hand, and in later experiments by an automatic X-ray film processor.

L. Densitometry

Estimations of relative amounts of nucleic acids detected by autoradiography were determined by densitometry. Autoradiographs were scanned on an LKB Ultrosan XL laser densitometer attached to an Epson FX-86e printer. Integration of the autoradiographic signals (area under the curve) was calculated by the densitometer, and relative amounts of nucleic acid were calculated. Efforts were made to scan at least two separate autoradiographic exposures from each individual experiment. Finally, due to the loss of linearity of the x-ray film, scanning overexposed autoradiographs was avoided (arbitrary absorbance units >2.5).

M. Filter De-Probing (Stripping)

Often filter membranes were stripped of the first hybridization probe to allow for a second hybridization of the same filter. Only membranes that remained damp can be

successfully stripped, hence the use of heat sealed bags for storage and autoradiography. Filters were stripped by immersing in a solution of TE, pH 7.5, 1% SDS heated to 90 to 100°C. The filters were heated in this solution for 30 to 40 minutes, then removed and blotted to dampness with Whatman 3 MM paper. The stripped filter then was autoradiographed to ensure complete removal of the probe.

Isolation of Total Cellular RNA and Measurement of Gene-specific mRNA

To minimize the activity and presence of RNAses, certain precautions were taken. Sterile, disposable plasticware was used whenever possible. Gloves were worn whenever tubes containing RNA were handled, or whenever solutions used for RNA preparation were handled. All solutions (except the guanidium lysis solution and ethanol) used for RNA preparation were treated with 0.1% diethyl pyrocarbonate for at least 12 hours at 37°C, followed by removal of the diethyl pyrocarbonate by autoclaving. Solutions were then sterile filtered through a 0.2 μm pore size filter.

The guanidium salt isolation procedure was used to purify total cellular RNA (Chirgwin et al., 1979). Control and drug-treated cells (15 to 20 x 10⁶) were pelleted by centrifugation in a Beckman Model TJ-6 table top centrifuge at 1800 rpm (800 x g) for 5 minutes at room temperature. The media was poured off and the cell pellet was washed with ice-cold phosphate

buffered saline (PBS). The cells were centrifuged and washed again in 1x PBS. The cell pellet was resuspended in 3.5 ml of a filtered (0.45 μm pore size) 4 M guanidium isothiocyanate, 0.02 M sodium acetate, 0.01 M dithiothreitol (DTT), 0.5% Sarkosyl solution. The chromosomal DNA was sheared by repeated passage through a 20 gauge syringe needle. The lysate was then either quick frozen in a dry ice/ethanol bath and stored at -85°C or centrifuged immediately. For RNA isolation, the lysate was layered onto a 5.7 M CsCl, 0.1 mM EDTA solution, and centrifuged in a Beckman SW-40Ti rotor at 35,000 rpm (218,000 x g), 20°C , for 16 hours. The supernatant was aspirated off, and the RNA pellet was resuspended in 350 μl of deionized water, transferred to a sterile 1.5 ml microfuge tube, and precipitated with 0.1 volume 3 M sodium acetate and 2.5 volumes ethanol. The RNA was pelleted in a Beckman Microfuge 11 by centrifugation at 12,000 x g for 30 minutes, at 4°C . The supernatant was removed, the RNA pellet was resuspended in a small volume of deionized water, and the RNA was quantitated by UV spectrophotometry. Equal amounts of total RNA isolated from control and drug-treated cells were size-fractionated by agarose gel electrophoresis, capillary blotted to nylon filter membranes, and probed for the expression of specific genes.

DNA Interstrand Crosslinking Studies

The induction and disappearance of nitrogen mustard-

induced DNA interstrand crosslinks were estimated within the genome overall and within specific genomic regions. These experiments were designed to first, determine if DNA interstrand crosslinks may be produced preferentially and/or removed from the transforming genes of human tumor cells, and second, to determine if DNA interstrand crosslinks may play a role in suppressing the production of mRNA transcripts from these genes.

Measurement of Gene-Specific DNA Interstrand Crosslinks

Gene-specific DNA interstrand crosslinks were measured using a DNA denaturation/renaturation gel electrophoresis plus Southern blot assay (Vos and Hanawalt 1987). With this technique, DNA isolated from drug treated and control cells was digested with a restriction enzyme, denatured under mild conditions and loaded onto a neutral agarose gel. The DNA restriction fragments that are crosslinked immediately renature and migrate as a double stranded molecule, but the non-crosslinked DNA migrates as single-stranded molecules. If the restriction enzyme used was chosen carefully, then an entire gene may be contained within a given fragment. Subsequent Southern blotting of the gel, followed by hybridization with a gene-specific probe allows for the detection of crosslinks within a specific gene. Furthermore, the frequency of gene-specific DNA interstrand crosslinks was calculated by densitometry by determining the fraction of

molecules migrating as a double-stranded molecule (crosslinked fragments) relative to the fraction migrating as single strand molecules (non-crosslinked fragments).

If it is assumed that HN2 produces DNA interstrand crosslinks randomly, then the frequency of DNA interstrand crosslinks in a given DNA fragment can be calculated from the Poisson distribution:

$$P_x = (m^x / X!) \times e^{-m}$$

where P_x is the probability of X interstrand crosslinks, m is the mean number of crosslinks per fragment and e is the natural constant. The fragments with no interstrand crosslinks is P_0 , and is the fraction of molecules that migrated as single-strand molecules. Conversely, the fraction of crosslinked fragments (fragments with one or more crosslinks which migrate as double-stranded molecules) corresponds to the complement of the zero class ($1-P_0$). Thus, the mean number of crosslinks per fragment is $-\ln P_0$ (Vos and Hanawalt 1987).

The nitrogen mustard-induced DNA lesion is a relatively unstable chemical adduct, and therefore efforts must be made to minimize the spontaneous degradation of this adduct, which can lead to a potential underestimation of DNA interstrand crosslinks. Earlier work (Kohn and Spears, 1967) demonstrated that the incubation of nitrogen mustard-crosslinked DNA in a 0.05 M sodium carbonate solution of $10 < \text{pH} < 10.8$ derivatizes nitrogen mustard-modified guanine bases to the formamido

pyrimidine form, and this form is essentially resistant to spontaneous degradation. The exposure of DNA to nitrogen mustard can cause spontaneous depurination of modified bases. The use of the standard DNA denaturant, NaOH, therefore creates potential problems because NaOH is known to cause DNA strand breaks at apurinic sites. NaOH-induced strand breakage will cause a decrease in double-stranded DNA fragments, and thus a decrease in the estimation of gene-specific DNA interstrand crosslinks.

In the present study, experiments were designed such that all cells from the various time points were lysed at the same time. This procedure obviated the concern that differential nitrogen mustard-induced spontaneous DNA degradation could occur, due to different periods of storage of the DNA from different time points. Another attempt to minimize drug-induced, spontaneous DNA degradation is to assay the DNA for interstrand crosslinks within 24 to 30 hours from the time of the cell lysis. Finally, to avoid the concern that NaOH denaturation may cause strand breaks in DNA isolated from drug-treated cells, a milder denaturing procedure was used; DNA was incubated at 65°C for five minutes in the presence of 65% formamide.

A. Isolation of High Molecular Weight DNA

Control and drug-treated cells (15 to 20 x 10⁶) were pelleted by centrifugation in a Beckman Model TJ-6 table top centrifuge at 1800 rpm (800 x g) for 5 minutes at room

temperature. The media was poured off, and the cell pellet was washed with ice-cold phosphate buffered saline (PBS). The cells were centrifuged and washed again in 1x PBS.

Following the second wash the cell pellet was resuspended in 7 mls of a cell lysis buffer consisting of 0.05 M $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$, 0.001 M EDTA, 0.5% N-Lauroyl Sarcosine (w/v), and 0.3 mg/ml proteinase K. The final pH of the lysis solution was 10.4, and was buffered well enough to maintain this pH throughout the cell lysis procedure. The cell lysate was incubated in a water bath at 37°C for 2 hours to ensure total cell lysis.

The nucleic acids were separated from the cell lysate by standard organic solvent extractions. The lysate was extracted once with an equal volume of phenol saturated with TE, pH 8.0, once with an equal volume of phenol/sevag (1:1), and once with an equal volume of sevag. The cell lysates were mixed with the organic solvents by gentle mixing for 5 to 10 minutes. The aqueous phase was separated from the various organic phases by centrifugation in a Beckman J-6M centrifuge at 2370 rpm (1600 x g), at room temperature for 10 minutes. In each extraction the aqueous phase was removed from the organic phase with a wide bore pipette. Following the final organic extraction, the nucleic acids were precipitated by the addition of 0.2 volumes of 11 M ammonium acetate and two volumes of ice cold, non-denatured 95% ethanol.

The precipitated nucleic acids were removed by spooling-

out with a pipette tip. Excess salt was removed from the nucleic acids by washing the pellet with 70% non-denatured ethanol. The nucleic acids were then resuspended in three ml of deionized water for one hour at 37°C. RNA was removed by digestion with 10 ug/ml RNase A (Sigma St. Louis, Mo.) for one hour, at 37°C. Following RNase A digestion, the protein was removed by one phenol/sevag and one sevag extraction, as described above. The DNA was then precipitated by the addition of 0.1 volumes of 3 M sodium acetate and two volumes of ice cold non-denatured ethanol.

The precipitated DNA was spooled-out and resuspended overnight in 350 μ l of deionized water at 4°C. To document that the isolation of high molecular weight DNA, aliquots of individual samples were electrophoresed on 0.4% agarose minigels in parallel with a known amount of intact lambda bacteriophage DNA. This allowed for the approximate determination of both the size and concentration of the sample DNA. DNA samples were used only if they are equal to, or larger than, the intact lambda phage DNA molecule (~49 kb).

B. Restriction Enzyme Digestion of Genomic DNA

The high molecular weight genomic DNA was then digested with a given restriction enzyme were done according to the manufacturer's specifications. The digestions were carried out for 2.5 to 3.0 hours at 37°C. At this time, complete restriction enzyme digestion was checked by electrophoresing an aliquot of each sample on a 0.8% agarose minigel. HindIII-

digested lambda phage was used as a DNA size marker, and was electrophoresed in parallel with the digested samples. Completely digested samples were extracted once with phenol/sevag and once with sevag. Phases were separated by centrifugation in a Beckman Model E microfuge for two minutes at top speed at room temperature. The DNA, recovered from the aqueous phase, was precipitated by the sequential addition of 0.1 volume of 3 M sodium acetate and two volumes of ice cold, 95% non-denatured ethanol.

C. DNA Denaturation-Renaturation Gel Electrophoresis and Southern Blot Analysis

The precipitated DNA was collected by centrifugation in a Beckman Model 11 microfuge for 30 minutes at 4°C. The supernatant was removed, and the DNA pellet was washed with 70% ethanol. The pellet was then dried in a Savant speed vac concentrator connected to a Virtis lyophilizer and a Precision vacuum pump. The dried DNA pellet was resuspended in a small volume of 0.01 M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ pH 7.0. Following spectrophotometric quantitation, the DNA samples were prepared for crosslinking analysis by agarose gel electrophoresis. Ten microgram samples of DNA isolated from control and drug treated samples were assayed by the DNA crosslinking gels. Samples were either electrophoresed as native, double-stranded DNA molecules, or were denatured immediately prior to loading onto the gel. DNA molecules that were not crosslinked migrate as single-stranded molecules, but those DNA fragments with

crosslinks immediately renature, and migrate as double-stranded molecules. To DNA samples electrophoresed as native double-stranded molecules, 0.1 volume of a 50% glycerol, 0.25% bromphenol blue gel loading dye was added. To DNA samples electrophoresed as single stranded DNA molecules, two-thirds volume of a denaturing loading dye was added, and samples were denatured by heating at 65°C for five minutes, and quick-chilled on ice prior to loading onto the gel. The DNA denaturing gel loading dye consists of 96% deionized formamide, 0.001 M EDTA, 0.1% bromphenol blue and 0.1% xylene cyanol. Native and denatured DNA samples were electrophoresed at 30 volts for 14 to 18 hours. Lambda phage DNA digested with HindIII was electrophoresed in parallel with the sample DNA, and served as a DNA size marker. The ethidium bromide-stained DNA was visualized on the UV transilluminator, and a photographic record was made of each gel. A fluorescent ruler was placed on the gel, next to the DNA size marker, and served as a measure of the relative mobility of the size marker fragments.

Following gel electrophoresis of the DNA, the gel was prepared for Southern blotting. The gel was first immersed in 1 liter of 0.25 M HCl and slowly rocked on a rocking platform for 15 minutes at room temperature. This served to depurinate the DNA in the gel matrix. The HCl was aspirated away from the gel, and was replaced with a DNA denaturation solution for 30 minutes at room temperature on the rocking

platform. This solution was comprised of 0.5 M NaOH, 0.4 M NaCl, and was used to fully denature the DNA and hydrolyze the DNA at apurinic sites. This treatment facilitates the transfer of large DNA molecules out of the gel matrix. After the alkaline wash, the solution was aspirated away and replaced with a neutralization solution for one hour. The neutralization solution was comprised of 0.5 M Tris, pH 7.5 and 3 M NaCl. During the post-electrophoresis gel treatments, the bromphenol blue and xylene cyanol in the gel loading dyes served as pH indicators, and their color changes are indicative that the solutions used penetrate the gel matrix. Bromphenol blue is yellow at acid pH, and blue at neutral and basic pH; xylene cyanol is blue at acid pH, and green at neutral and basic pH. Following the post-electrophoresis gel treatments, the DNA within the gel was transferred to nylon membranes by capillary blotting, and probed for sequences as described earlier.

Measurement of Genomic DNA Interstrand Crosslinks

The production and disappearance of DNA interstrand crosslinks in the total genome was measured by the crosslinking assay of the alkaline elution technique (Kohn et al., 1981). Exponentially growing Colo320 HSR are labelled by the addition of 0.02 $\mu\text{Ci/ml}$ ^{14}C -thymidine (sp. act. 56 mCi/mmol, NEN, Boston, Ma.) to their growth media for 24 hours. This was followed by growth in radioactive-free media

for 24 hours prior to drug treatment. This "cold chase" allows for all the ^{14}C label to be incorporated into long chain DNA. L1210 mouse leukemia cells, which were used as internal standards were labelled with $0.05 \mu\text{Ci/ml } ^3\text{H}$ -thymidine (sp. act. 20 mCi/mmol , NEN) 24 hours before use. L1210 cells have a doubling time of 12 hours, therefore the radiolabel was depleted from the media in the first 12 hours and the second 12 hours acted as the chase period.

Approximately 1.5×10^5 control, or drug-treated, Colo320 HSR cells (^{14}C) and 1.5×10^5 L1210 cells (^3H) were added to five ml of cold, complete media and irradiated with 300 rads of X-ray to introduce a fixed amount of DNA single strand breaks. The irradiated cells, which were kept on ice, were layered onto a $0.8 \mu\text{m}$ pore-size polycarbonate filter (Nucleopore, Ca.) using mild suction. The cells were immediately lysed with 2% SDS, 0.02 M EDTA, pH 10.0. DNA-protein cross links were removed by Proteinase K treatment (0.5 mg/ml), after cell lysis and prior to elution. For the alkaline elution of the DNA, a tetrapropylammonium hydroxide (RSA Corp. Ardsley, N.Y.) solution containing 0.02 M EDTA, 0.01% SDS, pH 12.1 was pumped through the filter at a rate of 2 ml/hr for 15 hours by a Gilson Minipuls 2 peristaltic pump. Five 3.0-hour fractions were collected, as was the liquid remaining in the lines or on the filter, and assayed for radioactivity by liquid scintillation counting using Beckman Ready Gel scintillation fluid and a Beckman LS 5800 liquid

scintillation counter.

After the amount of radioactivity of each fraction was determined, the fraction of ^{14}C retained on the filter was calculated and plotted on a double log scale against the fraction of ^3H retained on the filter. Thus, the ^3H -labelled L1210 cells serve as an internal control to monitor anomalies in pump speed and/or filters. Increasing amounts of ^{14}C retained on the filter relative to ^3H are indicative of DNA interstrand crosslinks. Apparent DNA interstrand crosslink frequencies (in rad equivalents) are calculated using the following formula:

$$\text{DNA Interstrand Crosslinks} = \{[(1 - r_0) / (1 - r)]^{1/2} - 1\} \times 300 \text{ rads}$$

where r_0 and r are the fractions of the ^{14}C -labelled DNA from drug treated and control cells remaining on the filter when 25% of ^3H -labelled DNA is retained on the filter (Kohn et al., 1981).

Measurement of Colo320 HSR DNA Synthesis

Experiments were performed to assess the effects of HN2 on ^3H -thymidine incorporation into the acid insoluble fraction of Colo320 HSR. This incorporation presumably reflects incorporation only into DNA (and not RNA) because thymidine was used. Initial experiments were performed to determine how

long cells maintain linear incorporation of the ^3H -thymidine, after the addition of the radioisotope into the growth media.

Exponentially-growing control Colo320 HSR cells ($4 - 5 \times 10^5$ cells/ml) were incubated with five μCi of ^3H -thymidine per ml of cell culture. Two aliquots of 1 ml were taken after incubation at 37°C for 30, 60, 90, and 120 minutes, respectively. Cells in the aliquots were pelleted by centrifugation (as described above), the media was removed, and the cells were resuspended in five mls of ice-cold 1x PBS, and placed on ice. The cells were then layered onto Whatman GF/C glass fiber filters, that had been previously wetted with ice-cold 1x PBS and placed on a Millipore vacuum manifold. The cells were then lysed by the addition of 10 mls of ice-cold 10% TCA, followed by 10 mls of ice-cold 2% TCA, and finally 10 mls of 95% ethanol. Filters were removed from the manifold, placed in scintillation vials, and dried for 15 minutes in an 80°C oven. Eight mls of Beckman Ready Gel scintillation fluid were added and the samples were counted in the Beckman LS 5800 liquid scintillation counter. These experiments demonstrated that ^3H -thymidine was incorporated into the DNA of exponentially-growing Colo320 HSR cells for at least 90 minutes after the addition of five μCi ^3H -thymidine per ml of growth media.

To assess the effects of HN2 on DNA synthesis in exponentially growing Colo320 HSR, cells were exposed to varying concentrations of HN2 for one hour at 37°C , and washed

free of drug. Cells were resuspended in warm media and 800 μ l aliquots of cells were combined with 200 μ l of warm media containing 5 μ Ci of ^3H -thymidine. Another 500 μ l sample was used to determine cell number. Cells were incubated for 60 minutes at 37°C, and then assayed for TCA-precipitable counts. This is done three times after drug exposure: from 0 - 1 hour after drug removal; 2.5 - 3.5 hours after drug removal; from 5 - 6 hours after drug removal. The ^3H CPM in the TCA-precipitable fractions was then determined. Values obtained for drug-treated samples were expressed as a percent of control corrected for differences in cell number.

In vitro N7 Guanine Alkylation Studies

A. Target DNA Preparation

Cesium Chloride purified, pGEM-3Z 420 c-myc (-8) DNA (Figure 3) was digested with PstI and the products of the reaction are electrophoresed on a 1.2% agarose gel. The 420 bp c-myc DNA fragment was eluted from the gel (as described above), and was 3' end-labeled by incubation (60 min, 37°C) with 15 units terminal deoxyribonucleotidyl transferase (Stratagene, La Jolla, Ca.) and 170 μ ci [α - ^{32}P] ddATP (5000 Ci/mmol; Amersham, Arlington Heights, IL) in a reaction buffer consisting of 0.140 M sodium cacodylate, 0.001 M cobalt chloride, and 0.0001 M dithiothreitol, pH 7.5 (Yousaf et al., 1984). Incorporation of the radioactive nucleotide was monitored by TCA precipitation. Following labelling and

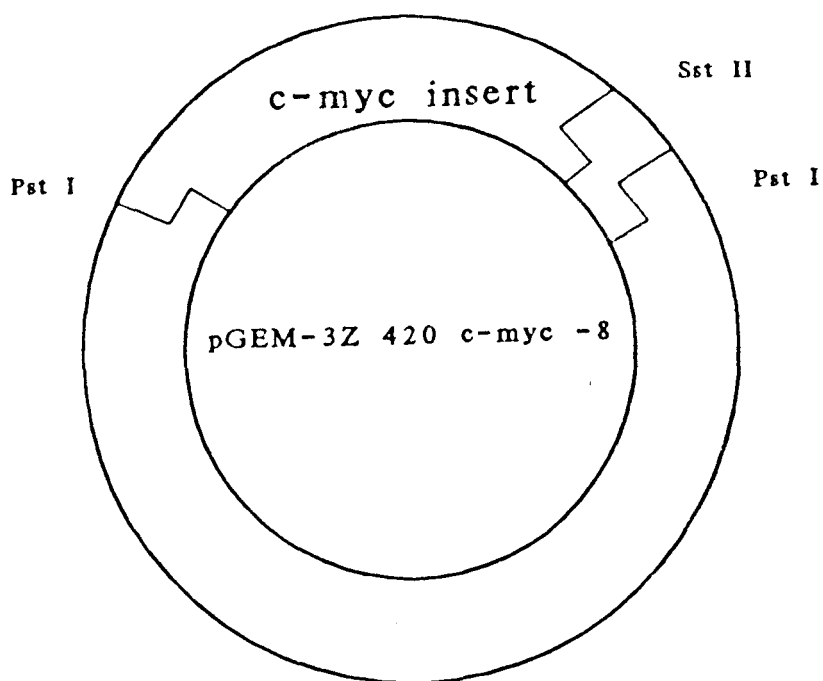


Figure 3. pGEM-3Z 420 c-myc +8 plasmid used for N7 guanine alkylation studies and Maxam and Gilbert DNA sequencing. Relevant restriction sites are noted.

ethanol precipitation, the DNA was digested with Sst II, resulting in 382 bp and 38 bp end labelled fragments. Unincorporated nucleotide and the the labelled 38 bp fragment were removed by G-50 gel filtration.

B. Chemical DNA Sequencing

DNA sequencing of the 382 bp 3' end-labeled c-myc fragment was accomplished using the chemical sequencing technique. The reaction conditions and components to which end-labelled DNA are exposed was exactly as previously described (Maxam and Gilbert, 1980). Briefly, all base-specific modification reactions are carried out at 15°C. The guanine-specific modification was carried out by the addition of 100% dimethyl sulfate (Kodak, Rochester, N.Y.) to a final concentration of 0.5% for seven minutes. Purine-specific modification was done by the addition of 90% formic acid (Sigma, St. Louis, Mo.) to a final concentration of 65% for 12 minutes. Pyrimidine-specific modification was accomplished by the addition of 100% hydrazine (Aldrich, Milwaukee, Wi.) to a final concentration of 50%. Cytosine-specific modifications also was performed by the addition of hydrazine to a final concentration of 50%, but reaction with thymine was suppressed by the addition of NaCl to a final concentration of 1 M. After base modification, stop solutions were added and nucleic acids were precipitated with three volumes of ice cold, non-denatured 95% ethanol, and stored at -70°C for five minutes. The base-modified DNA was pelleted by centrifugation

at 4°C for 15 minutes in the Beckman Model 11 microfuge. The pellet was resuspended in 250 μ l of ice cold 3 M sodium acetate, precipitated by the addition of three volumes of ice cold, non-denatured 95% ethanol, and stored at -70°C for five minutes. Base-modified DNA was collected by centrifugation, and the DNA pellet was washed free of residual salt by rinsing with 70% ethanol at room temperature. Strand breakage at sites of modified bases was achieved by incubation of the modified DNA with freshly-diluted 1 M piperidine (NEN, Boston, Ma.) at 90°C for 30 minutes. Piperidine was removed by lyophilization, and residual piperidine was removed by repeated washing of the pellet with deionized water and drying by lyophilization.

C. Drug Treatment of 420 bp c-myc Fragment in vitro

End-labeled, double-stranded DNA was added to TE pH 8.0 (final volume 100 μ l), and drugs were added to a final concentration of 0, 12.5, or 25 μ M L-PAM; 0, 2.5, or 5 μ M HN2; and 0, 50 or 100 μ M activated C2. The reactions then were incubated for 1 hr at 37°C. Because L-PAM and HN2 were stored as a stock in 0.1 N HCl, the pH was closely monitored to ensure a reaction pH of ~7 to 8. After drug incubation, 10 μ g tRNA and 0.1 volume of 3 M sodium acetate were added and nucleic acids were precipitated with three volumes of ice cold, non-denatured 95% ethanol, and stored at -70°C for five minutes. The drug-modified DNA was pelleted by centrifugation at 4°C for 15 minutes in the Beckman Model 11 microfuge. The

pellet was resuspended in 250 μ l of ice cold 3 M sodium acetate, precipitated by the addition of three volumes of ice cold, non-denatured 95% ethanol, and stored at -70°C for five minutes. Drug-modified DNA was collected by centrifugation, and the DNA pellet was washed free of residual salt by rinsing with room temperature 70% ethanol. The drug-modified DNA pellet then was lyophilized to dryness and resuspended in freshly-diluted 1 M piperidine. Cleavage of DNA at alkylated N7 guanines was accomplished by incubating the drug-modified DNA in the 1 M piperidine at 90°C for 15 minutes (Mattes et al., 1986). Piperidine was removed by lyophilization, and residual piperidine was removed by repeated washing of the pellet with deionized water and drying by lyophilization.

D. Polyacrylamide Gel Electrophoresis of DNA Damage and DNA Sequencing Products

End-labeled, drug-treated, chemically-cleaved DNA, as well as DNA products from the guanine-specific (G), purine-specific (G+A), pyrimidine-specific (C+T), and cytosine-specific (C) reactions of the Maxam-Gilbert protocol, were resuspended in 3 μ l loading solution (80% formamide, 0.01M NaOH, 0.001M EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue), heated at 90°C for 3 minutes, and chilled on ice for 1 minute. Approximately equal amounts from each group were loaded on a 6% denaturing polyacrylamide gel [acrylamide:bis-acrylamide (19:1), 8.3 M urea, in 1x TBE] and were electrophoresed for 3 hours at constant power (50 watts). The

duration of electrophoresis eliminated any DNA fragments derived from the end-labeled 38 bp Sst II-Pst I DNA fragment that were not removed by G-50 gel filtration. This resulted in adequate separation of end-labeled DNA fragments derived from the DNA region 60-200 nucleotides 5' to the labeled end of the 382 bp fragment. Following electrophoresis, the gel was dried under vacuum at 80°C on a Bio-Rad Model 483 slab dryer, and autoradiographed for appropriate periods of time at -70°C.

RESULTS

Effect of Nitrogen Mustard Exposure on Colo320 HSR Viability

Bifunctional alkylating agents are used clinically for their cytotoxic action. For this reason, in studies conducted in the context of this dissertation, drug exposures were chosen for their ability to reduce Colo320 HSR viability by at least 90%. Viability (or clonogenic survival) was measured by the use of the colony formation assay.

Initial attempts to use the colony formation assay were not successful. Untreated Colo320 HSR cells had a colony formation efficiency of only 10% ([cells that formed colonies/cells seeded] x 100). Colo320 HSR cells, capable of efficient colony formation in soft agar, were therefore selected by resuspending one of the colony-containing agar tubes in fresh media. Cells that grew from this initial seeding were cultured, and tested again for their ability to form visible colonies in soft agar.

These selected Colo320 HSR cells had a colony forming efficiency of approximately 75%. Thereafter, these "agar growing" Colo320 HSR cells were cultured. Aliquots of these cells were frozen in liquid nitrogen (cryovials labelled Colo320 HSR-A), and used for all subsequent studies.

The effects of bifunctional alkylating agents exposure on viability in exponentially-growing Colo320 HSR (treated at

$4-5 \times 10^5/\text{ml}$) were determined. The drug concentrations examined for L-PAM were from 0 - 35.4 μM , HN2 concentrations from 0 - 10 μM , and 4-HC concentrations from 0 - 100 μM . Figure 4 illustrates the concentration-dependent relationship between bifunctional alkylating agent concentration and Colo320 HSR viability. One hour exposure to 2.1 μM HN2, 12.5 μM L-PAM, and 40 μM 4-HC were approximately equitoxic and produced slightly greater than a one log cell kill (10% survival). The three log cell kill drug concentrations were 6.3, 25, and 80 μM for HN2, L-PAM, and 4-HC, respectively. These were the minimal exposures used for the studies described below.

In vitro Alkylation of N7 Guanine by Bifunctional Alkylating Agents

The N7 position of guanine in DNA is thought to be the critical site of reaction in the cell, its being the precursor to the cytotoxic DNA interstrand crosslink. These experiments were performed to determine the extent of alkylation of the N7 position of guanine in DNA by exposure to the bifunctional alkylating agents. This experimental approach allowed for the determination of potential differences or preferences of DNA sequence reactivity between the bifunctional alkylating agents.

The double-stranded 420 bp fragment of exon 2 of the c-myc oncogene was 3' end-labelled with ^{32}P -ddATP, and the DNA

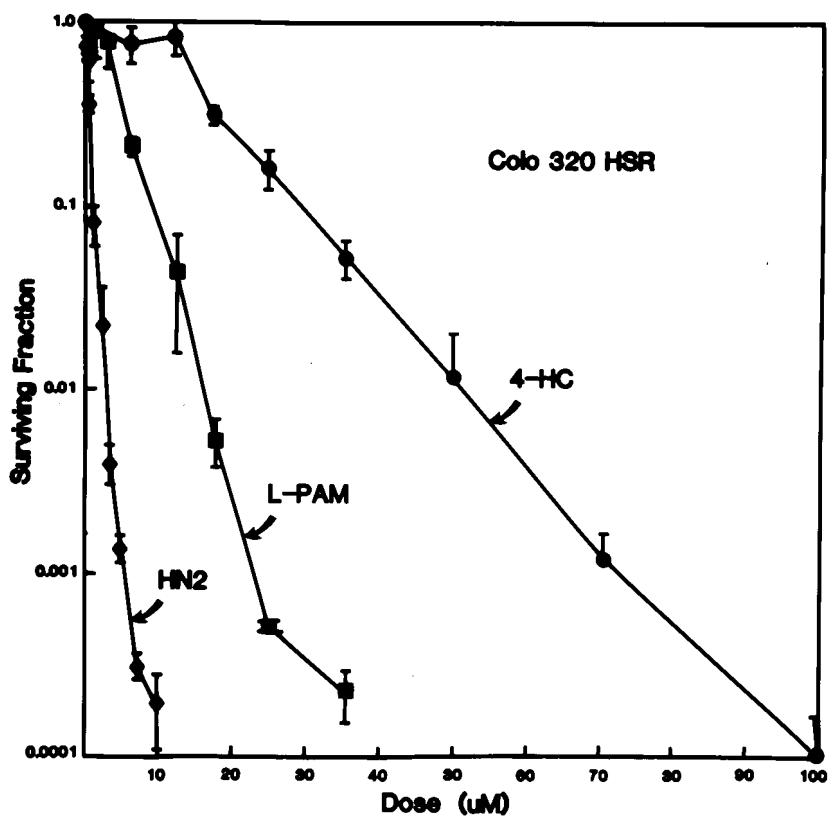


Figure 4. Colony formation ability of Colo320 HSR cells exposed to varying concentrations of HN2, L-PAM, and 4-HC for one hour at 37°C. Cells were washed free of drug, seeded into agar tubes, and colonies were scored 14 days later. Points and vertical bars are the means and standard deviations of three independent experiments.

fragment was exposed in vitro to 2.5 and 5.0 μM HN2, 12.5 and 25 μM L-PAM, or 50 and 100 μM C-2 for one hour at 37°C. The drug was removed, and the DNA was cleaved at sites of N7 alkylation by incubation with piperidine at 90°C. The resulting cleaved DNA fragments were electrophoresed in parallel with standard Maxam and Gilbert sequencing reactions. This allowed for the delineation of specific nucleotides attacked by the respective bifunctional alkylating agents along a portion of the sense strand (protein coding strand) of the c-myc gene. The autoradiograph of a representative experiment is shown in Figure 5. This figure illustrates the ability of the bifunctional alkylating agents to potentially attack any guanine nucleotide along the DNA template. It also is possible to get an indication of the relative reactivity of the bifunctional alkylating agents, based upon the amount (inferred from the autoradiographic signal) of full-length DNA molecules at the top of the gel because full length molecules indicate DNA that was not attacked by drug. Figure 6 shows an expanded view of the boxed area of Figure 5. This figure illustrates the sequence of the DNA template and the sites of N7 guanine alkylations from 106 to 131 nucleotides located 5' from the labelled 3' end of the c-myc sense strand. Clearly, every guanine in this tract was alkylated and no overt sequence specificity was discerned, however, differences did exist in overall reactivity of the drugs (L-PAM > HN2 > C-2).

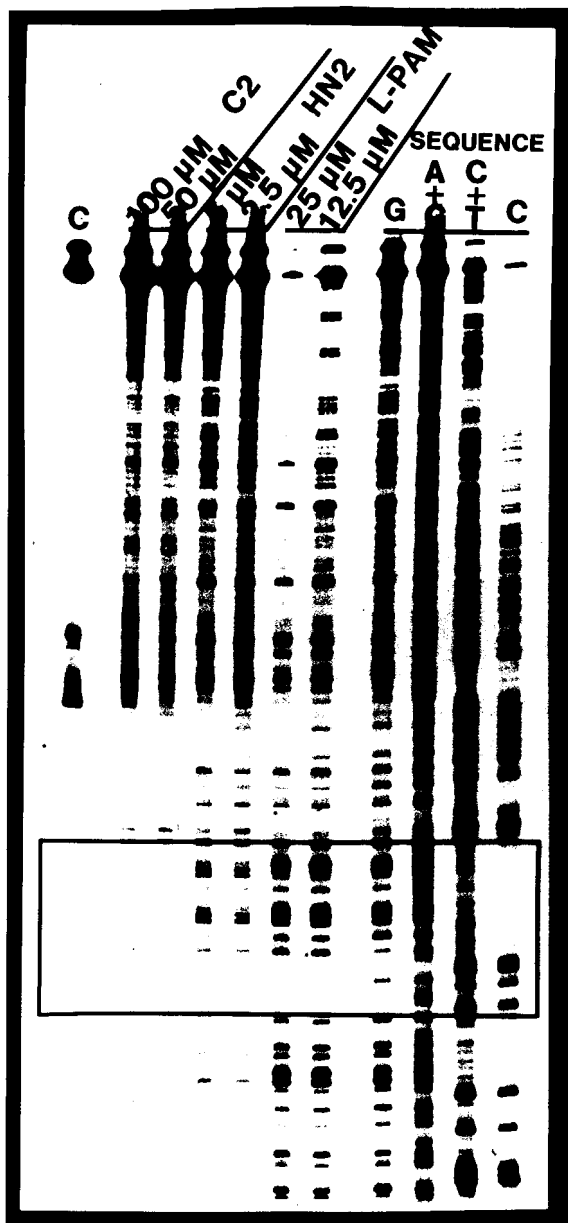


Figure 5. N7 guanine alkylations produced by C-2, HN2, and L-Pam *in vitro*. The 3'-end labelled 420 bp fragment c-myc exon 2 fragment was exposed to either 50 and 100 μM C-2, 2.5 and 5.0 μM HN2, or 12.5 and 25 μM L-PAM for one hour at 37°C. The drug-treated, end-labelled DNA was then cleaved at sites of N7 guanine alkylation by incubation with 1 M piperidine (90°C, 15 minutes). The resultant products then were electrophoresed in parallel with the standard Maxam and Gilbert DNA sequencing reactions (G, A+G, C+T, and C) on a 6% denaturing polyacrylamide gel. Following electrophoresis, the gel was vacuum-heat dried and autoradiographed.

Gene Expression Studies

Experiments were designed to test: 1) if bifunctional alkylating agents depress the steady state expression of proto-oncogene(s)/oncogene(s); and 2) if the three structurally-related compounds differ in their ability to elicit these changes.

Colo320 HSR cells were exposed, for one hour, to two concentrations of drug that produced one and three log cell kills. The drug was removed and the cells were resuspended in fresh media. Total cellular RNA was isolated from drug-treated cells immediately after drug removal (0 hour), and again 6, 12, and 24 hours after drug removal. Control total cellular RNA was obtained from untreated Colo320 HSR cells at the 0 hour time point. During the 24 hour time course, cell number was monitored. Drug-treated cell growth ceased; less than 20% increase in cell number was observed in any drug-treated sample. Approximately equal numbers of cells were lysed for total cellular RNA isolation at each time point.

The effect of nitrogen mustard exposure on the steady state expression of specific genes was assayed by Northern blot analysis. Relative amounts of gene-specific mRNA from drug-treated cells after drug removal were compared to mRNA levels in untreated, control cells. Equal amounts of total RNA from each sample were denatured with glyoxal and electrophoresed. Size-fractionated RNA was transferred by capillary action to nylon membranes, and hybridized with a ³²P-

labelled DNA probes specific for either c-myc, c-fos, N-ras, or β -actin mRNA. Following autoradiography of the hybridized filter, it was possible to get a reliable assessment of the relative amounts of specific mRNA in the respective samples, based on the signal intensity produced by the hybridized, labelled probe.

Precautions were taken to ensure reliable estimations of relative mRNA levels. From the outset, RNA quantitation must be precise and equal amounts of RNA must be carefully loaded onto the gel prior to electrophoresis. These manipulations were checked periodically. For an indication of RNA integrity and equality of RNA loading on the gel, RNA was electrophoresed, stained with ethidium bromide in the gel, and visualized by UV. Figure 7 is an example of such a gel. Colo320 HSR cells were treated with either 12.5 or 25 μ M L-PAM, and equal amounts (20 μ g/lane) of RNA from control and drug-treated cells were electrophoresed and stained. Examination of the 18s and 28s rRNA in the samples demonstrates that the RNA was intact and essentially equal amounts of RNA were loaded and electrophoresed. Additionally, after capillary blotting of the RNA, the homogeneity of transfer, integrity of the RNA and equality in gel loading was further checked by the visualization of transferred, filter-bound RNA with UV light.

To measure quantitatively signal intensity on autoradiographs, the concentration of the labelled probe in

the hybridization solution must be in excess with respect to the complementary target sequence on the filter. Otherwise, real differences in relative mRNA levels may not be observed. Experiments were conducted to determine if the probe concentration was in excess of the filter-bound sequences, under the hybridization conditions used in this study.

Incremental amounts of Colo320 HSR total cellular RNA were electrophoresed, transferred, and hybridized to ³²P-labelled probes specific for c-myc or N-ras. Figure 8 represents an autoradiograph of a filter with 5, 10 or 20 μ g of transferred, Colo320 HSR RNA per lane hybridized to a ³²P-labelled c-myc probe, under the hybridization conditions used in all subsequent experiments. Quantitation of the autoradiographic signal by scanning laser densitometry indicates that the c-myc probe is in excess of filter-bound c-myc mRNA contained in at least 20 μ g of total cellular Colo320 HSR RNA (figure 8b). Figure 9 is a picture of an autoradiograph of a filter with 20, 40, 60, or 80 μ g of transferred Colo320 HSR RNA per lane hybridized to a ³²P-labelled N-ras probe, under the hybridization conditions used in all subsequent experiments. Densitometry of this autoradiograph indicates that N-ras probe is in excess of filter-bound N-ras mRNA contained in at least 80 μ g of total cellular Colo320 HSR RNA.

Figure 10 shows the effects of L-PAM, 4-HC, and HN2 on c-myc steady-state transcripts, as measured by Northern blot

analysis. Each agent produced an initial increase in c-myc transcripts immediately following drug removal. Densitometric measurements of each autoradiograph indicate this to be an approximate 2 - 3 fold increase in steady state transcripts of this highly overexpressed gene. At 6 and 12 hours after drug removal, c-myc transcripts consistently were reduced following drug exposures which produced 1 and 3 log cell kills. These exposures produced approximately 2.5 and 5-fold reductions in transcript levels. By 24 hours, c-myc transcript levels approached control levels.

The effects of drug treatment on steady state transcripts of c-fos are shown in Figure 11. Like c-myc, c-fos expression was elevated immediately following drug removal. In this case, levels of c-fos transcripts were seen to increase 3- and 4-fold, for 1 and 3 log cell kill exposures, respectively. At 6 to 24 hours after drug exposure, c-fos levels returned to, or fell below, the very low basal levels observed in control cultures.

Rapid increases in c-myc and c-fos mRNA occur in many cell types following external stresses, and are most often associated with proliferative stimuli. In the experiments presented here, however, an increase is seen following exposure to cytotoxic agents with anti-proliferative actions. Experiments were performed to determine if these increases are as rapid as those seen following proliferative stimulation. Figure 12 illustrates the effects of 25 μ M L-PAM on c-myc and

c-fos expression after 15, 30, and 60 minutes following the addition of L-PAM. Increases in the steady state expression of both c-myc and c-fos can be seen within 15 minutes of drug addition. These increases, which were observed following cytotoxic insult, occur in the same time frame that increases in c-myc and c-fos occur following proliferative stimulation.

Because of cell manipulation during the 24 hour time course (i.e. washing of cells to remove drug, addition of fresh media), experiments were performed to determine if c-myc transcript levels changed after a mock drug treatment. Figure 13 shows Colo320 HSR c-myc mRNA levels over a 24 hour time course, after a mock drug treatment. Cells continued to grow exponentially over the 24 hour time course, but equal amounts of cells were lysed at each time point. Essentially no alterations in steady state c-myc mRNA levels were detected, and the small differences (< 20%) between the time points is probably due to experimental variability.

In Figures 14 and 15, the effects of drug treatment on N-ras and β -actin expression are shown. N-ras is expressed at low levels in untreated Colo320 HSR. The three alkylating agents employed in this study appeared to have no discernible effect on N-ras expression over the 24 hour time course. These compounds had little or no effect on the expression of the constitutively expressed "housekeeping gene" β -actin.

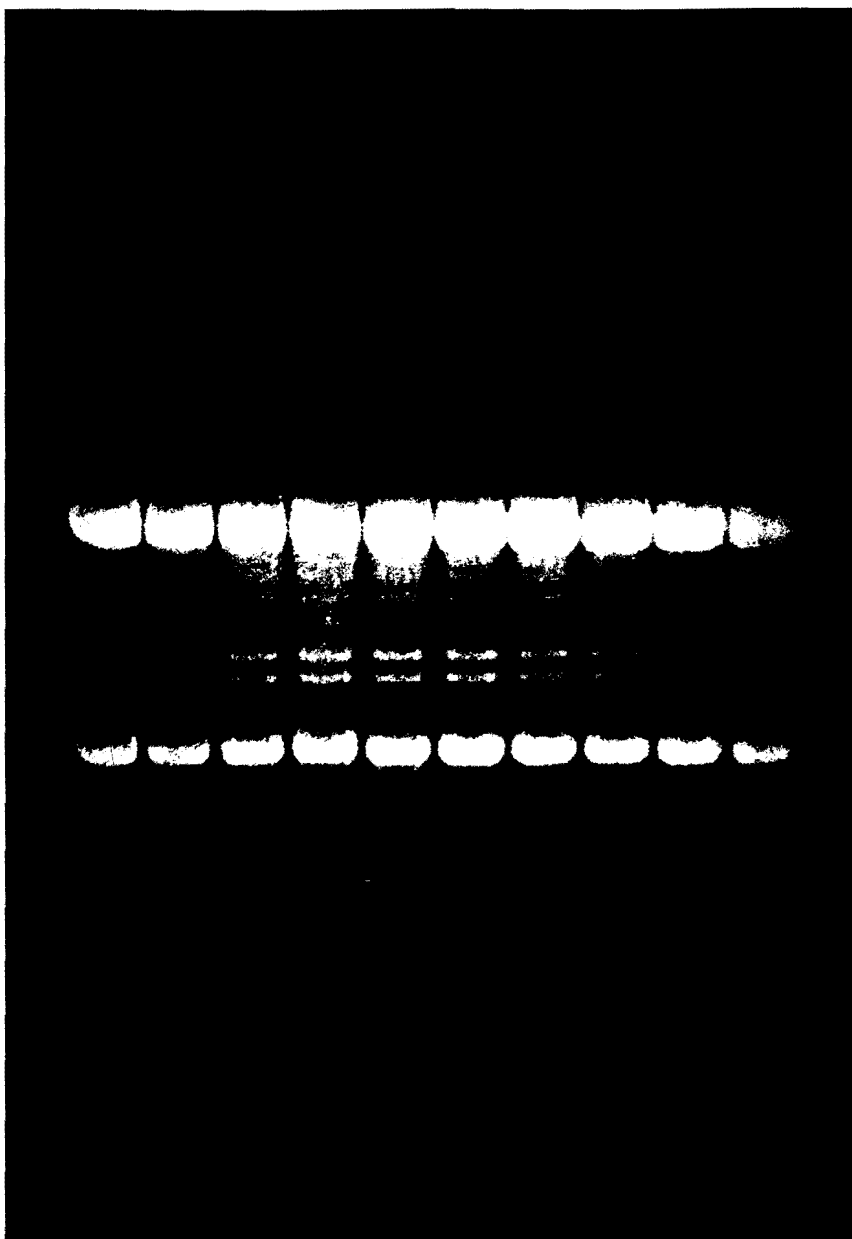


Figure 7. Ethidium bromide stained 1% agarose gel of 20 μg of glyoxalated total cellular RNA isolated from control and drug treated (L-PAM) cells. The gel lanes are in the same order as the subsequent Northern blot autoradiographs.

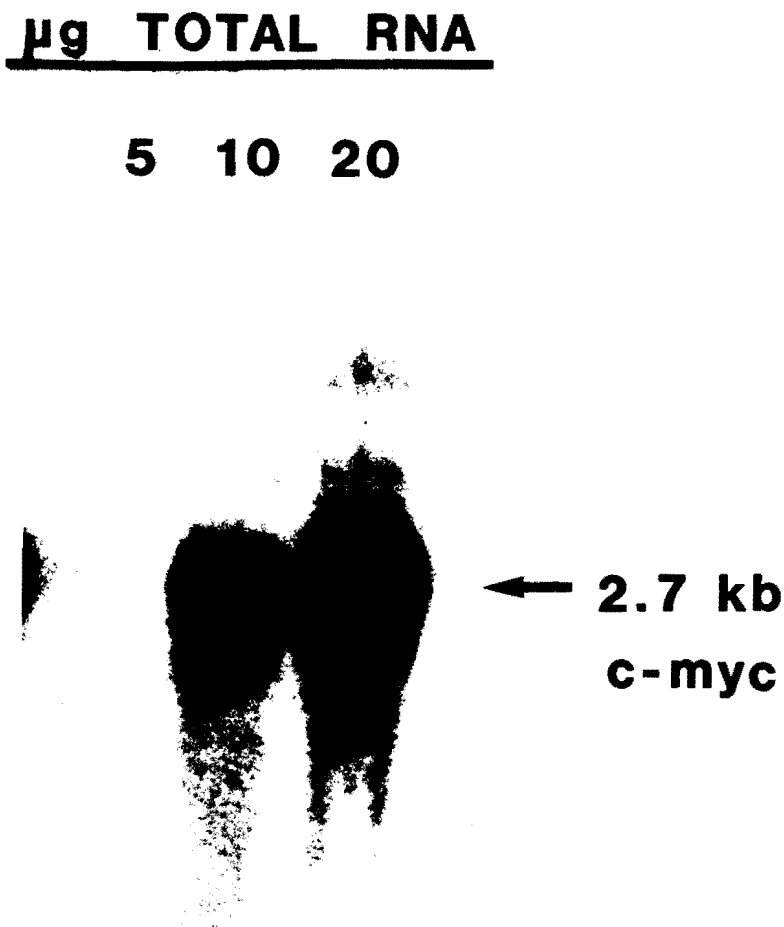


Figure 8. Northern blot analysis of relative levels of c-myc mRNA levels in varying amounts of total cellular RNA isolated from untreated Colo320 HSR cells. Either 5, 10, or 20 μ g RNA samples were size fractionated on a 1% agarose gel, capillary transferred to nylon filters, and probed with the 420 bp c-myc probe. Densitometry of c-myc autoradiographic signals in the different lanes indicated that the autoradiographic signal increased proportionally to the amount of filter-bound RNA in each lane.

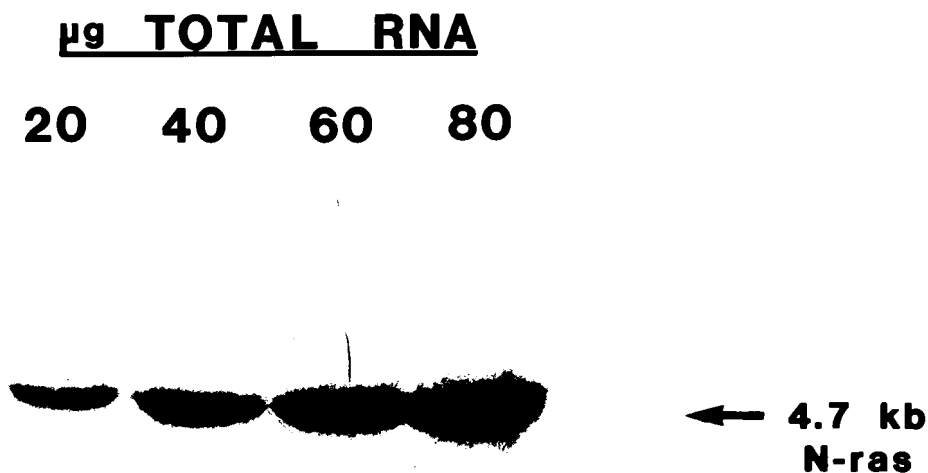


Figure 9. Northern blot analysis of relative levels of N-ras mRNA levels in varying amounts of total cellular RNA isolated from untreated Colo320 HSR cells. Either 20, 40, 60, or 80 μg RNA samples were size fractionated on a 1% agarose gel, capillary transferred to nylon membranes, and probed with the 350 bp N-ras probe. Densitometry of N-ras autoradiographic signals in the different lanes indicated that the autoradiographic signal increased proportionally to the amount of filter-bound RNA in each lane.

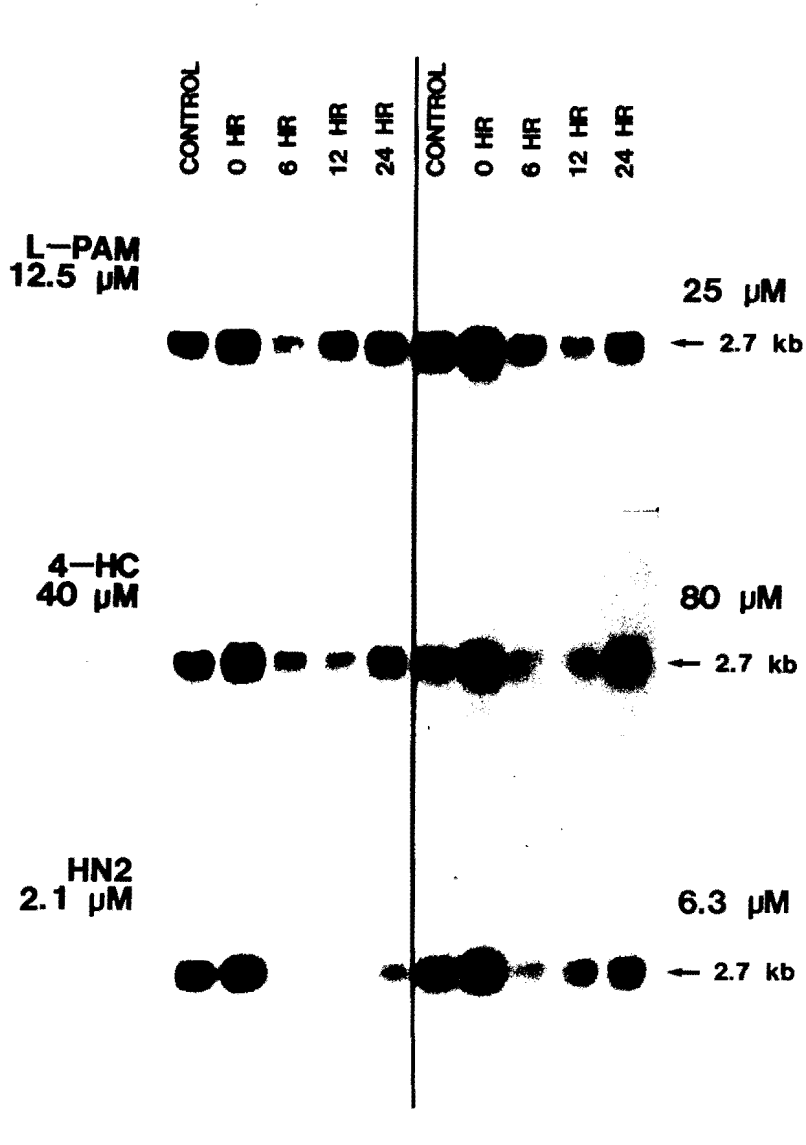


Figure 10. Northern blot analysis of the effect of alkylating agents on the expression of the 2.7 kb *c-myc* mRNA. Total cellular RNA was isolated at specified times after an one hour drug exposure. 20 μg RNA samples were denatured, size fractionated on 1% agarose gels, capillary transferred to nylon filters, and probed with the 420 bp *c-myc* probe. Exposure times of the autoradiographs was less than five hours using intensifying screens.

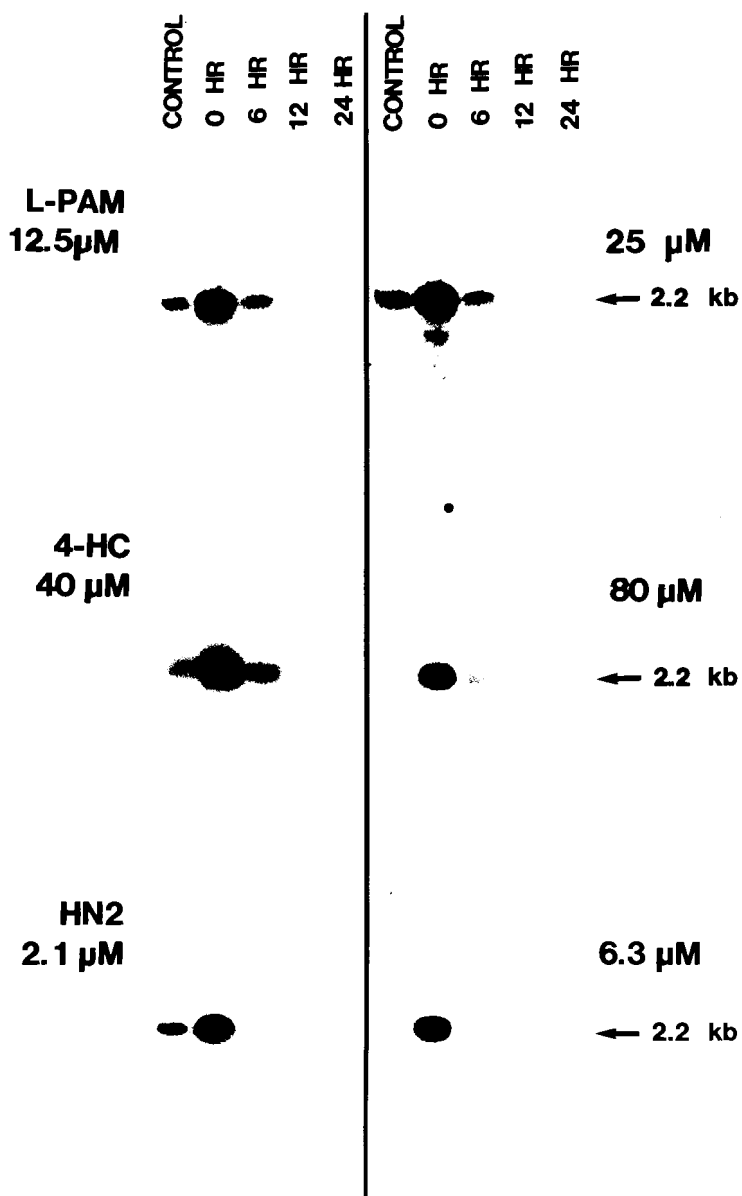


Figure 11. Northern blot analysis of the effects of alkylating agents on the expression of the 2.2 kb c-fos mRNA. Total cellular RNA was isolated at specified times after an one hour drug exposure. 40 μg RNA samples were denatured, size fractionated on 1% agarose gels, capillary transferred to nylon filters, and probed with the 1 kb v-fos probe. Exposure times of the autoradiographs was three to four days with intensifying screens.

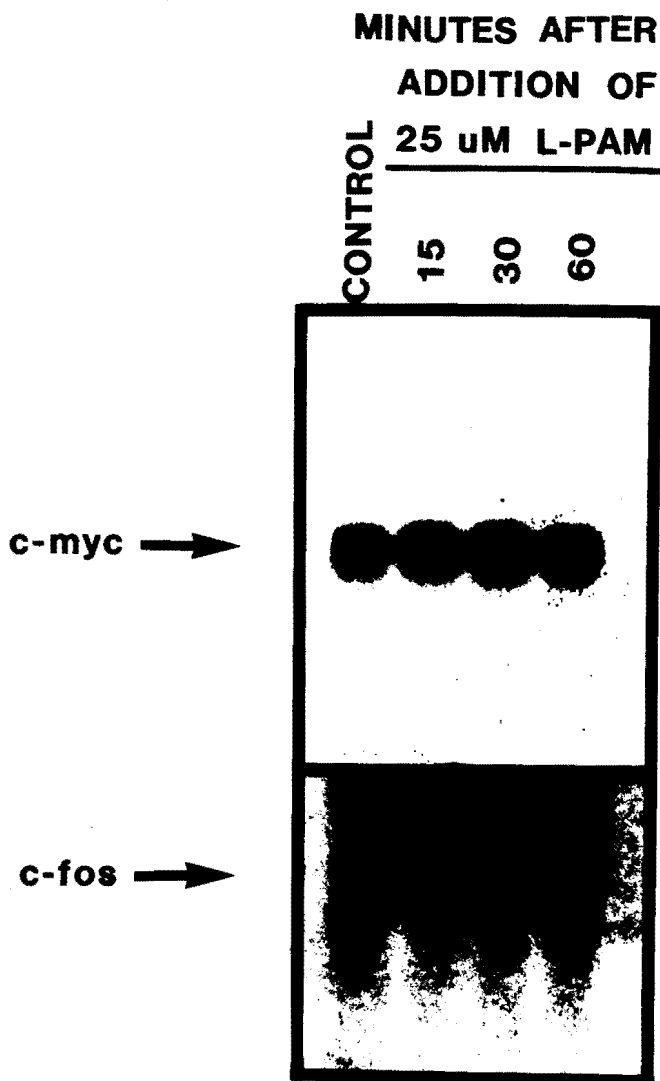


Figure 12. Northern blot analysis of the effects of L-PAM on the expression of c-myc and c-fos mRNA. Total cellular RNA was isolated at specified times after the addition of 25 μ M L-PAM. Either 20 μ g (c-myc) or 40 μ g (c-fos) RNA samples were denatured, size-fractionated on 1% agarose gels, capillary transferred to nylon filters and probed with either the 420 bp c-myc probe or the 1 kb v-fos probe. Exposure times of the autoradiographs was less than five hours for the c-myc northern blots and three days for the c-fos northern blots.

MOCK-TREATED COLO320 HSR

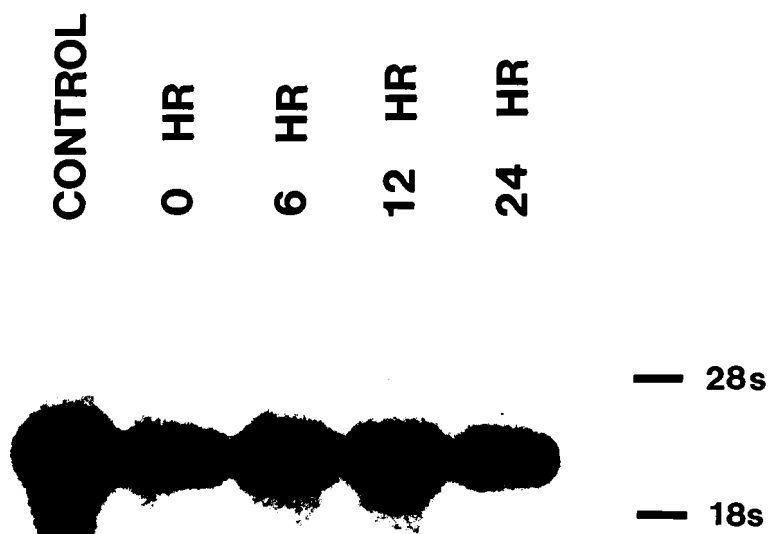


Figure 13. Northern blot analysis of c-myc mRNA levels over the 24 hour time course, following a mock-drug treatment. Total cellular RNA was isolated, and 20 μg RNA samples were size fractionated on 1% agarose gels, capillary transferred to nylon filters, and probed with the 420 bp c-myc probe. Exposure times of the autoradiographs was less than five hours with intensifying screens.

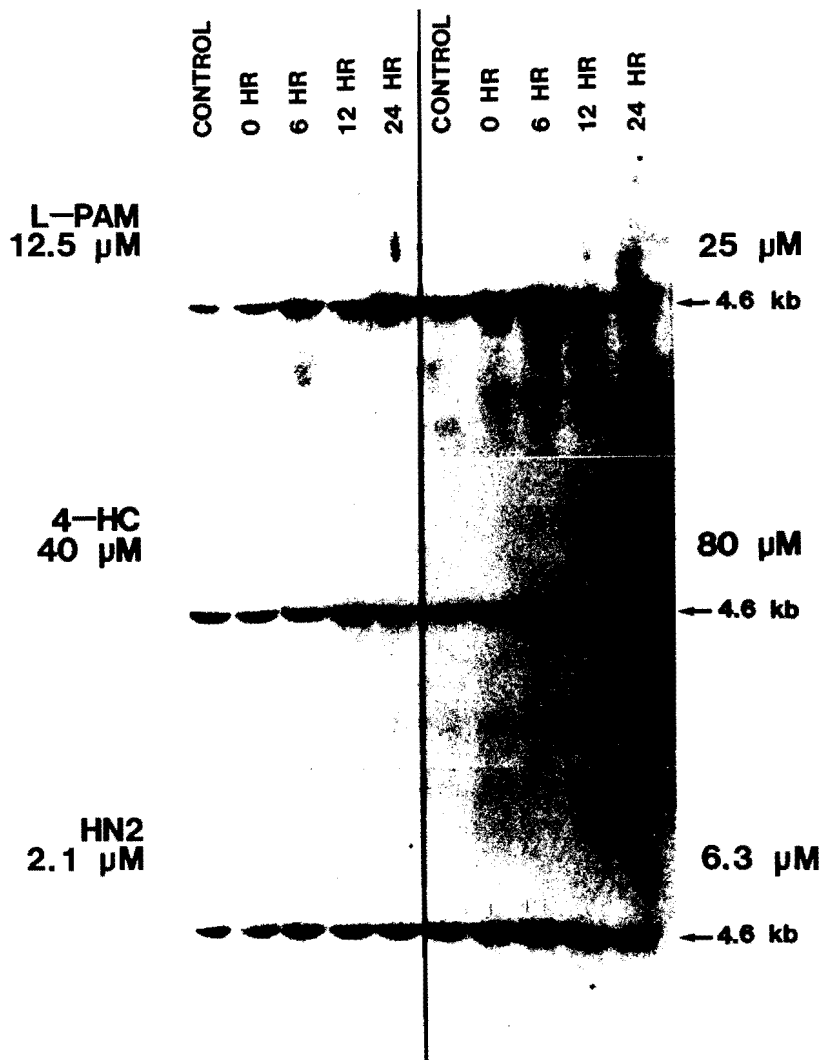


Figure 14. Northern blot analysis of the effects of alkylating agents on the expression of the 4.6 kb N-ras mRNA. Total cellular RNA was isolated at various times after an one hour drug exposure. 40 μ g RNA samples were size fractionated on 1% agarose gel, capillary transferred to nylon filters, and probed with the 350 bp N-ras probe. Exposure times of the autoradiographs was four to five days using intensifying screens.

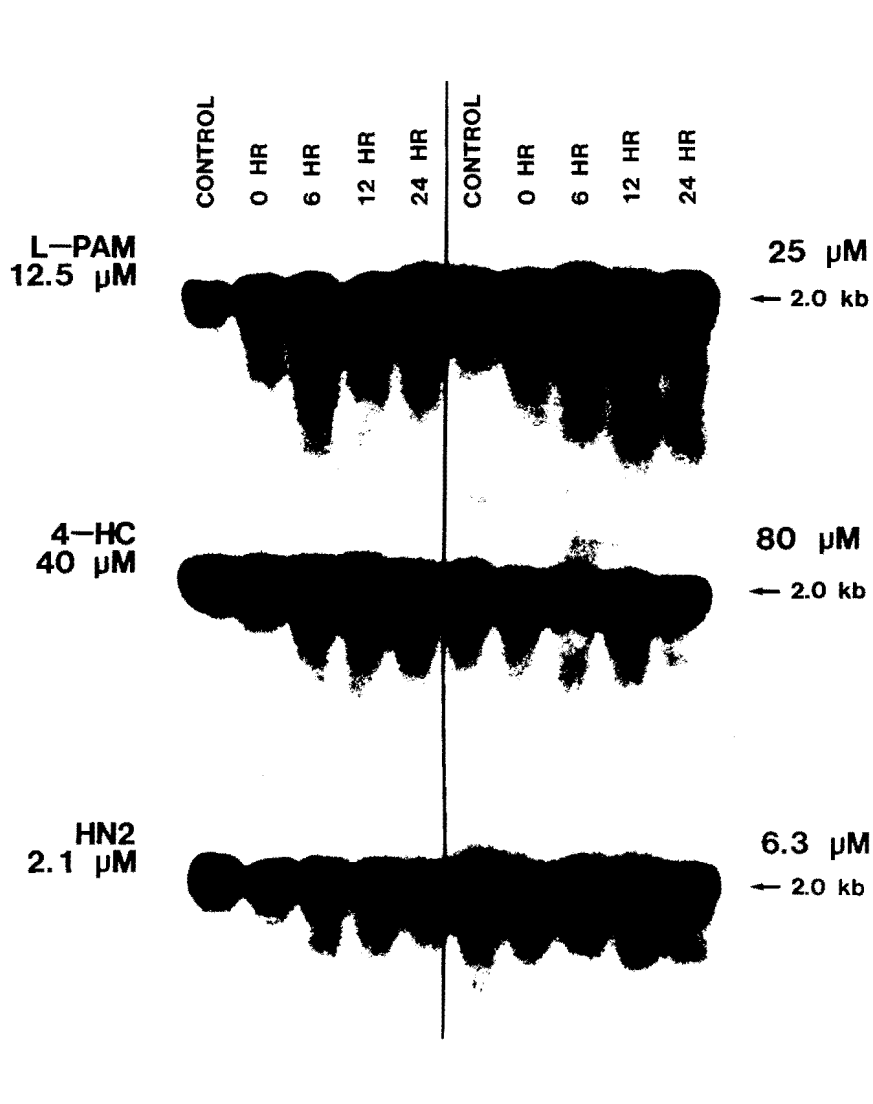


Figure 15. Northern blot analysis of the effects of alkylating agents on the expression of the 2.0 kb β -actin mRNA. Total cellular RNA was isolated at various times after an one hour drug exposure. 20 μ g RNA samples were size fractionated on a 1% agarose gel, transferred to nylon filters, and probed with the 770 bp β -actin probe. Exposure times of the autoradiographs were less than 12 hours using intensifying screens.

HN2-Induced DNA Interstrand Crosslinks in Colo320 HSR

The temporal relationship between the changes in oncogene expression, and the induction and removal of DNA interstrand crosslinks, was examined. Over the 24 hour time course, total genomic DNA interstrand crosslinks were measured by the alkaline elution technique. Gene-specific DNA interstrand crosslinks were measured by a Southern blot analysis which was modified by the use of DNA denaturation/renaturation gel electrophoresis.

Alkaline Elution Studies

Exponentially-growing Colo320 HSR cells (at $4-5 \times 10^5/\text{ml}$) were exposed to varying concentrations of HN2 (2.1, 6.3, or 12.6 μM); L-PAM (6.25, 12.5, or 25 μM); or 4-HC (25, 50, or 100 μM) for one hour. Control and drug-treated cells were resuspended in fresh media, and assayed for DNA interstrand crosslinks at 0, 6, 12 and 24 hours after drug removal.

In Figures 16 - 19, the relative levels of DNA interstrand crosslinks produced by different concentrations of L-PAM over the 24 hour time course are shown. These plots demonstrate that the level of DNA interstrand crosslinks produced is concentration dependent. Furthermore, visible differences in the relative levels of DNA interstrand crosslinks at the different time points are apparent, and indicate that length of post-drug exposure time is necessary in the production and removal of DNA interstrand crosslinks.

Figures 20 - 23 and 24 - 27 also are time- and concentration-dependent plots of the alkaline elution assay performed on Colo320 HSR exposed to varying concentrations of 4-HC and HN2, respectively. Like L-PAM, the relative levels of DNA interstrand crosslinks produced by 4-HC and HN2 are concentration-dependent, and are influenced by post-drug exposure incubation times.

The relative levels of DNA interstrand crosslinks were converted to quantitative rad equivalents. Rad equivalents are the apparent amount of X-rays (in rads) by which DNA interstrand crosslinks slow the elution rate of DNA from drug treated cells exposed to 300 rads compared to untreated cells exposed to 300 rads. Figure 28 shows the level of DNA interstrand crosslinks (in rad equivalents) produced by each of the three doses of the three drugs at 0, 6, 12, and 24 hours after drug removal. Clearly, these compounds produce DNA interstrand crosslinks in a concentration-dependent fashion. Both 4-HC and L-PAM produce their maximal number of DNA interstrand crosslinks in Colo320 HSR cells approximately 6 hours after drug removal. In contrast, HN2 produces its maximal number of DNA interstrand crosslinks immediately after drug exposure. Six hours after drug removal, however, Colo320 HSR cells exposed to HN2 had similar levels of DNA interstrand crosslinks (as measured by rad equivalents) as did the Colo320 HSR cells exposed to either 4-HC or L-PAM. By 12 hours after drug exposure HN2-induced DNA interstrand crosslinks were

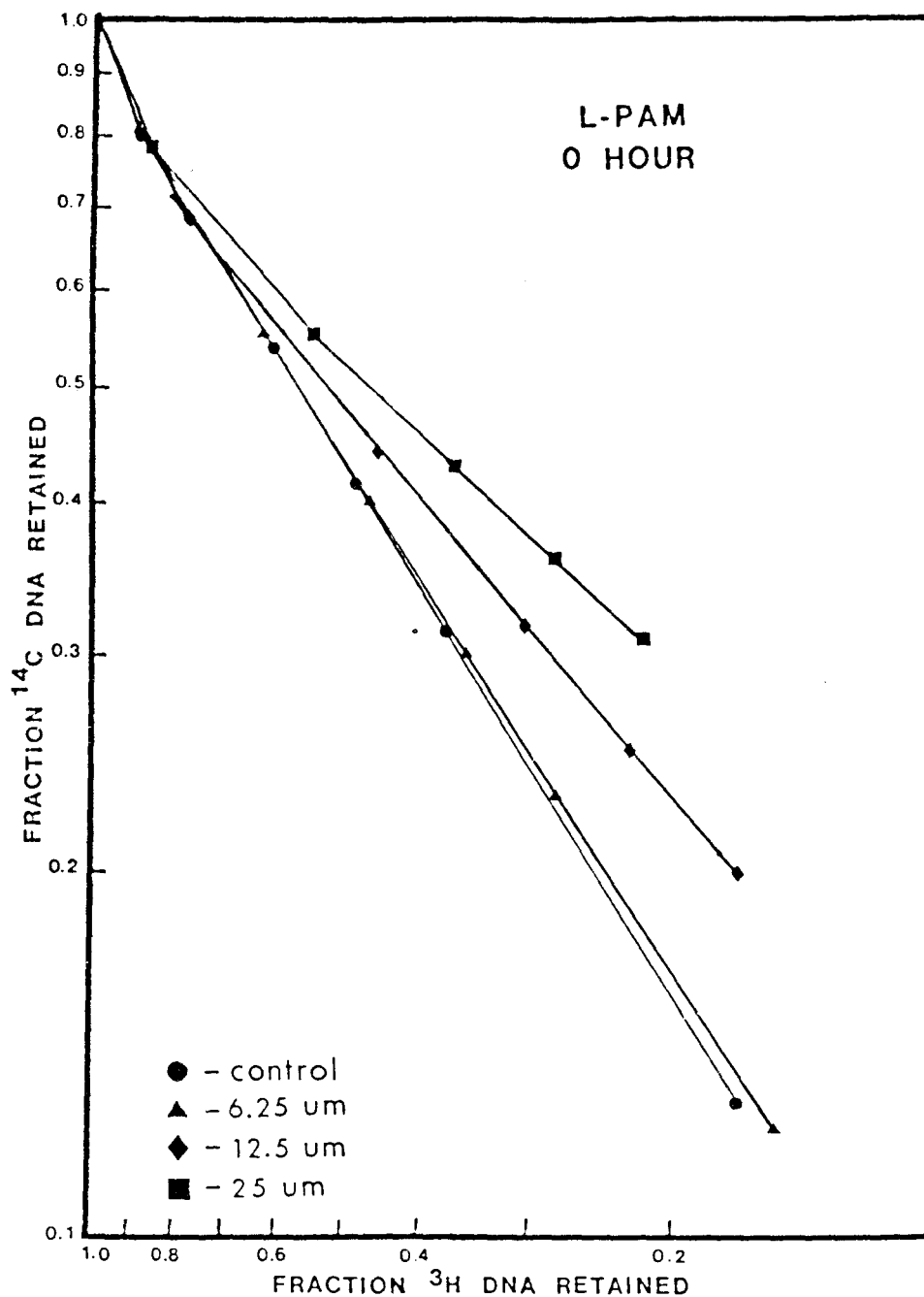


Figure 16. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of L-PAM, and assayed for DNA interstrand crosslinks at 0 hours after L-Pam exposure.

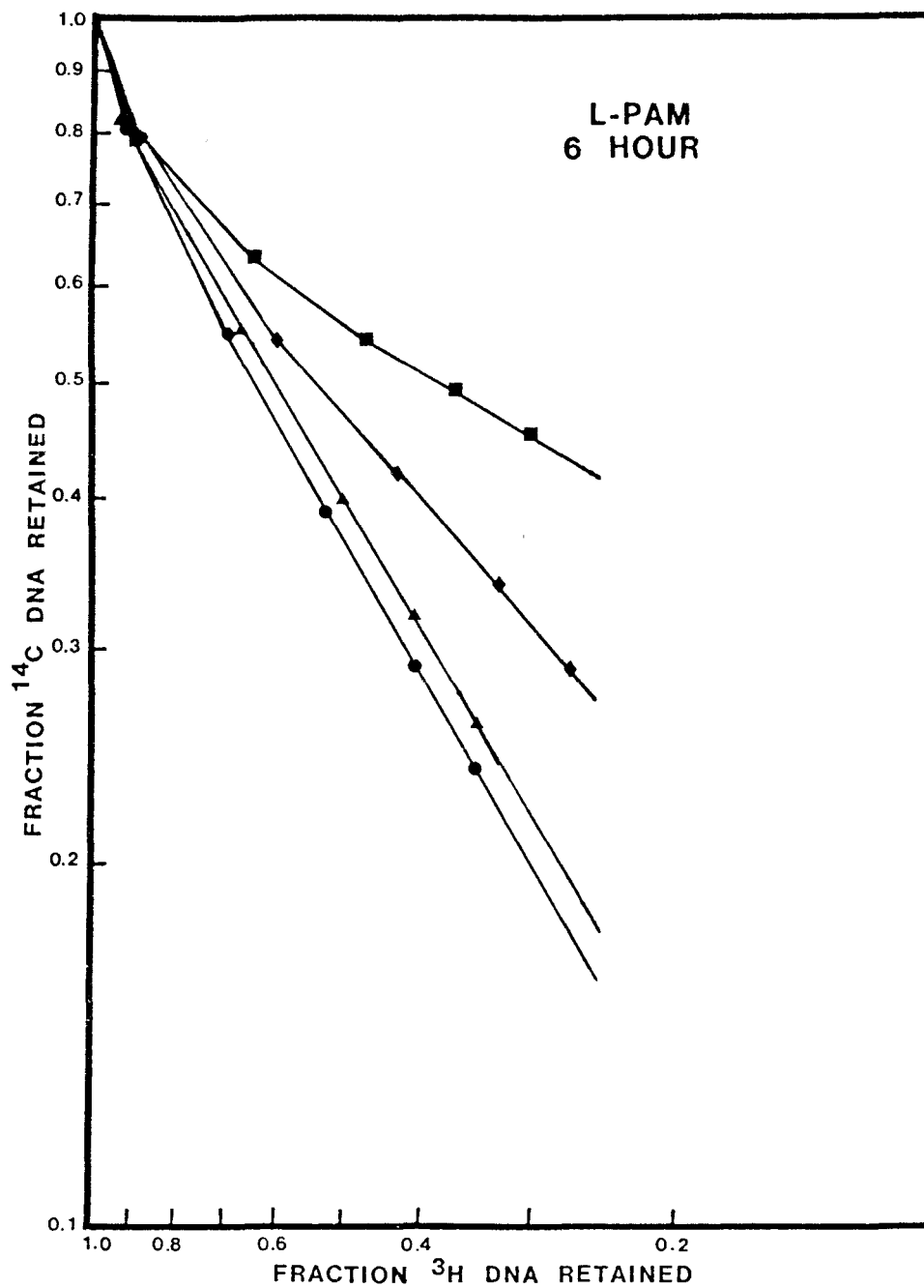


Figure 17. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of L-PAM, and assayed for DNA interstrand crosslinks at 6 hours after L-Pam exposure.

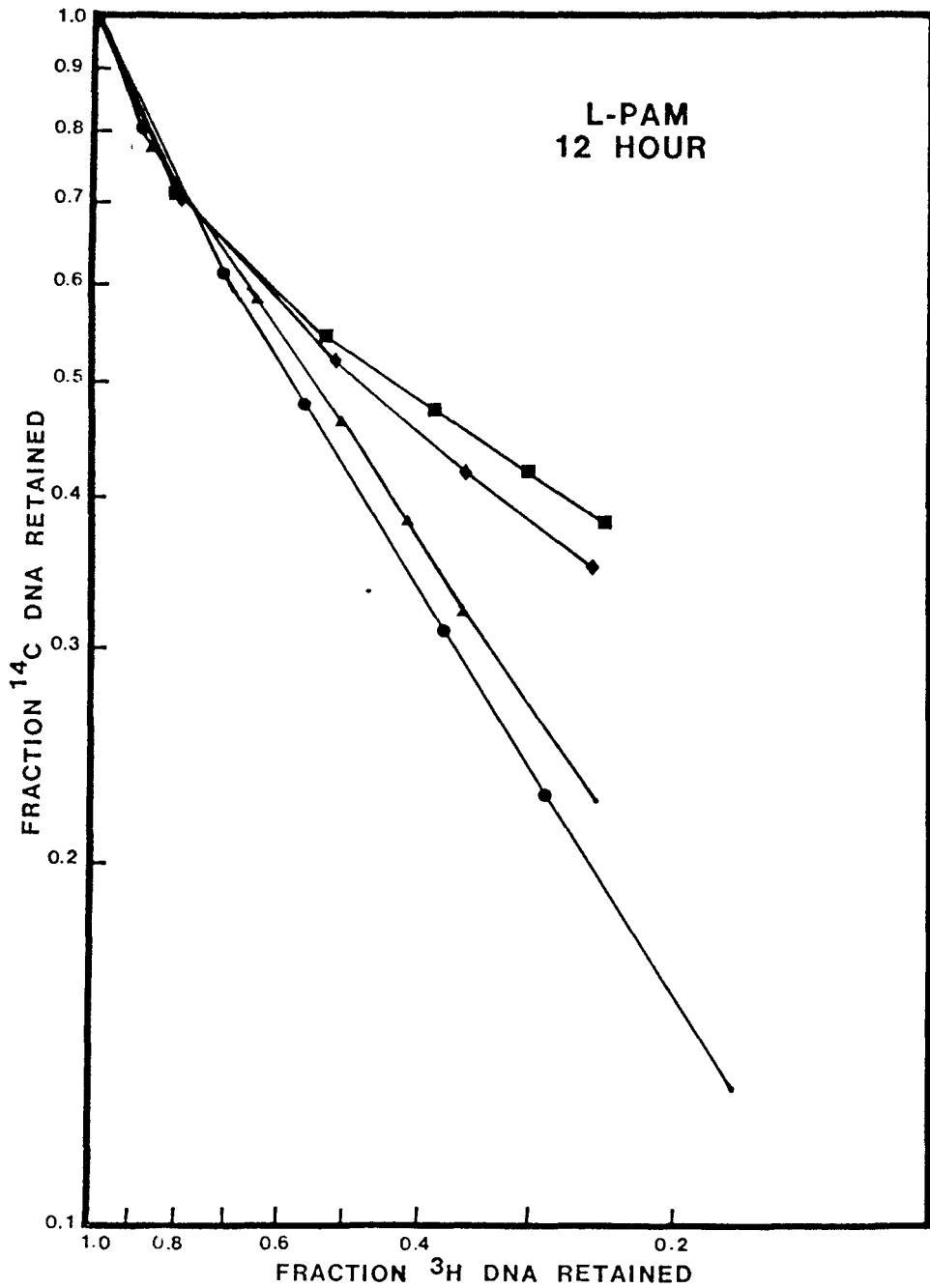


Figure 18. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of L-PAM, and assayed for DNA interstrand crosslinks at 12 hours after L-Pam exposure.

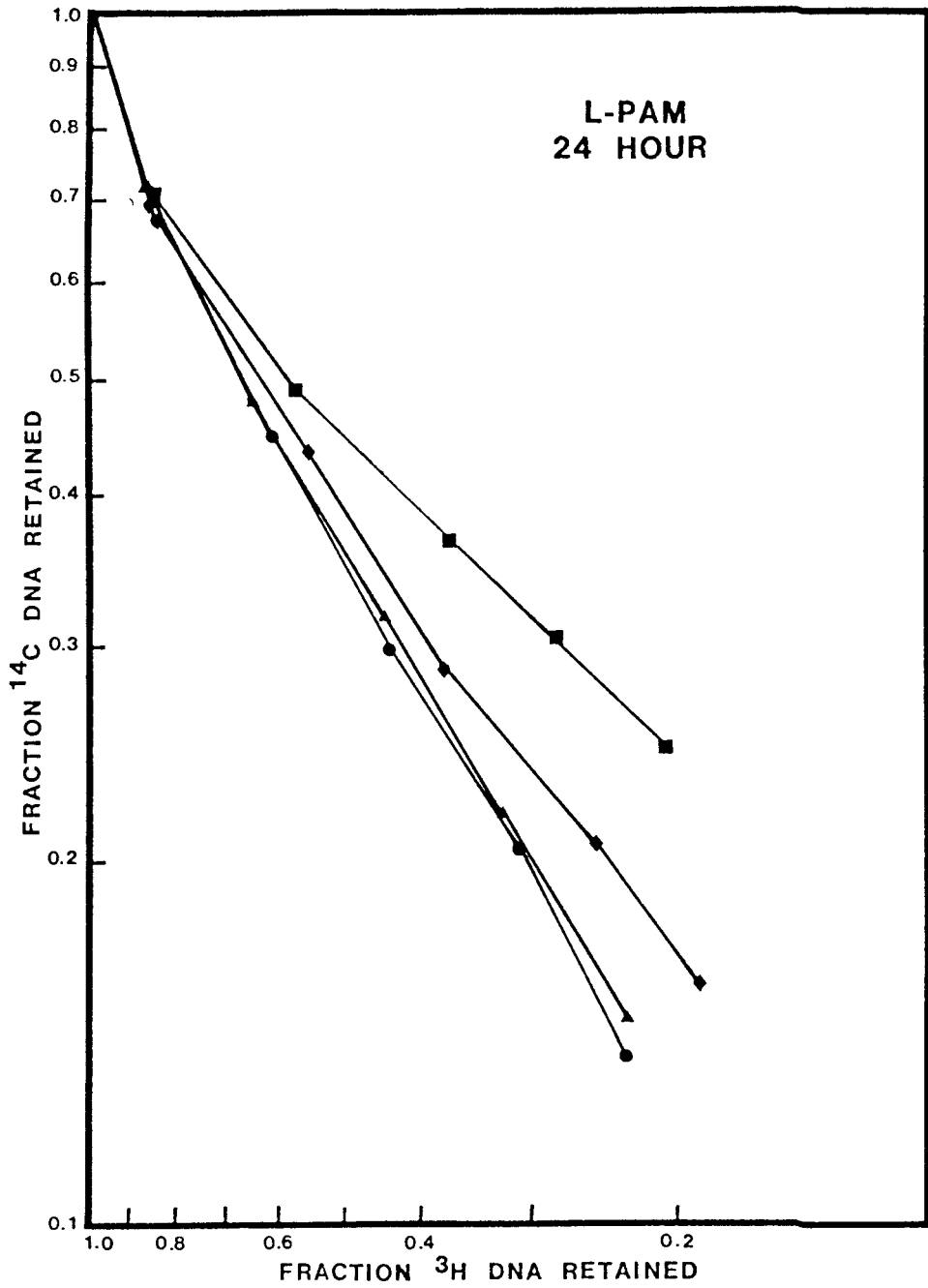


Figure 19. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of L-PAM, and assayed for DNA interstrand crosslinks at 24 hours after L-Pam exposure.

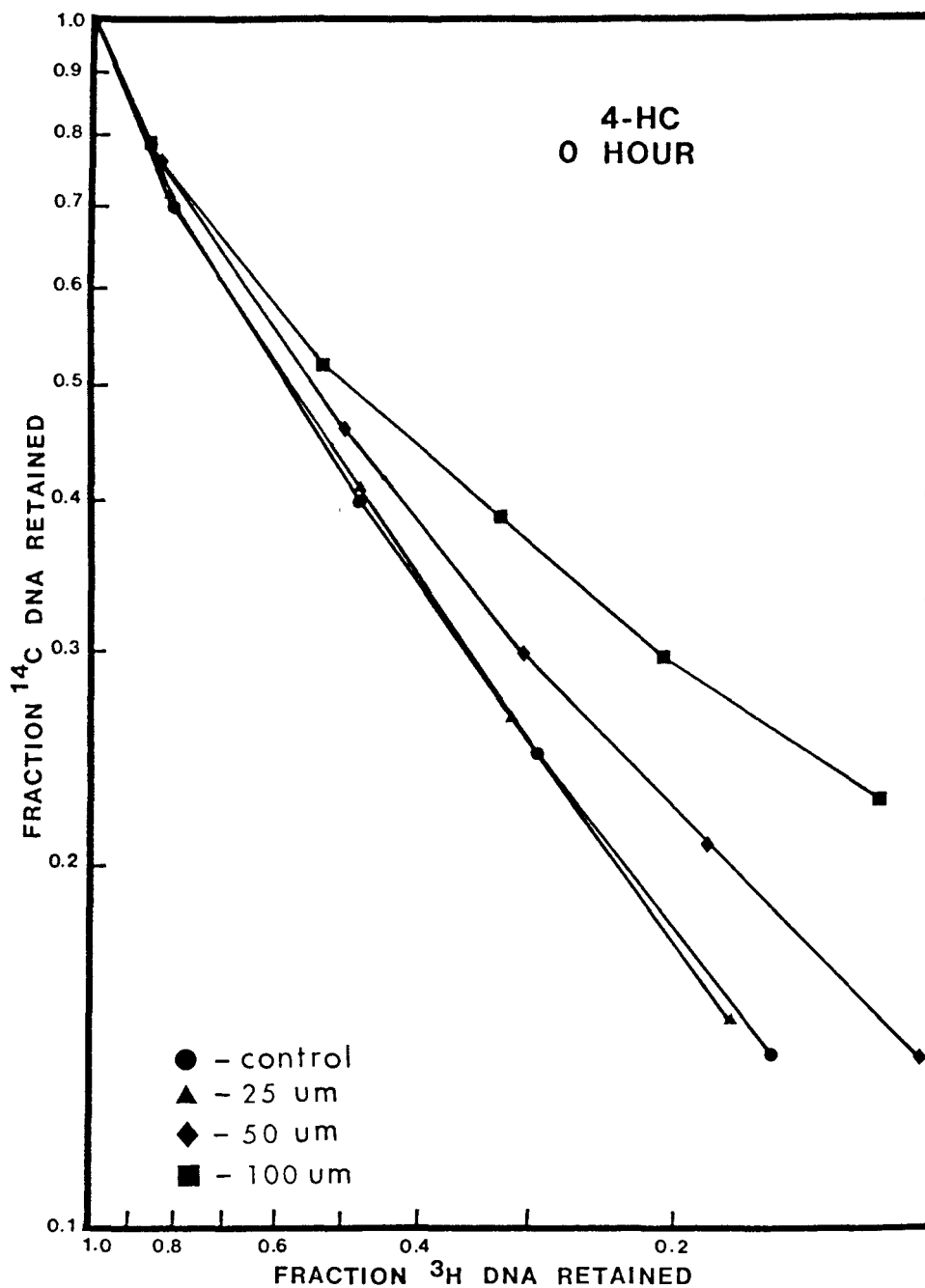


Figure 20. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of 4-HC, and assayed for DNA interstrand crosslinks at 0 hours after 4-HC exposure.

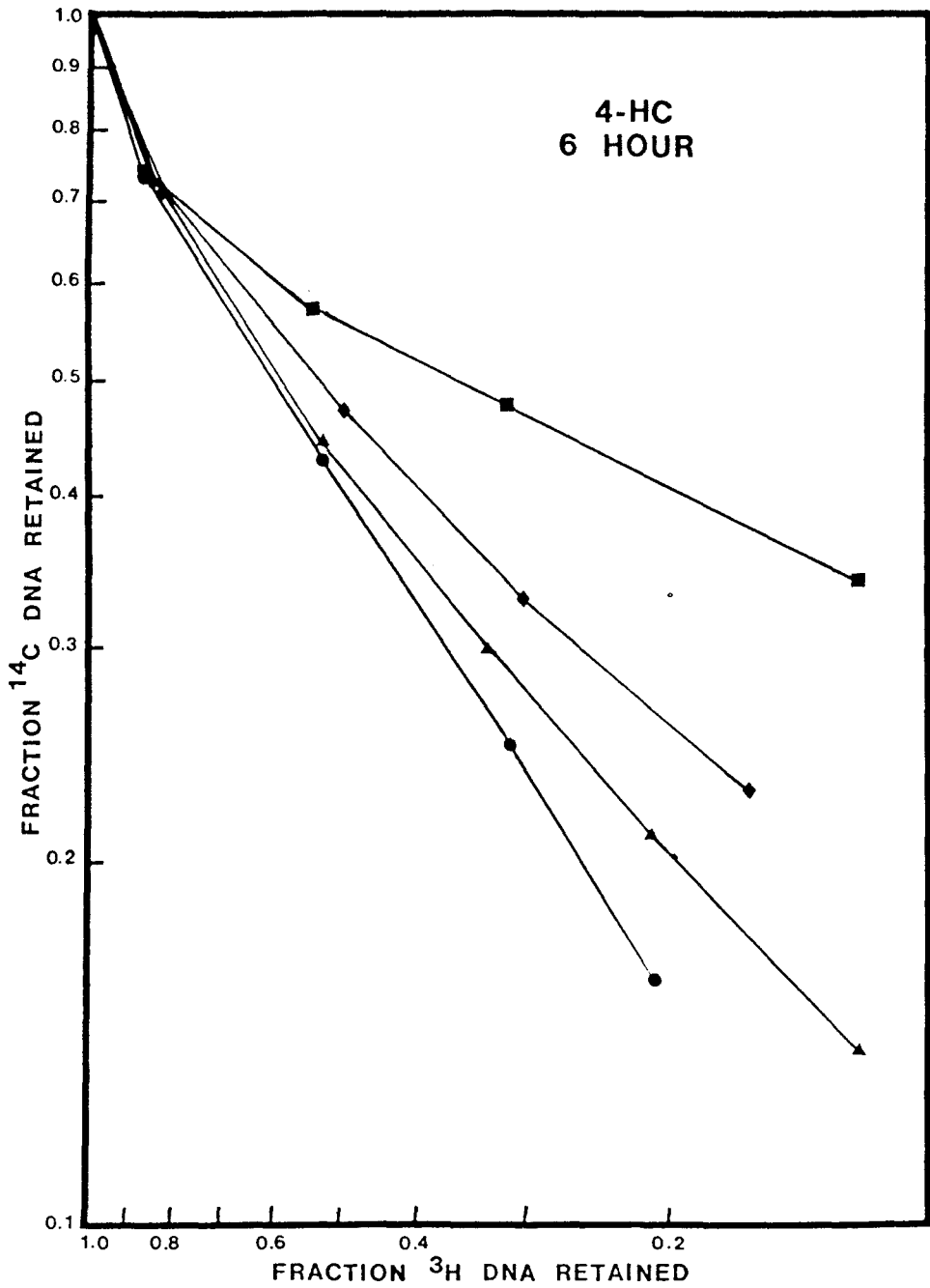


Figure 21. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of 4-HC, and assayed for DNA interstrand crosslinks at 6 hours after 4-HC exposure.

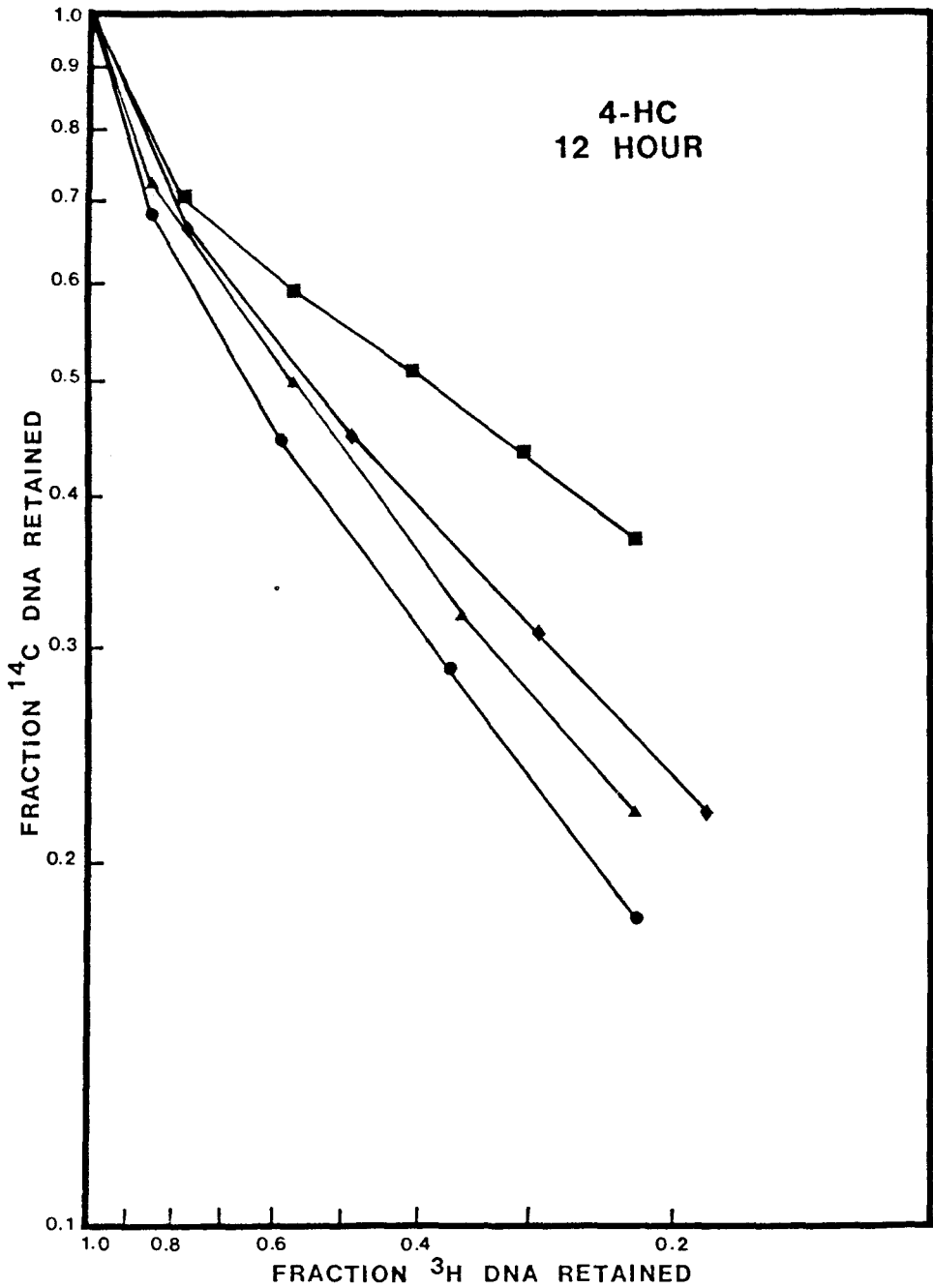


Figure 22. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of 4-HC, and assayed for DNA interstrand crosslinks at 12 hours after 4-HC exposure.

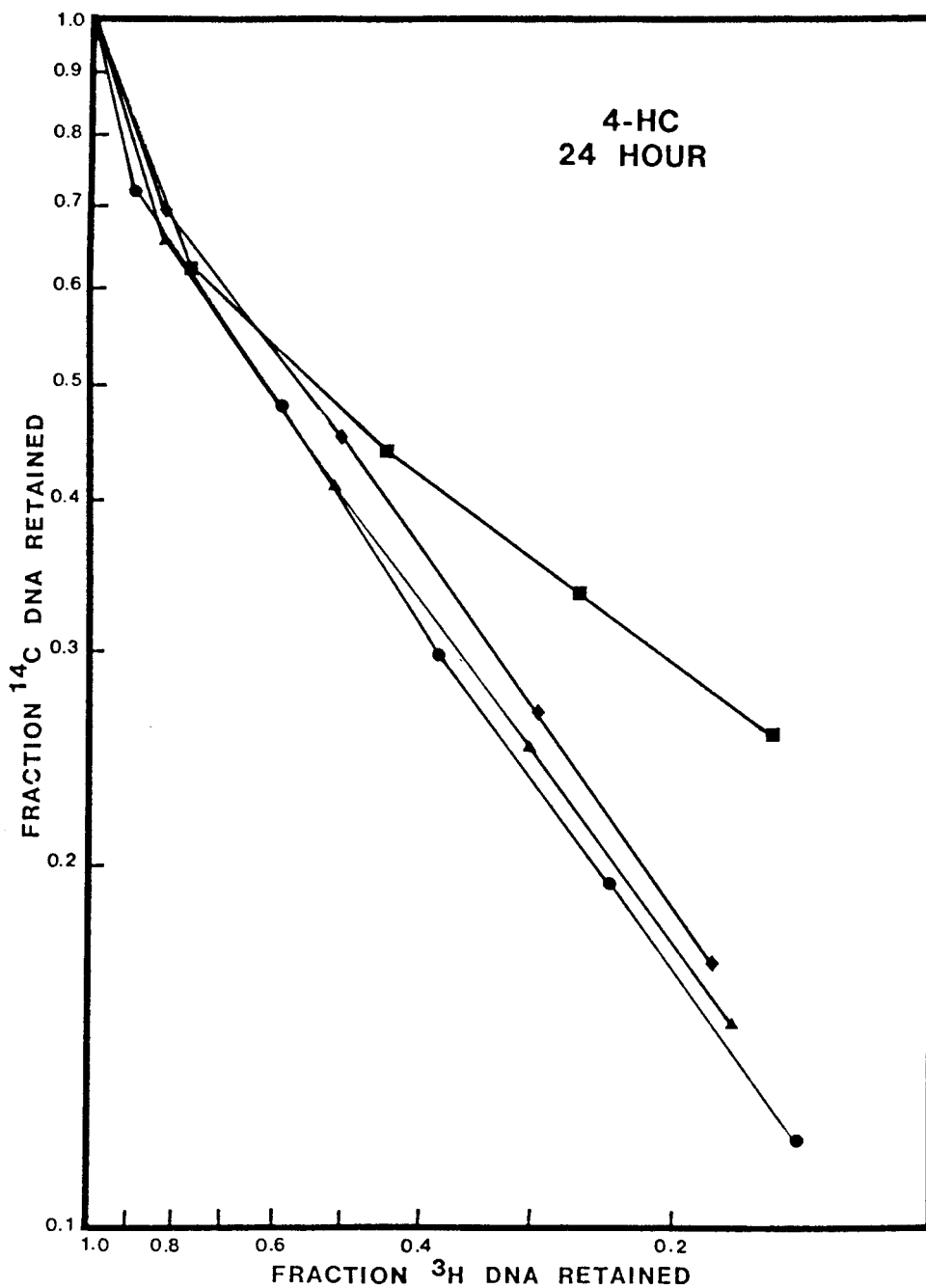


Figure 23. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of 4-HC, and assayed for DNA interstrand crosslinks at 24 hours after 4-HC exposure.

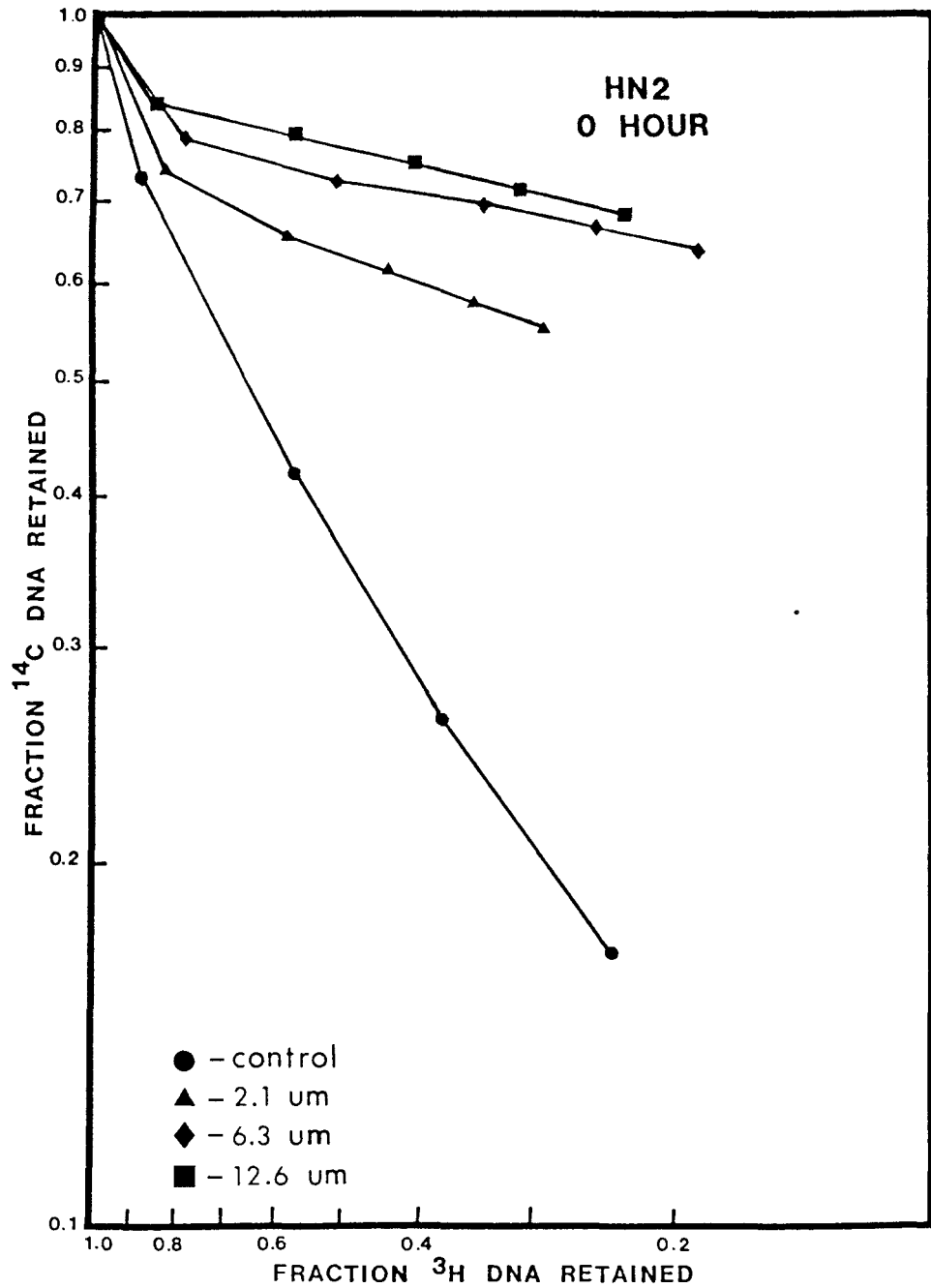


Figure 24. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of HN2, and assayed for DNA interstrand crosslinks at 0 hours after HN2 exposure.

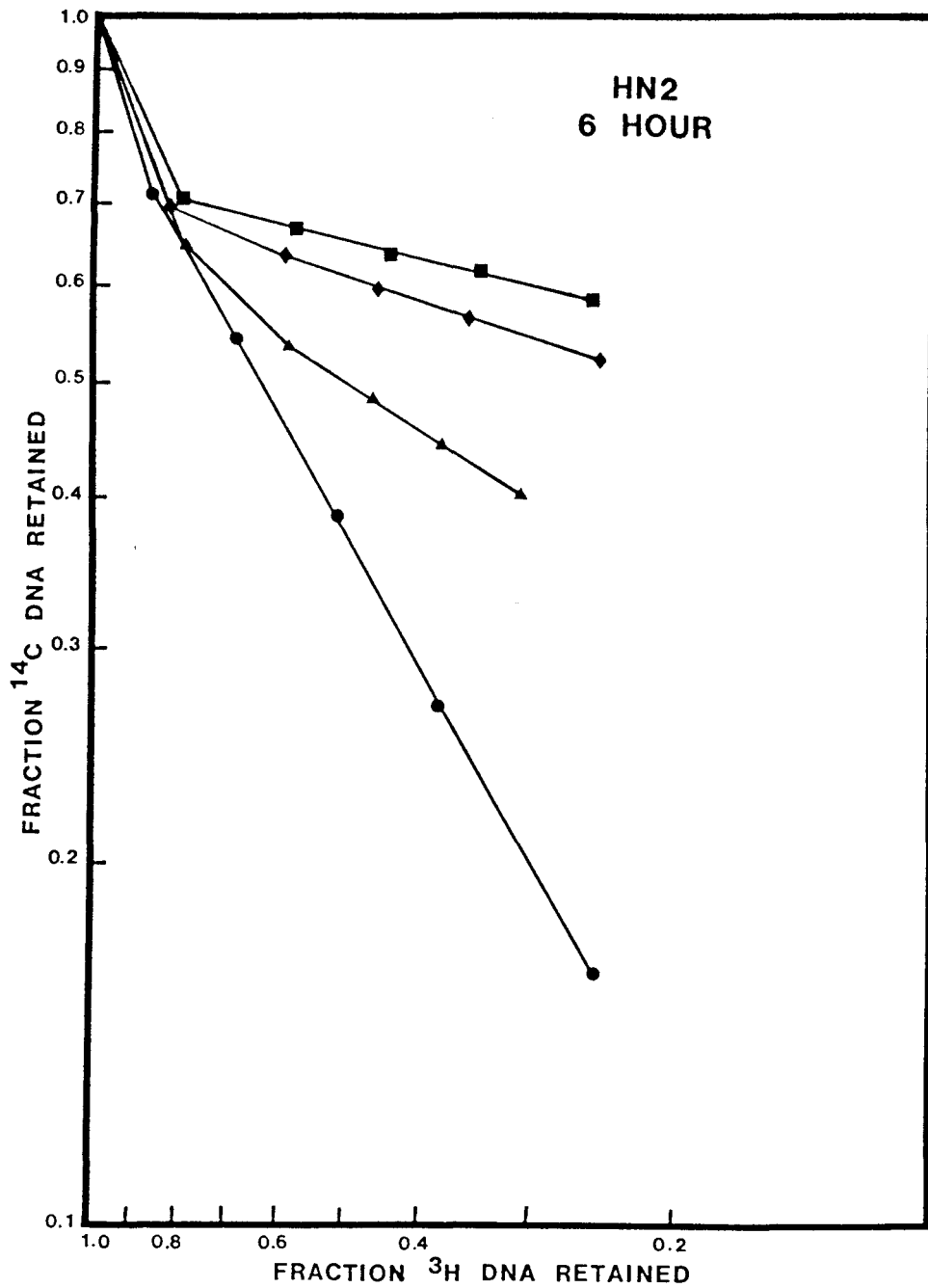


Figure 25. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of HN2, and assayed for DNA interstrand crosslinks at 6 hours after HN2 exposure.

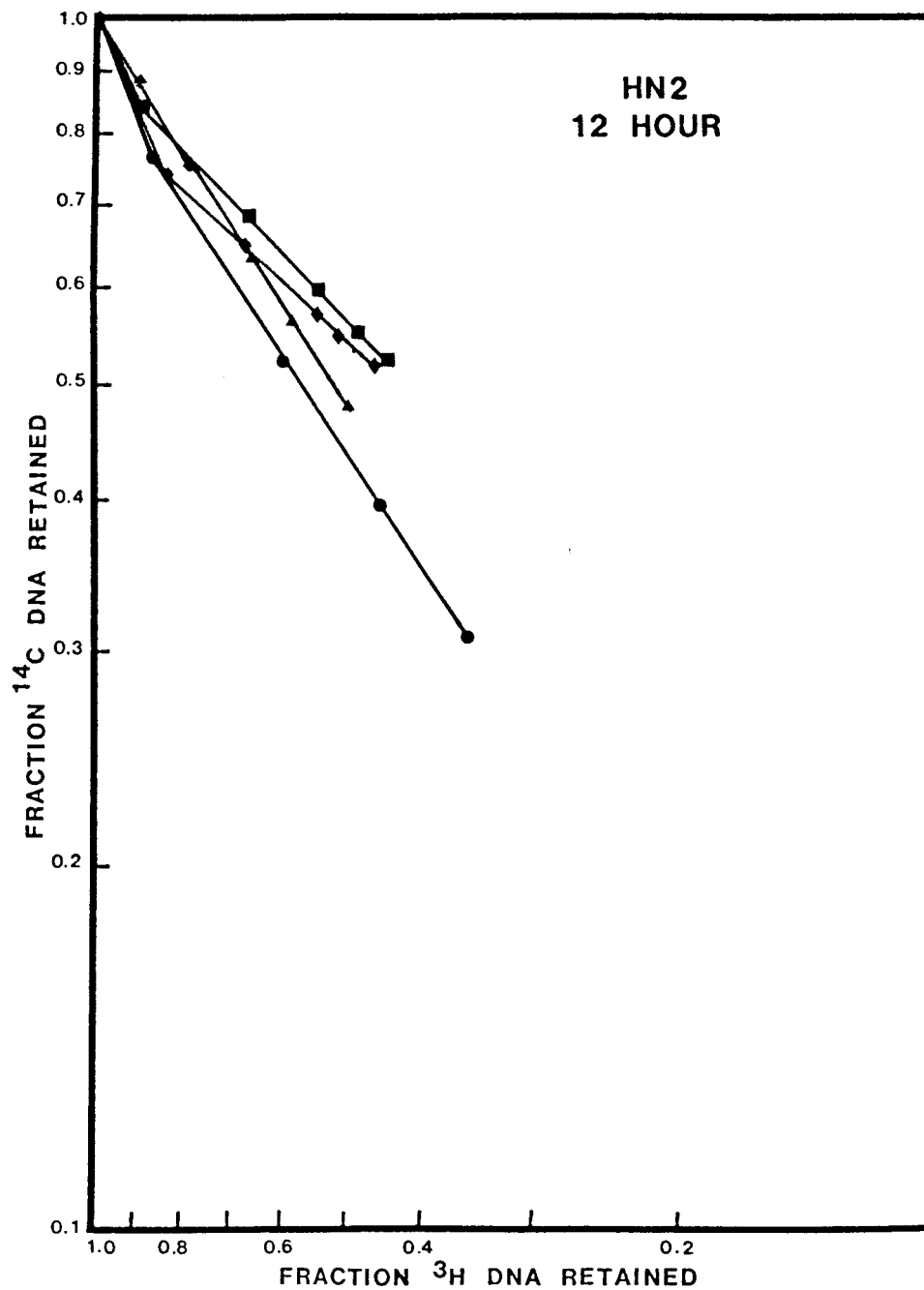


Figure 26. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of HN2, and assayed for DNA interstrand crosslinks at 12 hours after HN2 exposure.

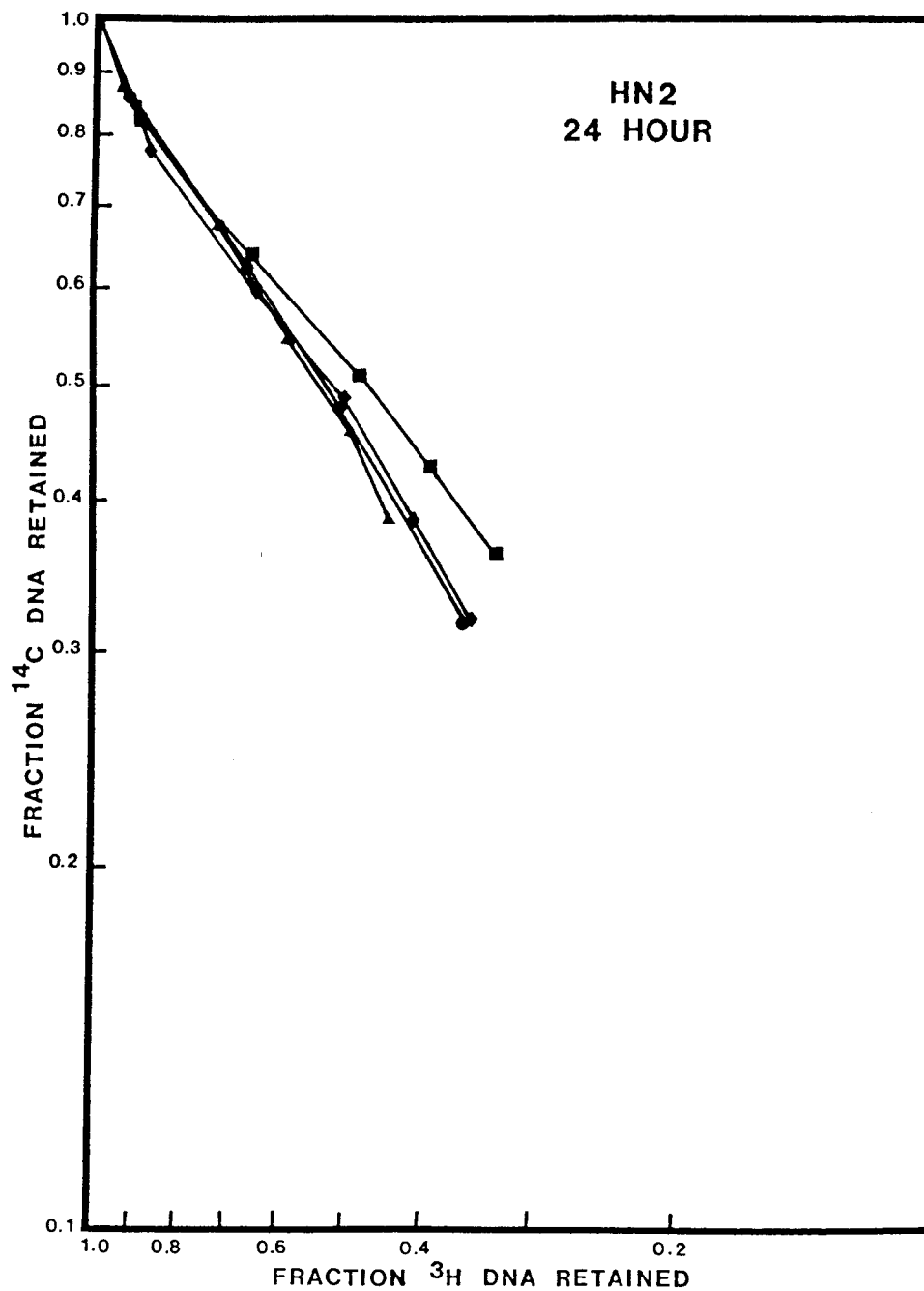


Figure 27. DNA alkaline elution profiles of Colo320 HSR cells exposed to varying doses of HN2, and assayed for DNA interstrand crosslinks at 24 hours after HN2 exposure.

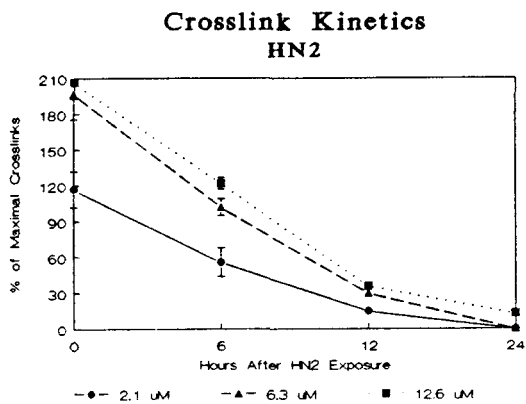
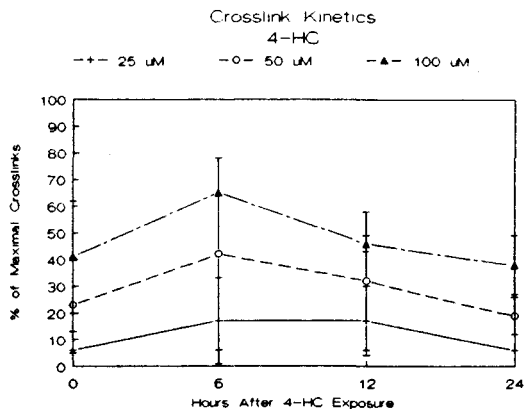
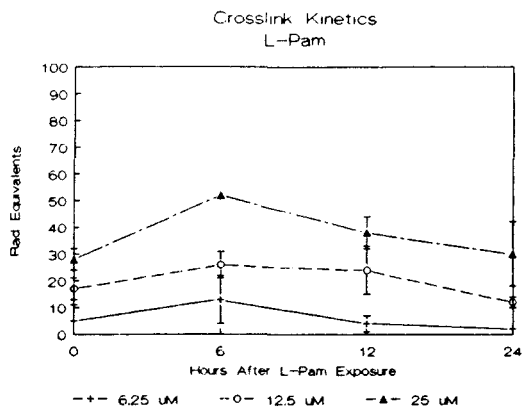


Figure 28. Crosslink kinetics of L-PAM, 4-HC, and HN2 at varying doses over the 24 hour time course in Colo320 HSR cells.

virtually gone, although significant levels of 4-HC and L-PAM induced crosslinks were still present. Approximately 50% of the maximal amount of DNA interstrand crosslinks produced by each concentration of 4-HC and L-PAM remained 24 hours after drug removal.

High levels of DNA interstrand crosslinks occur at the time of maximal inhibition of c-myc steady state transcript levels (six hours after drug removal). By 24 hours after drug removal most DNA interstrand crosslinks are gone, which is the time c-myc steady state transcript levels approach those seen in untreated controls. Thus, a viable possibility remains that gene-specific crosslinks are responsible for the inactivation of the c-myc gene, and the subsequent depression of steady state c-myc transcripts.

Measurement of Gene-Specific DNA Interstrand Crosslinks

Experiments were designed to determine if DNA interstrand crosslinks produced within the amplified c-myc oncogene were present at the time of maximal depression in the steady state c-myc mRNA. Exponentially-growing Colo320 HSR (at $4-5 \times 10^5$ /ml) were treated with either 2.1, 6.3, or 12.6 μ M HN2 for 1 hour. Genomic DNA was isolated from control and HN2-treated cells immediately after exposure to HN2 (0 hour), and after a 6 hour post-exposure incubation. Experiments were designed such that cells from both time points (0 and 6 hour) could be lysed for DNA preparation at the same time. Upon exposure to

HN2 cell growth ceased, and approximately equal numbers of cells were lysed for each time point.

Purified, high molecular weight DNA isolated from control and HN2-treated Colo320 HSR cells was digested with a restriction enzyme (Eco RI), and the DNA interstrand crosslinks were measured in defined genomic restriction fragments. The resulting restriction fragments were either denatured by the addition of formamide (final concentration 65%) and heated at 65°C for 5 minutes, or allowed to remain in native double-stranded form. Denatured and native DNA samples then were size-fractionated on a neutral agarose gel. Following electrophoresis, the gel was Southern-blotted and hybridized to probes specific for defined genomic restriction fragments. Hybridization with the 420 bp, exon 2 specific c-myc probe led to the recognition of a 12.5 kb EcoRI fragment containing the entire c-myc oncogene. Hybridization with the 350 bp exon 1 specific, N-ras probe led to the recognition of a 9.7 kb EcoRI fragment containing the entire N-ras oncogene. Finally, hybridization with the 1.7 kb α -satellite DNA probe led to the recognition of a 1.7 kb EcoRI fragment containing the α -satellite higher order array of chromosome 20.

To ensure accurate measurement of signal intensity on the autoradiographs, experiments were done to determine if the probe concentration was in excess of filter-bound sequences, under the hybridization conditions used. Figure 29 represents an autoradiograph of a filter with 2.5, 5.0, 10.0, or 15.0 μ g

of EcoRI-digested, Southern-transferred, control Colo320 HSR DNA per lane hybridized with a ^{32}P -labelled c-myc probe. Densitometry of the autoradiograph indicates that the c-myc probe is in excess of filter-bound c-myc DNA through 10 μg of total cellular DNA because the linear increase in the c-myc autoradiographic signal is maintained only through the 10 μg DNA sample. Thus, 10 μg of EcoRI-digested Colo320 HSR DNA was used for all subsequent experiments.

Figure 30, shows the formation and disappearance of HN2-induced DNA interstrand crosslinks in the 12.5 kb EcoRI restriction fragment containing the entire c-myc oncogene. Immediately after the removal of HN2, a concentration-dependent increase in DNA interstrand crosslinks within the c-myc oncogene was seen, however, by six hours after drug removal, no c-myc-specific DNA interstrand crosslinks could be detected.

Figure 31 is a graph which shows the frequency of DNA interstrand crosslinks in the c-myc oncogene calculated from the Poisson distribution, and is based on the the complement of the zero class ($-\ln P_0$). P_0 values used were the means from three independent experiments (\pm S.D.), and are as follows: 2.1 μM = 0.987 (0.001), 6.3 μM = 0.969 (0.008), and 12.6 μM = 0.942 (0.017).

Figure 32 represents an autoradiograph in which the N-ras oncogene was assayed for DNA interstrand crosslinks. No DNA interstrand crosslinks in this weakly transcribed gene

could be detected.

Figure 33 shows a picture of an autoradiograph in which DNA interstrand crosslinks within the heterochromatic α -satellite DNA were assayed. Like N-ras, no DNA interstrand crosslinks in this non-transcribed region were detected.

Effect of HN2 Treatment on DNA Synthesis in Colo320 HSR

Colo320 HSR cells were exposed to 0, 2.1, 6.3, or 12.6 μM HN2 for one hour, and ^3H -thymidine incorporation as a measure of DNA synthesis was assayed at varying times up to six hours after drug removal. DNA synthesis in drug-treated cells was then plotted relative to DNA synthesis in untreated control cells (corrected for cell number). Figure 34 illustrates the results of these experiments.

Exposure of Colo320 HSR cells to HN2 caused a concentration- and time-dependent inhibition of DNA synthesis. One hour after drug removal, ^3H -thymidine incorporation into Colo320 HSR cells exposed to 2.1 μM HN2 was 65.7% of control; in cells exposed to 6.3 and 12.6 μM HN2, ^3H -thymidine incorporation was 37.7% and 28.2% of control, respectively. Three and one-half hours after drug removal, ^3H -thymidine incorporation in cells exposed to 2.1, 6.3, and 12.6 μM HN2 was 28.5%, 13.0%, and 7.9% of control, respectively. Finally, 6 hours after drug removal, ^3H -thymidine incorporation in cells exposed to 2.1, 6.3, and 12.6 μM HN2 was 18.5%, 5.4%, and 2.9% of control, respectively. These results confirm that

HN2 inhibits DNA synthesis, and suggests that little DNA replication was occurring following drug exposure.

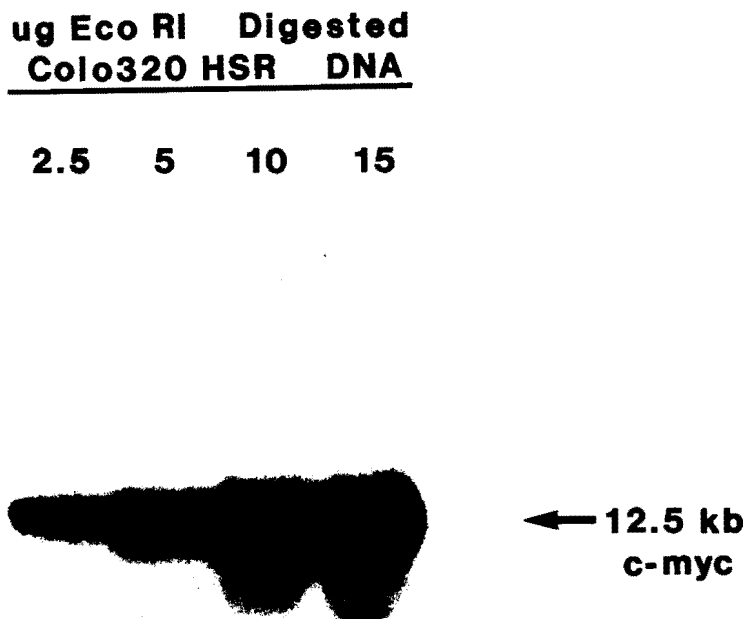


Figure 29. Southern blot analysis of the relative levels of the *c-myc* gene in varying amounts of genomic DNA isolated from untreated Colo320 HSR cells. DNA was digested with the restriction enzyme *EcoRI*, and varying amounts of DNA (2.5, 5, 10, and 15 μg) were size fractionated on a 0.5% agarose gel, capillary transferred to a nylon filter, and probed with the 420 bp *c-myc* probe. Densitometry of the *c-myc* autoradiographic signal indicated the signal increased proportionally to the amount of filter-bound DNA from 2.5 μg to 10 μg ; thereafter the filter-bound DNA was in excess of the probe concentration, and the proportional increase in autoradiographic signal was lost.

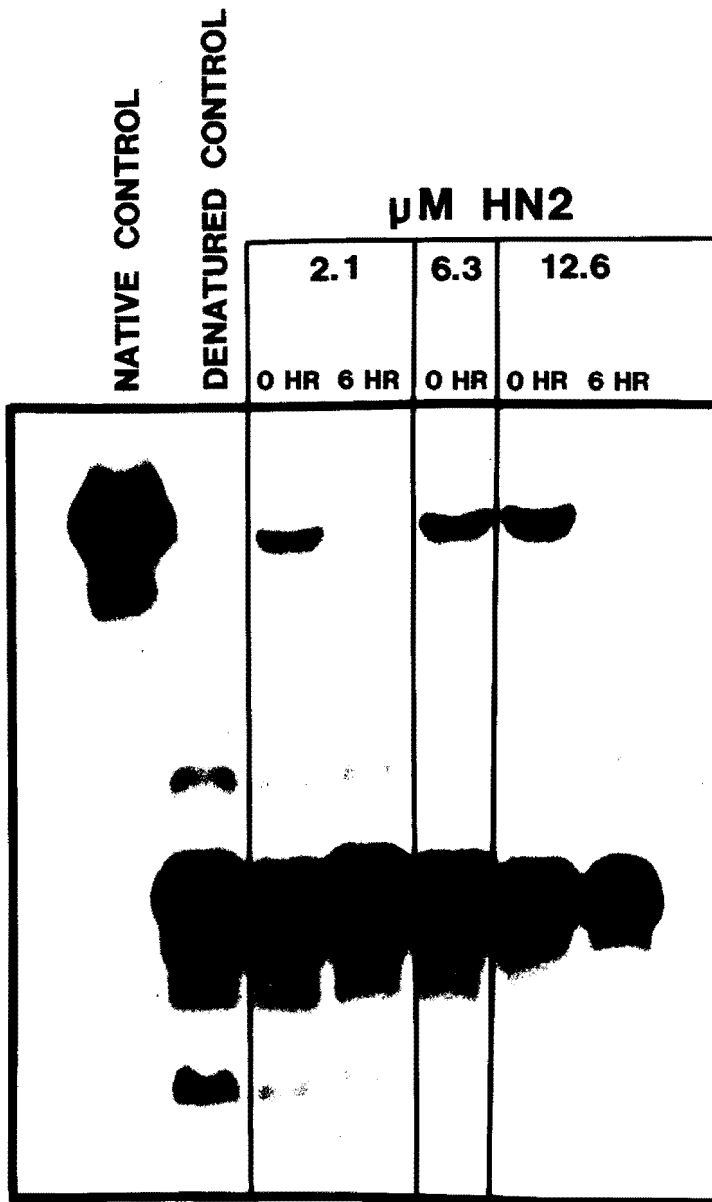


Figure 30. Detection and disappearance of DNA interstrand crosslinks in the c-myc oncogene by DNA denaturation/renaturation gel electrophoresis and Southern blot analysis. Colo320 HSR cells were exposed to varying concentrations of HN2 for one hour. Genomic DNA was isolated at 0 and 6 hours following HN2 exposure, and digested with EcoRI. Denatured and native DNA samples (10 μg /lane) were size-fractionated on 0.5% neutral agarose gels, capillary transferred to nylon membranes, and probed with the 420 bp c-myc probe. Nylon membranes were autoradiographed, with intensifying screens for appropriate periods of time.

Frequency of DNA Interstrand Crosslinks/ c-myc Oncogene following HN2 Exposure

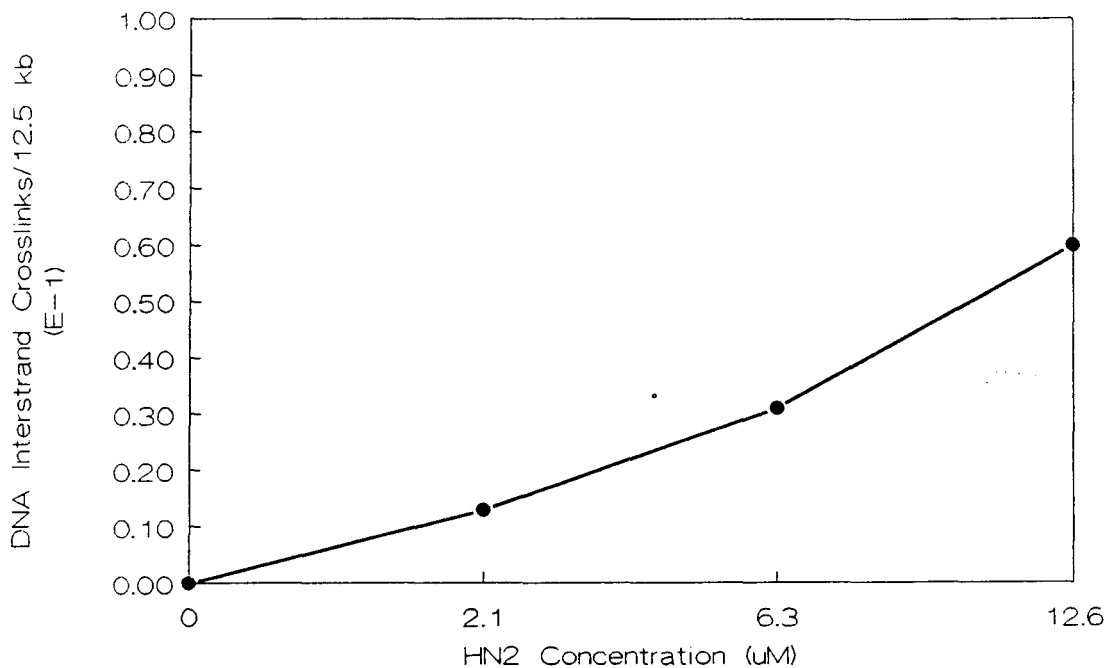


Figure 31. The frequency of DNA interstrand crosslinks in the c-myc oncogene immediately following a one hour exposure to 2.1, 6.3, or 12.6 μM HN2. Calculations of interstrand crosslinks were determined from the Poisson distribution, and ignore the small fraction of DNA molecules that would have more than one DNA interstrand crosslink. The value of the zero class (P_0) used in the calculations was the mean of three independent experiments.

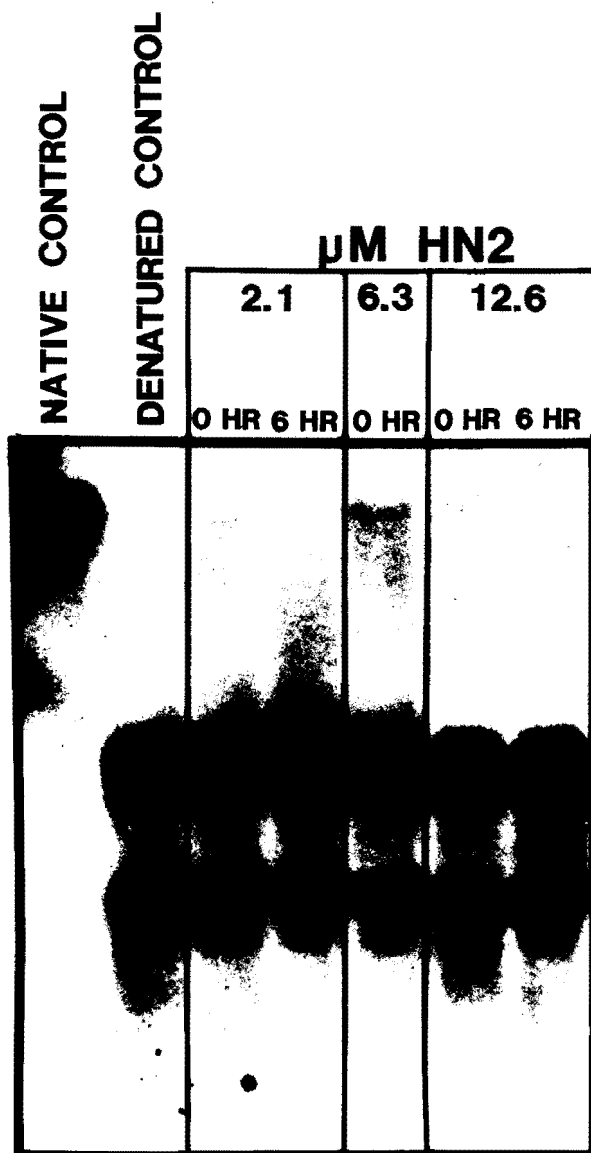


Figure 32. Absence of DNA interstrand crosslinks in the N-ras oncogene as assayed by DNA denaturation/renaturation gel electrophoresis and Southern blot analysis. Colo320 HSR cells were exposed to varying concentrations of HN2 for one hour. Genomic DNA was isolated at 0 and 6 hours following HN2 exposure, and digested with EcoRI. Denatured and native DNA samples (10 $\mu\text{g}/\text{lane}$) were size-fractionated on 0.5% neutral agarose gels, capillary transferred to nylon membranes, and probed with the 350 bp N-ras probe. Nylon membranes were autoradiographed, with intensifying screens for appropriate periods of time.

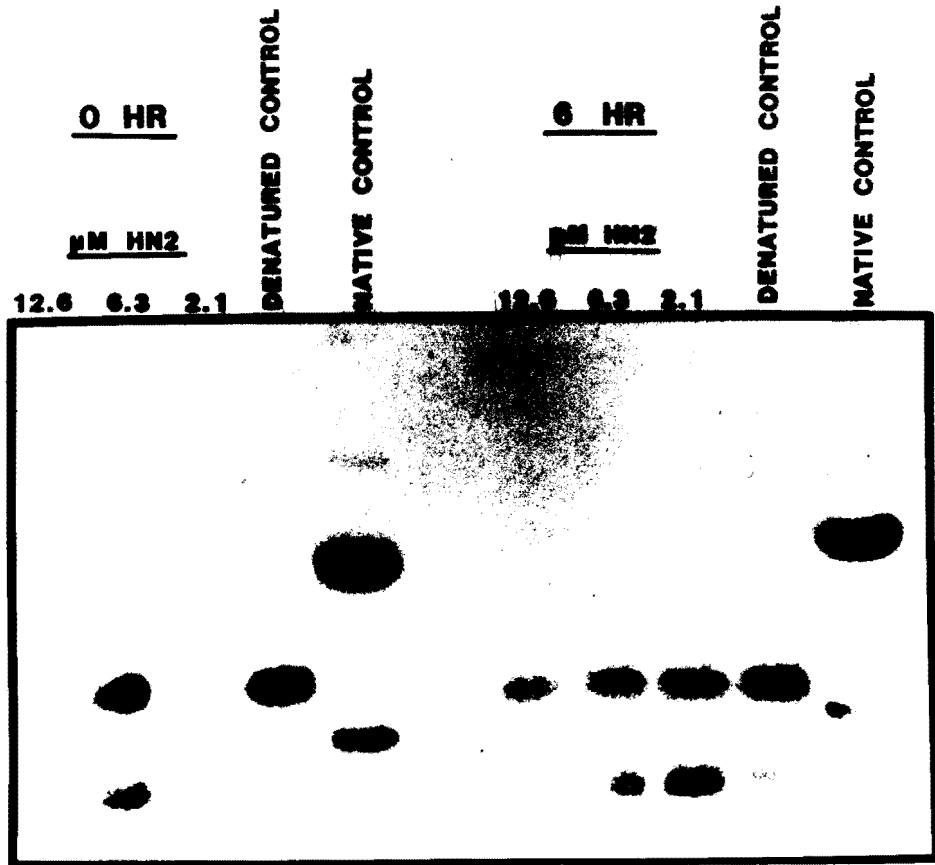


Figure 33. Absence of DNA interstrand crosslinks in α -satellite DNA from chromosome 20 as assayed by DNA denaturation/renaturation gel electrophoresis and Southern blot analysis. Colo320 HSR cells were exposed to varying concentrations of HN2 for one hour. Genomic DNA was isolated at 0 and 6 hours following HN2 exposure, and digested with EcoRI. Denatured and native DNA samples (10 μ g/lane) were size-fractionated on 0.5% neutral agarose gels, capillary transferred to nylon membranes, and probed with the 1.7 kb α -satellite probe. Nylon membranes were autoradiographed, with intensifying screens for appropriate periods of time.

Effect of HN2 on Colo320 HSR DNA Synthesis

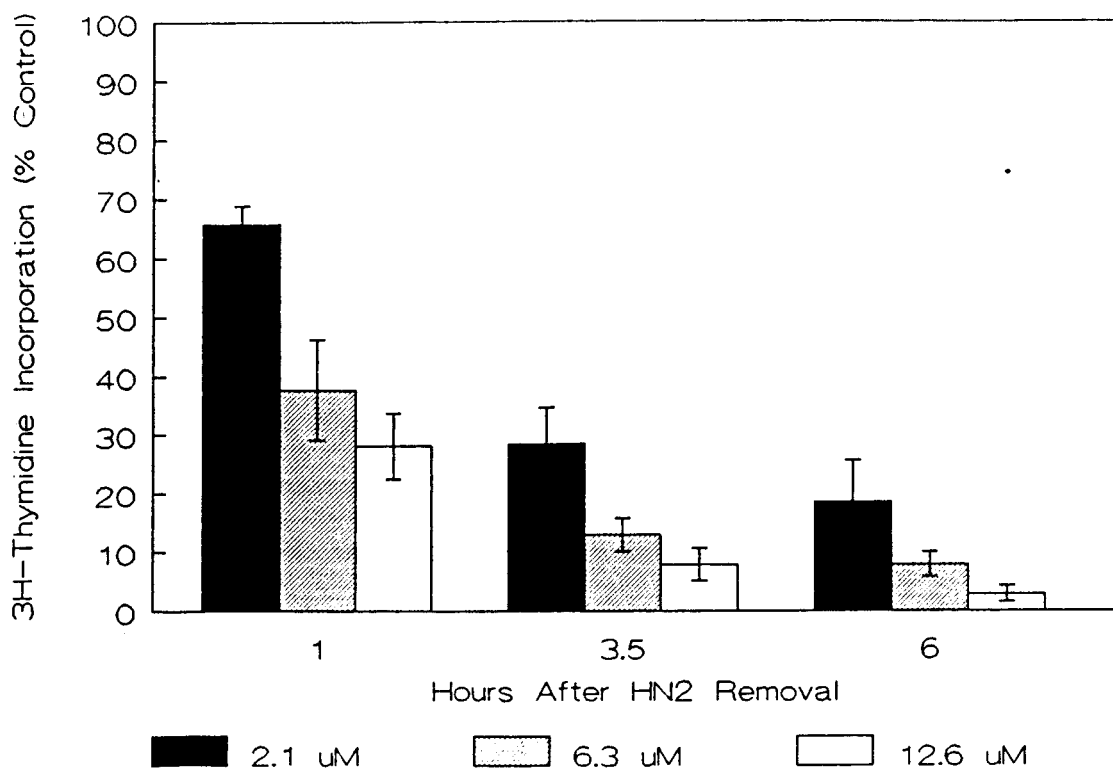


Figure 34. Measurement of Colo320 HSR ³H-thymidine incorporation, at various time points, following a one hour exposure to 2.1, 6.3, or 12.6 μM HN2. Values are expressed as the percent of the mean incorporation (+/- S.D.) of ³H-thymidine as compared to untreated control cells.

DISCUSSION

In this dissertation, an attempt was made to delineate the effects of bifunctional alkylating agents on the structure and function of particular oncogenes in a human tumor cell line. Studies presented here confirm that bifunctional alkylating agents can affect specifically the expression of the c-myc and c-fos oncogenes. Subsequent experiments were performed to determine if nitrogen mustard-induced DNA interstrand crosslinks in the c-myc oncogene were responsible for the decreased expression observed in this gene 6 to 12 hours after drug exposure. The experimental results indicate that there is not a temporal relationship between the production of c-myc-specific DNA interstrand crosslinks and decreased gene expression; however, it is possible that the induction of DNA interstrand crosslinks within a transcribed gene is responsible ultimately for the transcriptional inactivation of that gene. The results further suggest that nitrogen mustard-induced DNA interstrand crosslinks may preferentially modify highly transcribed sequences, and that, overall the lesions in highly transcribed sequences were removed more rapidly than from the genome.

N7 Guanine Alkylation Studies

Initial studies were designed to examine if HN2, L-PAM, or C-2 demonstrated any sequence-specific differences in their

reaction with a defined, double-stranded DNA fragment in vitro. Although in this assay, sequence specificity of the nitrogen mustards is assessed by their ability to react with naked DNA, it has been demonstrated that HN2 will modify the same DNA sequences in intact cells; albeit to a far lesser extent (Grunberg and Haseltine, 1980).

A 420 bp PstI restriction fragment from exon 2 of the human c-myc oncogene, originally cloned from Colo320 HSR, was used as the defined DNA fragment, and was exposed to varying concentrations of HN2, L-PAM, or C-2. When this DNA fragment was assayed for drug-induced N7 guanine alkylations, none of the compounds demonstrated differences in their ability to react with sequences of alternating guanines and cytosines - the sequence necessary for the formation of the DNA interstrand crosslink. All three compounds displayed a slight preference for contiguous runs of three or more guanines. This previously was reported for a number of other DNA-damaging anti-tumor agents (cis-Pt, BCNU, HN2)(Mattes et al., 1988), and is even seen for dimethyl sulfate, which is used for the guanine-specific reaction in the standard Maxam and Gilbert DNA sequencing procedure. Differences did exist, however, in the relative reactivity of these compounds with the DNA (L-PAM > HN2 > C-2). This does not necessarily mean L-PAM reacts more frequently with cellular DNA than the other two compounds. A number of cellular processes may negate this enhanced reactivity seen in naked DNA. For instance,

decreased drug influx (or increased drug efflux) or differential reactivity with cellular drug scavengers (e.g. glutathione) may decrease the amount of drug available to react with the DNA (Bellamy et al., 1988, Lee et al., 1989).

Although these bifunctional alkylating agents do not display differences in their sequence selectivity in reacting with the N7 position of guanine, recent work suggests that other, less frequent, lesions may be involved in terminating RNA transcription. Using an in vitro transcription system, it was demonstrated that HN2 produced transcription-terminating lesions at select guanine pairs along the DNA template, L-PAM produced transcription terminating lesions at adenine pairs, but C-2 produced no transcription terminating lesions (Pieper et al., 1989). This assay does not directly address the potential effects of the DNA interstrand crosslink, but does demonstrate differences between the bifunctional alkylating agents in their potential ability to disrupt transcription in intact cells.

Gene Expression Studies

The exposure of bifunctional alkylating agents to Colo320 HSR was able to modulate the expression of specific oncogenes in Colo320 HSR, as measured by Northern blot analysis. The steady state expression of c-myc and c-fos were affected, but the steady state expression of N-ras and β -actin were not. Furthermore, each compound elicited similar responses.

In the 24 hours following exposure to bifunctional alkylating agents, little or no change in the steady state levels of N-ras and β -actin was observed. This is not surprising since most other compounds have had little or no effect on the expression of these two genes. Furthermore, changes in the levels of β -actin may not be readily discernible because of the long $t_{1/2}$ of the β -actin mRNA (Watanabe et al., 1985, Mitchell et al., 1986, Kharabanda et al., 1989).

The mRNA levels of c-fos and c-myc were elevated immediately following drug exposure. c-fos mRNA can be increased rapidly by a disparate group of stimuli, and recent reports indicate that DNA damaging agents, other than bifunctional alkylating agents, can increase c-fos expression (Fornace et al., 1989). The reason for this increase is not understood, but the c-fos protein is a transcriptional regulator which plays a role in stimulating or repressing the transcription of genes under the influence of the AP-1 inducible enhancer (Chiu et al., 1988, Distel et al., 1987). It also has been reported that genes inducible by the DNA damaging agents Mitomycin C and UV light are also AP-1-inducible genes (Angel et al., 1986, 1987a, 1987b). From these observations it is possible to speculate that the bifunctional alkylating agents are inducing a cellular response to DNA damage mediated through the induction of the c-fos gene.

The rapid increases in c-fos and c-myc expression are seen consistently in the cellular mitogenic response (Armelin et al., 1984, Muller et al., 1984). Thus, the increases in expression of c-myc and c-fos seen after nitrogen mustard exposure are surprising, since the cellular response to nitrogen mustard exposure is both rapid and cytotoxic, as evidenced by the experiments measuring DNA synthesis and the colony formation assays. If c-myc and c-fos increases are due to increased transcription, the intracellular signalling pathways mediating these respective increases may be independent of one another, and/or different from the pathways mediating the proliferative response. This is consistent with observations demonstrating that the induction of c-fos and c-myc transcription can occur via a number of different intracellular signalling pathways (Hall and Stiles, 1987, Grausz et al., 1986, McCaffrey et al., 1987).

Finally, it is also possible that the increase in c-fos and c-myc mRNA levels is due to a drug-induced stabilization of these mRNA. While possible, this type of effect may ultimately lead to the same biological consequences as increased transcription.

Each of the bifunctional alkylating agents tested were able to suppress the level of c-myc mRNA transcripts at 6 and 12 hours following drug exposure. Suppression of c-myc transcripts by a variety of other compounds previously has been reported. In each case decreased levels of c-myc transcripts

was associated with cessation of cellular proliferation and/or cellular differentiation.

Specifically, agents such as dibutiryl cyclic-AMP, DMSO, and 1,25,-dihydroxy vitamin D₃ reduce c-myc expression in human hematologic tumor cell lines prior to terminal differentiation (Watanabe et al., 1985, Trepel et al., 1987, Reitsma et al., 1983). The anti-tumor agents 6-thioguanine and 1-β-D-arabinofuranosylcytosine given at doses that induce terminal differentiation in human hematologic tumor cell lines caused decreases in c-myc mRNA (Mitchell et al., 1986, Schwartz and Eninger, 1989). Finally, exposure to interferon α caused growth arrest in the Burkitt lymphoma cell line Daudi, and interferon gamma alone, or in cooperation with tumor necrosis factor, caused growth arrest in HeLa cells (Yarden and Kimichi, 1986, Einat et al., 1985).

Like the agents listed above, the depression of c-myc mRNA observed following nitrogen mustard exposure may be due to a cellular response specific for growth arrest or perhaps cell death. Alternatively, it also is possible that the suppression of c-myc transcript levels may be due to the accumulation of DNA damage (DNA interstrand crosslinks, in particular) within the c-myc oncogene.

DNA Interstrand Crosslinking Studies

Total genomic DNA interstrand crosslinks produced by HN2, L-PAM, and 4-HC were measured by the DNA alkaline elution

technique. The results obtained in Colo320 HSR cells were consistent with results in similar studies using other cell lines (Erickson et al., 1980). The production of drug-induced DNA interstrand crosslinks increased in a concentration-dependent fashion and the levels of DNA interstrand crosslinks were related directly to levels of drug-induced cytotoxicity.

Peak levels of DNA interstrand crosslinks were observed 6 hours after exposure to 4-HC and L-PAM. Levels of crosslinks produced by HN2 were comparable to 4-HC and L-PAM at 6 hours, however, interstrand crosslinks peaked immediately after exposure to HN2 (0 hour). Following the respective peak times of DNA interstrand crosslinks, the levels of interstrand crosslinks decayed over the remainder of the 24 hours.

High levels of DNA interstrand crosslinks at six hours following drug treatment suggested gene-specific DNA interstrand crosslinks may be responsible for the decreased expression of the c-myc oncogene. This possibility was tested by measuring HN2-induced DNA interstrand crosslinks in defined DNA restriction fragments isolated from HN2-treated Colo320 HSR cells. The defined restriction fragments were specific for the entire human c-myc oncogene, the entire human N-ras oncogene, and the α -satellite DNA specific for human chromosome 20.

DNA interstrand crosslinks were observed in the c-myc oncogene immediately following exposure to HN2. Moreover, the levels of crosslinks in this highly transcribed gene increased

in a concentration-dependent fashion. No HN2-induced DNA interstrand crosslinks could be detected in the N-ras oncogene, which is transcribed at low levels, nor the non-transcribed α -satellite sequences.

These results suggest that the HN2-induced DNA interstrand crosslinks may display intragenomic preferences in reactivity. The absence of DNA interstrand crosslinks in α -satellite DNA may reflect the fact that it is ~60% A-T; based on its consensus sequence (Willard, 1987). Additionally, in this sequence few sites exist where a DNA interstrand crosslink can form (only 10 G-C doublets in the 171 bp consensus sequence). The formation of the DNA interstrand crosslink also may be inhibited. In support of this possibility, is the observation that PUVA-induced DNA interstrand crosslinks were not formed in α -satellite DNA, however, monoadducts were formed in α -satellite DNA as readily as in bulk DNA. It has been suggested that the inhibition of crosslink formation is due to physical constraints imparted by the condensed chromatin structure of α -satellite DNA (Zolan et al., 1982).

Chromatin structure also may serve to explain observed differences between the DNA interstrand crosslinks formed in the c-myc and N-ras oncogenes because both genes are high in G-C content and, therefore, ideal targets for the formation of DNA interstrand crosslinks (Mattes et al., 1988). Based on the Northern blot analysis, c-myc is expressed at very high

levels while N-ras expression barely is detectable. This suggests differences in their transcriptional states, and perhaps their chromatin structure. Evidence from other studies indicates that various DNA damaging carcinogens and chemotherapeutic agents preferentially modify and crosslink the transcriptionally active fraction of the genome. Finally, from the Poisson distribution and the DNA interstrand crosslinks observed in the c-myc oncogene, there is one interstrand crosslink approximately every 200 kb of DNA in Colo320 HSR cells exposed to 12.6 μM HN2 for one hour (ignoring the small fraction of fragments with greater than one crosslink). Based on 6.6×10^9 bp per human diploid genome (probably far less DNA than the aneuploid Colo320 HSR cells contain), 12.6 μM HN2 would produce approximately 33,000 DNA interstrand crosslinks. This is in great excess of the 100 - 1000 DNA interstrand crosslinks thought to occur in tumor cells exposed to cytotoxic concentrations of nitrogen mustards, however, it is not contradictory if intragenomic heterogeneity exists in the formation of HN2-induced DNA interstrand crosslinks.

Six hours after exposure to HN2, all DNA interstrand crosslinks in the c-myc oncogene have disappeared. This disappearance of DNA interstrand crosslinks in the c-myc oncogene does not appear to be a dilutional effect produced by synthesis of damage-free replicated DNA because DNA synthesis was effectively inhibited by drug exposure. By

contrast, > 50% of the DNA interstrand crosslinks remained in the total genome. Although the assays used to measure DNA interstrand crosslinks in specific genes and the total genome were not the same, the large differences observed between the c-myc oncogene and the total genome indicates there may be heterogeneity in the removal of HN2-induced DNA interstrand crosslinks. Again, DNA topology and gene activity may explain the removal of DNA interstrand crosslinks from the c-myc oncogene. A growing body of work demonstrates that transcriptional activity and chromatin structure are important factors in the preferential removal of aflatoxin B₁ adducts, PUVA-induced DNA interstrand crosslinks, and UV-induced pyrimidine dimers (Leadon, 1986, Dean, 1989, Leadon and Snowden, 1988). In fact, it has been suggested that high levels of transcription are necessary for the removal of many types of DNA damage (Mellon and Hanawalt, 1987). If transcription is necessary for the removal of at least some DNA lesions, then this may explain the rapid disappearance of DNA interstrand crosslinks in the c-myc oncogene. The increased levels of c-myc mRNA transcripts observed immediately following drug exposure are consistent with this possibility.

The absence of DNA interstrand crosslinks in the c-myc oncogene 6 hours following HN2 exposure indicates that gene-specific DNA interstrand crosslinks are not responsible temporally for the inactivation of this gene. It does not,

however, exclude the possibility that the persistence of other DNA interstrand crosslinks in other areas of the genome are indirectly responsible for the inhibition of c-myc expression, or the initial DNA interstrand crosslinks formed in the c-myc oncogene are responsible for the inactivation of this gene.

Finally, it should be mentioned that the removal of DNA interstrand crosslinks, as measured by the assays used in this dissertation, reflects the disappearance of the lesion, but not necessarily the complete repair of the damage. Like many other repair pathways of DNA damage, repair of the interstrand crosslink is probably multi-step and multi-enzymatic. The first step in the repair process is thought to be the "unhooking" of one arm of the interstrand crosslink (Reid and Walker, 1969). This is the event observed as the disappearance of DNA interstrand crosslinks in the assays used. If inhibition of strand separation is the critical cytotoxic event, then the "unhooking" step may be the critical first step in the repair event.

In summary, these experiments demonstrate that HN2, L-PAM, and 4-HC can affect the expression of a subset of active genes. The immediate increases in c-fos and c-myc expression observed in these studies imply a possible cellular response to the drug challenge. The depression of c-myc expression following exposure to bifunctional alkylating agents is similar to the responses observed following exposure to non-DNA-damaging, therapeutic compounds, and is consistent with

the observed growth arrest produced by these compounds. These results, and the knowledge that c-myc plays a participative role, if not a causal role, in many human malignancies, suggests that inhibition of c-myc expression may ultimately be a targeted therapeutic endpoint or a prognostic indicator. Indeed, depression of c-myc expression in leukemic patients following chemotherapy has been correlated with a good response to treatment.

The observed depression of c-myc expression 6 hours after exposure to HN2 does not appear to be due to the presence of c-myc-specific DNA interstrand crosslinks at this point in time, but the formation of DNA interstrand crosslinks in the c-myc gene immediately after drug exposure may be responsible for transcriptional inactivation of c-myc. Furthermore, experimental evidence implies that HN2-induced DNA interstrand crosslinks are produced and processed in the genome in a heterogeneous fashion. Analysis of observed subgenomic differences in the formation and disappearance of DNA interstrand crosslinks may ultimately aid in the delineation of specific genomic regions necessary for the formation and maintenance of critical cellular lesions.

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