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Effects of bifunctional chemotherapeutic agents on gene expression in the human tumor cell line Colo320HSR

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EFFECTS OF BIFUNCTIONAL CHEMOTHERAPEUTIC AGENTS ON GENE EXPRESSION IN THE HUMAN TUMOR CELL LINE COLO320HSR

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DEDICATION

To my husband, Fengdao Liu, for his unconditional love, understanding and support.

To my parents, Huijie Jiang and Yaping Dong, for their belief in me and their encouragement.

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VITA

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

μCi	microcurie
μg	microgram
μl	microliter
μM	micromolar
APS	ammonium persulfate
BAP	bacterial alkaline phosphatase
bp	base pair(s)
cisplatin	cis-diamminedichloroplatinum (II)
cm	centimeter
CM	conditioned medium
cpm	counts per minute
ddNTP	2',3'-dideoxyribonucleoside 5'-triphosphate acid
DEPC	diethyl pyrocarbonate
DHFR	dihydrofolate reductase
DM	double minute
DMSO	dimethyl sulfoxide
dpm	disintegrations per minute
ds	double stranded
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor

FM	fresh medium
g	gram
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
h	hour(s)
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HN2	mechlorethamine, nitrogen mustard
HSR	homogeneously staining region
HU	hydroxyurea
IPTG	isopropylthio-ß-D-galactoside
kb	kilobase(s)
L	liter
lb	pound
M	molar
mCi	millicurie
mg	milligram
min	minute(s)
ml	milliliter
mm	millimeter
mM	millimolar
mmol	millimole
mRNA	messenger RNA
N	normal
ng	nanogram
nt	nucleotide(s)
OD	optical density

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pmol	picomole
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
sec	second(s)
sq. in.	square inch
ss	single stranded
SSC	standard saline citrate
TBE	Tris-borate-EDTA buffer
TCA	trichloroacetic acid
TE	Tris-EDTA buffer
TEMED	*N,N,N',N'*-tetramethylethyl-enediamine
Tris	Tris-[hydroxymethyl]-aminomethane
U	unit(s)
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X g	times gravity
°C	degrees Celsius

INTRODUCTION

Cancer arises when abnormal cells in the body multiply without restraint and produce a group of descendants that invade surrounding tissues. The molecular basis underlying cancer development has not been completely clarified. However, increasing evidence has shown that cancer is a result of an accumulation of multiple genetic alterations, which involve activation of oncogenes and inactivation of tumor suppressor genes.

The treatment of cancer depends on surgery, chemotherapy, radiotherapy, and immunotherapy. Chemotherapy plays an important role in the treatment of cancer by utilizing several groups of cytotoxic chemical agents. The predominant mechanism by which some cytotoxic drugs exert their toxicities generally is thought to involve DNA damage. Prior to 1980, most of our knowledge regarding DNA damage and repair was gained under the assumption that these events occur in the genome randomly. Recent advances in the study of DNA damage and repair at the gene level reveal a heterogeneity of these processes in mammalian cells. Little information is known regarding the interaction between DNA damaging anti-tumor

agents and individual genes, and changes of gene expression in response to DNA damage.

The goal of this dissertation is to elucidate the relationship between DNA damage and repair at the gene level, and alterations of gene expression in response to DNA damage induced by the DNA cross-linking agents, HN2 (mechlorethamine, nitrogen mustard) and cisplatin (*cis*-diamminedichloroplatinum (II), *cis*-Pt). The hypothesis that DNA cross-linking agents (HN2, cisplatin) preferentially produce DNA interstrand cross-links in transcribed genes and suppress their expression was investigated. Attempts were made to address the following questions:

*** Are DNA interstrand cross-links induced by HN2 or cisplatin detectable at the gene level?

*** Is oncogene expression suppressed by either HN2 or cisplatin?

*** Is the transcriptional state of a gene correlated with its accessibility to HN2-induced DNA damage?

This study was carried out in a human tumor cell line (Colo320HSR), which harbors an amplified and overexpressed c-*myc* oncogene. The effects of HN2 and cisplatin on c-*myc* gene expression was studied in order to reveal responses of an activated oncogene to DNA cross-linking agents. The GAPDH gene served as a control gene because it is a single copy

number gene and presumably constitutively expressed. The c-*fos* gene, which is silent in exponentially growing Colo320HSR cells, was used as the control in HN2-induced DNA interstrand cross-linking experiments. The histone H3.3 and ß-actin genes served as constitutively expressed controls for gene expression studies. Different control genes were used in each set of experiments due to the measurability of each gene in different assays.

The effects of the DNA cross-linking agents, HN2 and cisplatin, on gene expression were assessed at both the transcriptional and steady state mRNA levels. The accessibility of a gene to HN2-induced DNA interstrand cross-links was correlated with its relative transcription rate.

REVIEW OF RELATED LITERATURE

Oncogenesis

It is becoming increasingly clear that tumor formation arises as a consequence of alterations in the control of cell proliferation and differentiation. Some of these control points have been identified and have undergone intensive investigation. Multiple genetic changes observed in tumors support the concept that oncogenesis must be a multi-step process. The genetic changes found in tumor cells are of two types: induction, such as the activation of proto-oncogenes; and suppression, such as the inactivation of tumor suppressor genes.

Cellular proto-oncogenes encode proteins that play an essential role in normal biochemical processes with three main sites of action: the cell-surface membrane, the cytoplasm, and the nucleus (Bishop, 1987; Weinberg, 1985). Approximately 60 oncogenes have been identified to date, including genes encoding growth factors (e.g., *sis, int-2, hst*), receptor and nonreceptor protein-tyrosine kinases (e.g., *src, erb*B, *neu*), membrane-associated G proteins (e.g., *ras, gsp, gip*), cytoplasmic protein-serine kinases (e.g., *mos, pim*-1), and nuclear transcription factors (e.g. *myc, fos, jun*) (Varmus

1989; Hunter, 1991). The activation of proto-oncogenes involves a variety of mechanisms, resulting in either overexpression of a normal cellular protein, or the expression of an altered form of a normal cellular protein. For instance, the c-*myc* proto-oncogene is activated in many tumors by overexpression of the normal protein (Hayward *et al.*, 1981; Alitalo *et al.*, 1983; Perry, 1983; Leder *et al.*, 1983). The product of *ras* oncogenes also can transform cells when point mutation occurs in their alpha subunits (Bourne *et al.*, 1990). Mutations in codon 12, 13, or 61 of one of the three *ras* genes, H-*ras*, K-*ras*, and N-*ras*, convert these genes into active oncogenes (reviewed in Bos, 1989).

Transformation of a normal cell also is facilitated by recessive mutations of certain genes. These are tumor suppressor genes whose products are involved in negative regulation of cell proliferation. In contrast to the oncogenes discovered, only a small number of tumor suppressor genes have been identified to date. Known tumor suppressor genes include the Rb gene in retinoblastoma (Lee *et al.*, 1987), WT1 gene in Wilms' tumors (Rose *et al.*, 1990), p53 gene in various tumors (Rotter and Prokocimer, 1991; Hollstein *et al.*, 1991; Levine *et al.*, 1991), DCC and MCC genes in colorectal carcinoma (Fearon *et al.*, 1990; Kinzler *et al.*, 1991a), and APC gene in familial adenomatosis polyposis (Kinzler *et al.*, 1991b). Although much remains to be learned about the function of each tumor suppressor gene, it is clear

that loss of the normal function of these genes is important in tumorigenesis.

In general, the current model for loss of normal cellular growth control includes inactivation of one or more tumor suppressor loci inhibiting proliferation, and/or activation of one or more oncogenes to give a positive signal for proliferation (Marshall, 1991). The cooperation of two or more oncogenes and tumor suppressor genes appears to be required for tumorigenic transformation (Land *et al.*, 1983; Hunter, 1991).

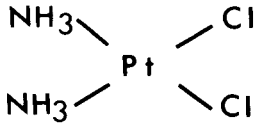
DNA Cross-linking Agents

Chemical cross-linking agents include the nitrogen and sulfur mustards (Brookes and Lawley, 1961), nitrosoureas (Ludlum *et al.*, 1975; Kohn, 1977), mitomycin C (Iyer and Szybalski, 1963), and various platinum derivatives (Roberts and Pascoe, 1972), and certain intercalating furocoumerins (e.g. 8-methoxypsoralen) in a photocatalytic reaction (Cole, 1970). These agents form covalent cross-links between the two complementary strands of DNA, which may pose a block to DNA replication or RNA transcription. HN2 and cisplatin are among the most efficacious DNA cross-linking anti-tumor agents in chemotherapy (Kohn, 1983). These agents were chosen as the drugs for this study because of their common ability to produce DNA interstrand cross-links.

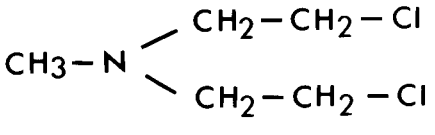
Nitrogen mustard was the first nonhormonal agent to show significant anti-tumor activity in humans (Gilman and Philips, 1946). The discovery of anti-tumor activity of HN2 led to the development of a wide variety of anti-tumor agents clinically used today, and HN2 remains a major drug used in the treatment of lymphoma.

The presence of the bis-(2-chloroethyl) group makes HN2 a highly reactive electrophilic compound (Figure 1). HN2 produces a wide spectrum of DNA lesions, including monoadducts, intrastrand adducts, and interstrand cross-links (Colvin, 1982). HN2 alkylates primarily at the N-7 position of guanine (Mattes *et al.*, 1986 and 1988; Kohn *et al.*, 1987 and 1988; Hartley *et al.*, 1988), and interacts in GC-rich regions to form DNA interstrand cross-links (Brooks and Lawley, 1961; Kohn *et al.*, 1966; Chun *et al.*, 1969). Although HN2 can form covalent linkage (alkylation) with various nucleophilic moieties, the presence of two chloroethyl groups is required for its anti-tumor activity, suggesting that DNA cross-links are effective lesions (Kohn *et al.*, 1966; Chun *et al.*, 1969; Mattes *et al.*, 1986).

Platinum complexes were discovered serendipitously by Rosenberg in the early 1960s due to their ability to inhibit bacterial division (Rosenberg *et al.*, 1965). Cisplatin (Figure 1) was found to be active against a wide variety of tumors, such as head and neck, cervical, testicular, and ovarian carcinomas (Roberts and Thomson, 1979; Rosenberg,



cis-Pt



HN2

Figure 1. Chemical structures of cisplatin and nitrogen mustard.

1985; Sherman and Lippard, 1987). Cisplatin also forms monoadducts, intrastrand adducts and interstrand cross-links in DNA. The major lesions of cisplatin are d(GG) and d(AG) 1,2-intrastrand adducts (Sherman and Lippard, 1987; Eastman, 1987). DNA interstrand cross-links represent less than 10% of total adducts, which are preferentially formed at the d(GC) sites (Lemaire *et al.*, 1991).

Extensive studies have shown that DNA interstrand cross-linking induced by HN2, or cisplatin, plays an important role in cell killing. Direct evidence for the presence of DNA interstrand cross-links was provided by a number of physical techniques, including velocity sedimentation studies and denaturation-renaturation studies (Brooks and Lawley, 1961; Horacek, 1971). In addition, the DNA alkaline elution method has provided a very sensitive assay to detect DNA cross-linking in cells exposed to minimally cytotoxic doses of a variety of DNA cross-linking agents. By using this method, the kinetics of DNA interstrand cross-link formation and disappearance in the overall genome of the cells can be studied (Kohn *et al.*, 1976). Theoretically, formation of DNA cross-links induced by a bifunctional agent requires a sequence of two steps: the reaction of the first functional group to form a monoadduct, followed by the reaction of the second functional group to form a cross-link. The time required by each agent to complete second step reaction varies. In the case of HN2, little or no lag in cross-linking

was detected in mammalian cells at 37°C (Ewig and Kohn, 1977; Ross *et al.*, 1978), but for cisplatin there is a lag of 6 to 12 h in the formation of interstrand cross-links (Zwelling *et al.*, 1978, 1979). The levels of interstrand cross-links formed in tumor cells induced by either drug were inversely proportional to cell survival (Ewig and Kohn, 1977; Ross *et al.*, 1978; Plooy *et al.*, 1985; Zwelling *et al.*, 1979 and 1981; Laurent *et al.*, 1981; Erickson *et al.*, 1981). A substantial amount of evidence indicates that DNA interstrand cross-links induced by either HN2 or cisplatin contribute significantly to the cytotoxicity (Colvin, 1982; Roberts and Thomson, 1979). However, since the cisplatin intrastrand adduct is the predominant lesion, many investigators believe this type of adduct also is the lethal lesion (Eastman, 1987).

Overall, DNA damage generally is considered the predominant mechanism by which these two drugs exert their toxicity. However, some recent studies have indicated that other potential mechanisms exist (Epstein, 1990). One mechanism that has been implicated for several anti-tumor agents is inhibition of RNA transcription (Pieper *et al.*, 1989). HN2- or cisplatin-induced DNA lesions have been shown to block RNA polymerase transcription *in vitro* at selected guanine pairs in the HN2-treated DNA template, and at d(GG), d(AG), and d(GC) sites of cisplatin-treated DNA template (Pieper *et al.*, 1989; Lemaire *et al.*, 1991; Gray *et al.*, 1991). The cisplatin adducts at the d(GC) sites were identified as interstrand

cross-links. HN2 also has been shown to alter steady state mRNA levels of the c-*myc* oncogene, the expression of which is critical for cell proliferation (Futscher and Erickson, 1990). Further study of the effects of DNA cross-linking agents on gene expression in tumor cells may offer some insights to the factors contributing to anti-tumor activity induced by these agents.

Heterogeneous DNA Damage and Repair

DNA repair processes are cellular responses associated with the restoration of the normal nucleotide sequence of DNA after damage. The repair proficiency is usually assumed to be uniform throughout the genome. However, recent studies of DNA repair at the gene level have revealed that the DNA repair processes in the mammalian genome overall are heterogeneous (Bohr *et al.*, 1987; Bohr, 1991). This phenomenon was suggested in studies evaluating UV-survival characteristics in rodent and human fibroblasts (Ganesan *et al.*, 1983). Twenty-four hours after irradiation, the human cells removed 80% of the UV-induced pyrimidine dimers, while rodent cells removed less than 20% of these lesions. However, the rodent cells survived as well as human cells despite their low overall repair efficiency. This "repairadox" was explained by the possibility that rodent cells may selectively repair genes essential for cell survival. The finding that B-11 CHO cells remove UV-induced pyrimidine dimers from the dihydrofolate

reductase (DHFR) gene much more efficiently than from the overall genome supports this hypothesis (Bohr *et al.*, 1985). In these cells, the expression of the DHFR gene is essential for survival, since the cells were maintained in methotrexate-containing medium. Differential DNA repair also was noted when the repair rates of two proto-oncogenes (c-*abl* and c-*mos*) in Swiss mouse 3T3 cells were compared. UV-induced dimers were removed faster in the transcriptionally active c-*abl* gene than in the transcriptionally inactive c-*mos* gene (Madhani *et al.*, 1986). Furthermore, the transcribed strand of the DHFR gene was selectively repaired relative to the non-transcribed strand following UV irradiation (Mellon *et al.*, 1987).

In contrast to the homogeneous distribution of UV-induced pyrimidine dimers (Williams and Friedberg, 1979), extensive studies have revealed that DNA damage produced by many anti-tumor agents exhibits a non-random pattern in the mammalian genome. For instance, nitrogen mustards react predominantly with guanines by alkylating their N-7 position in GC-rich regions to form DNA interstrand cross-links (Brooks and Lawley, 1961; Kohn *et al.*, 1966, 1987 and 1988; Chun *et al.*, 1969; Mattes *et al.*, 1986 and 1988; Hartley *et al.*, 1988). Some DNA damaging agents appear to interact preferentially with transcribed genes or DNA associated with the nuclear matrix where transcription and replication are thought to occur (D'Andrea and Haseltine, 1978; Beckmann *et al.*, 1987; Tulius and Lippard, 1981; Chiu *et al.*, 1982; Ciejek *et al.*,

1983; Irvin and Wogan, 1984; Gupta *et al.*, 1985; Wassermann, 1990; Futscher *et al.*, 1992). These data link the accessibility of a gene to DNA damaging agents with the primary sequence and the functional state of the gene. However, the relative transcription activity of active genes has not been directly measured to determine whether or not the transcriptional state of a gene is related to its accessibility to DNA damaging agents.

The production and disappearance of DNA interstrand cross-links induced by DNA cross-linking agents can be assessed at the gene level by a novel assay developed by Vos and Hanawalt (1987). Using this technique, Futscher *et al.* (1992) demonstrated that HN2-induced DNA interstrand cross-links distribute in a heterogeneous fashion in human Colo320HSR cells. DNA interstrand cross-links are preferentially formed in the c-*myc* oncogene, which is amplified and overexpressed in this cell line. No DNA interstrand cross-links were detected in the lowly expressed N-*ras* oncogene, or the silent c-*fos* gene. These data are consistent with the notion that DNA damage in the mammalian genome overall is heterogeneous, and this heterogeneity may be coupled with the transcriptional activity of a gene.

The relationship between the transcriptional state of a gene and its accessibility to HN2-induced DNA interstrand cross-links is studied in this dissertation. The relative

transcription rates and the production of DNA interstrand cross-links in different genes are compared.

Colo320HSR Cell Line

The Colo320HSR cell line is used for this dissertation study, mainly because it contains an amplified and overexpressed c-*myc* oncogene and the characteristics of several other genes has been well documented in our laboratory (Futscher and Erickson, 1990; Futscher *et al.*, 1992). Colo320 cell line was isolated from the tumor of a 55-year-old Caucasian female patient with colon carcinoma before the initiation of chemotherapy (Quinn *et al.*, 1979). Two sublines have been established due to their karyotype differences. Colo320HSR carries a chromosomal homogeneously staining chromosomal region (HSR), and Colo320DM has numerous double minute (DM). Colo320 cells (HSR or DM) possess amine precursor uptake and decarboxylation properties, such as ectopic production of norepinephrine, epinephrine, serotonin, adrenocorticotropic hormone, and parathyroid hormone. Colo320 cells neither produce carcinoembryonic antigens, nor do they morphologically resemble other known carcinoma cell lines. These data suggest that Colo320 cells are of neuroendocrine origin rather than of colonic adenoepithelial origin (Quinn *et al.*, 1979).

By *in situ* hybridization analysis, the homogeneously staining region of the Colo320HSR marker chromosome was found

to contain a highly amplified c-*myc* oncogene. In addition, the amplification of c-*myc* has been accompanied by translocation of the gene from its normal position on chromosome 8 to a distorted X chromosome (8q24) (Alitalo *et al.*, 1983). Colo320HSR cells have at least 15 to 20 copies of an apparently normal c-*myc* allele and 1 to 2 copies of an abnormal c-*myc* allele lacking exon 1. These cells express high levels of normal c-*myc* mRNA (Schwab *et al.*, 1986).

Although the tumorigenic etiology of Colo320HSR cells is not completely delineated, studies on oncogene expression have revealed that c-*myc* appears to be the only oncogene whose expression is enhanced in this cell line, suggesting that c-*myc* amplification and overexpression may play a causal role in the development of colon carcinoma Colo320HSR (Alitalo *et al.*, 1983). The N-*ras* gene is expressed at a very low level, and the c-*fos* gene is silent in this cell line (Futscher and Erickson, 1990).

The c-*myc* Oncogene

The c-*myc* gene belongs to a family of related genes that include N-*myc* and L-*myc*. There is a large body of information concerning oncogenic activation and the intricacies in the regulation of *myc* expression, as well as compelling evidence for the involvement of Myc protein in cell proliferation, mitogenesis, and differentiation.

A. The c-*myc* Gene Structure

All normal c-*myc* transcription units are composed of three exons, the second two encoding the major c-*myc* proteins. Figure 2 provides summaries of a c-*myc* genomic DNA map, transcripts and open reading frames. A long untranslated exon 1 is present in c-*myc*-related genes, however, these exons have little homology to each other (Kohl *et al.*, 1986; Stanton *et al.*, 1986; DePinho *et al.*, 1987; Downs *et al.*, 1989). These observations lend support to the notion that an important structural or regulatory role of the c-*myc* leader regions exists via a sequence-independent mechanism. The first exon has been postulated to be the site of translational (Parkin *et al.*, 1988) and mRNA stability control (Pei and Calame, 1988). Near the end of exon 1, there is a site of transcriptional elongation blockage known to modulate c-*myc* expression in a number of cell types (Bentley and Groudine, 1986a, Bentley *et al.*, 1989; Nepveu and Marcu, 1986; Eick and Bornkamm, 1986).

Another conserved feature of the c-*myc* transcription unit is the presence of multiple transcription start sites. There are two major c-*myc* promoters, P1 and P2, located 161 bp apart in the human gene (Battey *et al.*, 1983; Watt *et al.*, 1983a). In normal cells, the P2 promoter predominates, giving rise to 75-90% of steady state c-*myc* mRNA. P1-initiated transcripts account for 10-25% of c-*myc* mRNA (Stewart *et al.*, 1984; Bentley and Groudine, 1986a; Taub *et al.*, 1984a and b; Spencer *et al.*, 1990). Minor promoters lacking canonical RNA

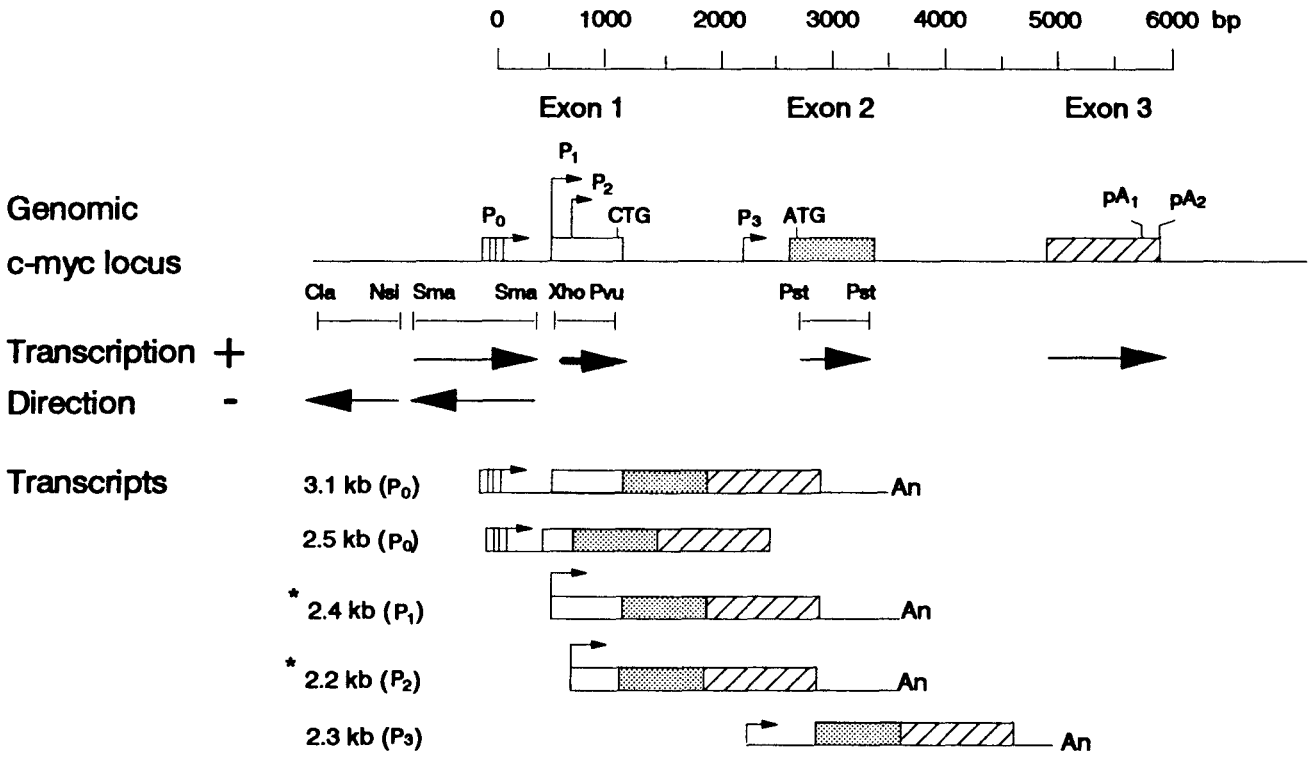


Figure 2. The c-myc gene structure and its transcripts.

Adapted from Spencer and Groudine (1991), and Bentley and Groudine (1986). * indicates major transcripts.

polymerase II TATA sequences have also been identified. P0 is located 550 to 650 bp upstream of P1. P3 is positioned near the 3' end of intron 1 of the human gene. Transcripts initiated from these two promoters contribute less than 5% of steady-state c-*myc* RNA (Bentley and Groudine, 1986b; Ray and Robert-Lézénès, 1989).

The sizes of the normal c-*myc* mRNA species are approximately 2.2 and 2.4 kb for major species initiating at the P2 and P1 promoters, respectively, and 3.1 and 2.3 kb for those minor transcripts initiating at P0 and P3 promoters (Battey *et al.*, 1983; Bentley and Groudine, 1986b; Ray and Robert-Lézénès, 1989). In some cell types, a 2.5 kb RNA has been detected that initiates near P0 with a major portion of exon 1 removed by RNA processing (Bentley and Groudine, 1986b). The half life of c-*myc* mRNA is about 10 to 30 minutes depending on the cell type (Dani *et al.*, 1984).

Antisense transcription in several regions of the human c-*myc* gene has been detected by nuclear run-on transcription assays as depicted in Figure 2 (Bentley and Groudine, 1986a). There is, however, no evidence of stable antisense c-*myc* transcripts in human cells. The functional role of the antisense transcription is unknown.

B. The c-Myc Protein

There are two major c-Myc proteins, P64 and P67, derived from alternative initiation codons but sharing the same

reading frame in exons 2 and 3 (Hann *et al.*, 1988). The ORF begins at an AUG codon at the 5' end of exon 2 and has been demonstrated to code for P64, a major *in vivo* c-myc protein (Hann and Eisenman, 1984; Ramsay et al, 1984). The second primary translation form, p67, is translated from an open reading frame beginning at a CUG initiation codon at the 3' end of exon 1 and thus contains an additional 15 amino acids at amino terminus (Hann *et al.*, 1988).

c-Myc proteins are short-lived nuclear phospho-proteins modified by phosphorylation on serine and threonine residues in a region spanning the exon 2/exon 3 boundary. Despite close to 3000 publications relating to the Myc family genes, there is no strong consensus as to their molecular function. Over the last few years, two competing models for Myc function have emerged and have received varying degrees of experimental support. These include a role in DNA replication and regulation of gene expression at transcriptional and post-transcriptional levels (for review, see Lüscher and Eisenman, 1990).

The structure of c-Myc protein has several significant features. The carboxyl terminus of Myc family proteins contains a basic region and a helix-loop-helix leucine zipper motif (BR-H-L-H-Zip) (for review, see Lüscher and Eisenman, 1990). The leucine zipper is a 20- to 30-residue-long amphipathic α-helix with a leucine residue situated at every second helical turn (i.e., every 7 residues) (Landschulz *et*

al., 1988). The function of the zipper as a protein-protein interaction domain appears in many cases to be closely coupled to a DNA-binding activity. In c-Myc, N-Myc, and L-Myc, the region of helix-loop-helix motif (H-L-H) also preceded by a basic region covers a block of ~55 amino acids located ~30 residues before their carboxyl termini. The remaining 30 carboxy-terminal amino acids possess the leucine zipper motif. These two features are common structure motifs found in transcriptional regulators (Jones, 1990; Olson, 1990). Many studies indicate that the homology region has a functionally bipartite structure with the H-L-H segment involved in homo- and heterotypic protein-protein interactions, while the basic regions are involved with DNA binding (Lassar *et al.*, 1989; Murre *et al.*, 1989a and b; Davis *et al.*, 1990). The inability to demonstrate specific DNA binding, or to find conclusive evidence for the ability of Myc to form homotypic and/or heterotypic complexes with known transcription factors, argues against the possibility that c-Myc may function as a transcription regulator. However, the discovery of Max, a myc-associated-protein, which also possesses a BR-H-L-H-Zip domain and binds to c-Myc in a manner that depends on the integrity of the c-Myc carboxyl terminus, may shed some light on Myc function (Blackwood and Eisenman, 1991).

The strong correlation between cell proliferation and expression of c-Myc has made the possibility of the involvement of Myc in DNA replication rather attractive.

Antisense oligonucleotides to c-*myc* resulted in a cessation of proliferation, and inhibited the entry of cells into the S phase (Heikkila *et al.*, 1987; Holt *et al.*, 1988). In addition, some studies have shown that c-Myc protein can bind and promote DNA replication at replication origins of both SV40 and c-*myc* itself (Lüscher and Eisenman, 1990). Overall, experimental results are consistent with the notion that c-Myc might be involved in replication, but it remains unclear as to the mechanism by which Myc acts.

C. Regulation of c-*myc* Expression

Studies on the regulation of c-*myc* gene expression revealed a complex array of mechanisms, including both transcriptional and post-transcriptional regulation. Steady state c-*myc* mRNA levels have been correlated with the cellular growth state. In general, levels of c-*myc* RNA are 10 to 40-fold higher in growing cells than in quiescent cells (Kelly *et al.*, 1983; Dean *et al.*, 1986). Initially, it had also been suggested that c-*myc* RNA levels might be regulated during the cell cycle, but it was subsequently shown that both RNA and protein levels are constant in cycling cells (Hann *et al.*, 1985; Thompson *et al.*, 1985). In contrast to the constant c-*myc* RNA levels in proliferating cells, decreased c-*myc* RNA levels are associated with some cells exiting the cell cycle, or undergoing terminal differentiation (Bentley and Groudine, 1986a; Simpson *et al.*, 1987; Siebenlist *et al.*, 1988).

Control of c-*myc* transcription initiation is mediated through a composite of positive and negative *cis*-acting transcription initiation elements (for review, see Spencer and Groudine, 1991). *In vivo*, steady state c-*myc* RNA is synthesized from P0, P1, P2, and P3 promoters by RNA polymerase II (Spencer and Groudine, 1990). Control over the elongation phase of transcription in the c-*myc* gene provided the first example of a eukaryotic gene regulated transcriptionally by an attenuation mechanism (Bentley and Groudine, 1986a; Eick and Bornkamm, 1986). Nuclear run-on analysis revealed a molar excess of exon 1 relative to exon 2 transcription in HL60, Hela, and Colo 320 cells, indicating the existence of a block to transcription elongation near the 3' end of exon 1 (Bentley and Groudine, 1986a). Furthermore, modulation in the half-life of c-*myc* mRNA and protein provides a commonly observed mechanism of c-*myc* regulation (reviewed in Piechaczyk *et al.*, 1987).

D. Oncogenic Activation of the c-*myc* Gene

The c-*myc* proto-oncogene is activated by several different mechanisms that result in the unrestrained growth of tumor cells. The mechanisms of activation include proviral insertion, chromosomal translocation, and gene amplification (Varmus, 1984; Rabbits, 1985; Cole, 1986; Alitalo *et al.*, 1987). The first demonstration that cellular proto-oncogenes could be activated in tumor cells was the finding that avian

leukosis virus was integrated into the c-*myc* locus in viral-induced B-cell lymphomas (Hayward *et al.*, 1981). Consistent chromosomal translocations in c-*myc* are characteristic of murine plasmacytomas (Adams *et al.*, 1983; Crews *et al.*, 1982), human Burkitt's lymphomas (Taub *et al.*, 1982; Dalla-Favera *et al.*, 1983) and human T cell tumors (Hollis *et al.*, 1988; Finver *et al.*, 1988). Amplification of c-*myc* occurs in a number of human tumors, such as promyelocytic leukemia (Collins and Groudine, 1982; Dalla-Favera *et al.*, 1982), some small-cell lung carcinomas (Little *et al.*, 1983; Krystal *et al.*, 1988), breast sarcomas (Escot *et al.*, 1986), colon carcinoma (Alitalo *et al.*, 1983), and osteosarcomas (Bogenmann *et al.*, 1987). Each of these mechanisms involves DNA rearrangements that lead to constitutive or elevated levels of c-*myc* expression. Thus, c-*myc* promotes tumor-cell growth via quantitative increases in c-Myc protein level, rather than by qualitative changes in the c-Myc protein. On the other hand, a consistent feature of tumors containing an activated c-*myc* gene is that the level of c-*myc* RNA is invariably higher (10 to 50 fold) than the low level found in quiescent cells (Keath *et al.*, 1984). These observations have led to a consensus that it is deregulation of c-*myc* expression, or an inability to turn the gene off, that is essential for activation.

GAPDH Gene

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.2.12)is a key enzyme in the control of glycolysis, catalyzing the conversion of glyceraldehyde-3-phosphate to 1, 3-diphosphoglycerate (Dandliker and Fox, 1955; Fox and Dandliker, 1956; Harris and Perham, 1963). The GAPDH protein is a tetramer composed of identical subunits of 37,000 molecular weight (Fox and Dandliker, 1956; Harris and Perham, 1963).

GAPDH belongs to a large multiple gene family. This gene family contains 150 or more GAPDH-like sequences, many of which are processed pseudogenes (Piechaczyk *et al.*, 1984a; Tso *et al.*, 1985). Only one gene coding for GAPDH is known to be functional in human, mouse, rat and chicken (Herbschleb-Voogt *et al.*, 1978; Bruns *et al.*, 1978 and 1979; Serville *et al.*, 1978; Alevy *et al.*, 1984). This gene has been localized to chromosome 12 in humans (Bruns *et al.*, 1978; Serville *et al.*, 1978). The protein sequence of GAPDH is highly conserved among six different organisms studied to date (Dayhoff, 1978). Comparison of GAPDH sequences between human, rat and chicken revealed a high degree of conservation at both the cDNA and protein levels (Tso *et al.*, 1985; Fort *et al.*, 1985).

The functional human GAPDH gene was isolated and characterized by Ercolani *et al.* (1988). The gene consists of 9 exons and 8 introns with eukaryotic signals necessary for the transcription and translation of GAPDH mRNA. Stable

transfection of rodent cells with the intact human GAPDH gene resulted in the expression of a correctly initiated human GAPDH mRNA and an enzymatically active human GAPDH polypeptide, indicating that the gene contains a functional promoter and intact coding sequences. Although many GAPDH pseudogenes and GAPDH-like sequences are present in the human genome, the single copy functional GAPDH gene can be identified on a Southern blot by using an intron segment of the gene (Ercolani *et al.*, 1988).

c-*fos* Oncogene

The human c-*fos* proto-oncogene was found as the cellular homolog of oncogenes carried by two murine retroviruses, FBR and FBJ osteogenic sarcoma viruses (Van Beveren *et al.*, 1983; Van Straaten *et al.*, 1983). The c-*fos* gene maps to the chromosome region 14q21-q31 (Barker *et al.*, 1984). It was cloned as a 9.0 kb genomic DNA fragment from a human lymphoblast cell line and has four exons which cover about 3.4 kb (Curran *et al.*, 1983). The polyadenylated c-*fos* mRNA is approximately 2.2 kb in size and encodes a 380 amino acids long protein (Curran, 1988).

The c-*fos* proto-oncogene is expressed at low or undetectable levels in most exponentially growing cells, although it can be activated transiently in response to numerous stimuli. The best characterized experimental system is quiescent fibroblasts which are induced to grow upon the

addition of growth factors. c-Fos protein accumulates quantitatively and rapidly in the nucleus, peaks at 1-2 h after stimulation, and disappears totally within 3-8 h, the length of each phase being cell type dependent (Kruijer *et al.*, 1984; Müller *et al.*, 1984; Vosatka *et al.*, 1989). Normal c-Fos protein is oncogenic both *in vitro* (Miller *et al.*, 1984) and *in vivo* (Rüther *et al.*, 1989) when expressed at an inappropriate level. To date, no rearrangements in the c-*fos* gene have been identified in DNA from human tumors (Curran, 1988).

Regulation of c-*fos* gene expression is complex and involves both transcriptional and post-transcriptional mechanisms. At the level of transcription initiation, several *cis*-acting elements of the gene bind *trans*-acting factors, which are responsible for the basal expression of the promoter (Treisman, 1985 and 1986; Gilman *et al.*, 1986; Greenberg *et al.*, 1987), or the induction by various agents such as serum (Treisman, 1985 and 1986), phorbol esters (Hayes *et al.*, 1987; Gilman, 1988; Sheng *et al.*, 1988; Siegfried and Ziff, 1989), cAMP (Sassone-Corsi *et al.*, 1988; Fisch *et al.*, 1989), and DNA damaging agents (Hollander and Fornace, 1989; Futscher and Erickson, 1990). At the post-transcriptional level, c-*fos* mRNA is highly unstable, thus providing a rapid shut-off of gene activity. Several defined regions appear to contribute to the rapid degradation of the mRNA, the use of each depending on the physiological conditions (Shyu *et al.*, 1989).

The c-Fos protein is a short-lived nuclear phosphoprotein. It is capable of stimulating gene expression not by direct binding to DNA but by interaction with transcriptional factors such as the members of the Jun family in the AP-1 complex (Chiu *et al.*, 1988; Franza *et al.*, 1988; Halazonetis *et al.*, 1988).

Histone H3.3 Gene

Histones are a class of basic proteins that are complexed with DNA to form the eukaryotic chromosome. Histone genes are transcribed into non-polyadenylated mRNAs, and no intron is found in most of the genes in histone family. In vertebrates, the regulation of histone genes is typically cell-cycle-dependent, with the expression being linked to ongoing DNA replication (Reviewed in Maxson *et al.*, 1983).

In contrast to the classic histone genes, the H3.3 histone gene is expressed in a replication-independent manner (Wu and Bonner, 1981; Wu *et al.*, 1983). The gene contains 3 introns and spans greater than 8 kb of genomic DNA. The processed histone mRNA is about 1.2 kb long, and contains unusually long 5' and 3' untranslated regions, and has a 3' polyadenylated tail (Wells and Kedes, 1985; Wells *et al.*, 1987). This variant basal histone somehow escapes the cell-cycle regulation and its protein function is unknown.

ß-actin gene

Cytoplasmic actins (ß-actin and τ-actin) constitute the major component of the microfilamentous structures and participate in a variety of cell functions. ß-actin is one of the most abundant cellular proteins in mammalian non-muscle cells (for review, see Clarke and Spudich, 1977).

The ß-actin gene contains six exons and five introns (Gunning *et al.*, 1983; Nakajima-Iijima *et al.*, 1985). The nucleotide sequences of the ß-actin exons are highly conserved among human, rat and chicken. The amino acid sequence deduced from the human ß-actin gene is exactly the same as that of the chicken ß-actin gene and differs by only one amino acid from that of rat ß-actin gene (Nakajima-Iijima *et al.*, 1985).

The expression of ß-actin gene is controlled by several sequences resided in the 5' flanking and first intron regions, such as a TATA box, a CCAAT box, and a serum response element (Sugiyama *et al.*, 1988; Frederickson *et al.*, 1989; Orita *et al.*, 1989). However, the regulation of expression of the ß-actin gene is a complicated process which requires further elucidation.

MATERIALS AND METHODS

Cell Culture

The cell line Colo320HSR was purchased from the American Type Culture Collection. Cells were maintained in suspension at 37°C in an atmosphere of 95% air/5% CO_2 in RPMI 1630 medium (Irvine Scientific, CA) containing 15% heat-inactivated bovine calf serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 50 units penicillin, and 50 μg/ml streptomycin (JRH Biosciences, Lenexa, KS). Cells were passed once a week before they reached the density of 1 X 10^6 cells/ml. Cell number was determined by counting on a Coulter Counter Model ZBI (Coulter Electronics, Inc., Hialeah, FL).

To obtain a serum-starved population of Colo320HSR, cells were seeded at 1 X 10^5 cells/ml and grown to their maximal density of 1.0 to 1.2 X 10^6 cells/ml over a period of 4-5 days in complete medium, and kept in stationary phase for three days without changing medium. These serum-starved cells were pelleted, and incubated in either fresh warm medium, or the same medium in which the cells were grown (conditioned medium) at a density of 5 X 10^5 cells/ml for 3 h before drug treatment.

Reagent Preparation

I. DNA Cross-linking Agents

HN2 and cisplatin were obtained from the Drug Development Branch, National Cancer Institute. HN2 was prepared as a 10 mM solution in sterile filtered 0.1 N HCl and stored at -20°C. Cisplatin was dissolved in complete conditioned medium (RPMI 1630 plus 15% calf serum) as a 500 μM solution by stirring at 37°C for 30 min just before use. Conditioned medium was defined as the same medium the cells were grown in before drug treatment.

II. General Chemical Agents

Glyoxal was obtained as powder and dissolved in DEPC-treated (diethyl pyrocarbonate) water as a 7 M stock solution (Sigma Chemical Co., St. Louis, MO). To facilitate the melting of glyoxal, the solution was heated to 55°C for 30 min. Glyoxal stock solutions were deionized by mixing with resin RG 501-X8 (Bio-Rad Laboratories, Richmond, CA) at room temperature until the pH of the solution was greater than 5.0. Small aliquots were stored at -80°C in tightly capped microfuge tubes. Each aliquot was used only once and discarded.

Formamide was purchased from BRL as a 100% solution. Formamide was deionized twice by stirring with RG 501-X8 resin (5 g/100 ml) at room temperature for 30 min. The solution was

filtered through 3 MM Whatman paper and stored at -20°C in aliquots.

Phenol was purchased as a frozen stock (BRL, Gaithers-burg, MD). Following melting at 68°C in a water bath, hydroxyquinoline was added to phenol with a final concentration of 0.1%. Hydroxyquinoline protects phenol from oxidization and adds a slight yellow color to the colorless phenol. Phenol was equilibrated to a pH >7.8 by mixing with an equal volume of 1 M Tris-HCl (pH 8.0) several times, and finally with 0.1 M Tris-HCl (pH 8.0). The phenol solution was stored at 4°C for up to a month.

All chemical solutions used for RNA preparation were either treated with DEPC or dissolved in DEPC-treated water. DEPC treatment was carried out by adding 100% DEPC to a final concentration of 0.1-0.2%, stirring at room temperature for at least 12 h, and autoclaved for 40 min at 15 lb/sq. in. pressure using liquid cycle.

Drug Treatment

To study effects of DNA cross-linking agents on gene expression, exponentially growing cells were seeded into individual flasks at a density of 2.5 X 10^5/ml and grown at 37°C overnight. 24 h after seeding, cells were exposed to various concentrations of either drug or vehicle (0.1 N HCl for HN2, or conditioned medium for cisplatin) for 1 h at 37°C. The drug was removed by washing the cells twice with warm

medium. Finally, the cells were incubated in warm fresh complete medium (RPMI 1630 plus 15% heat-inactivated bovine calf serum) for 0, 6, 12, 24, or 36 h. After incubation, cells were collected and analyzed. In addition, mock samples were set up for the corresponding time point control to monitor normal fluctuations of c-*myc* expression.

For studies on the accessibility of various genes to HN2-induced DNA interstrand cross-links, exponentially growing cells were set up at the same density as controls for serum-starved samples. Cells were exposed to 6.25 μM HN2, or vehicle for 1 h at 37°C. Then cells were washed twice with ice-cold 1 X PBS to remove the drug and harvested immediately for DNA, RNA and nuclei isolation.

Colony Formation Assays

Cytotoxicity induced by either HN2 or cisplatin was determined by soft agar colony formation assays as described by Chu and Fisher (1968). After drug treatment, resuspended cells were counted and 10^2 to 10^5 cells were seeded in triplicate into sterile tubes (Falcon 2054, 12 X 75 mm style, Becton Dickinson and Company, Lincoln Park, NJ) containing noble agar with a final concentration of 0.1% (DIFC Laboratories, Detroit, MI). Cells were grown for 14 days to produce clones of appropriate size for visual counting. The surviving fraction was calculated in relation to the control (cells without drug exposure).

General Methods

I. Nucleic Acids Isolation

A. Plasmid DNA Isolation

i). Large Scale Preparation of Plasmid DNA

To obtain large quantities of plasmid DNA for hybridization studies, equilibrium centrifugation in cesium chloride-ethidium bromide gradients was used. Bacteria carrying the plasmid of interest were grown overnight in 1 L Luria-Bertani medium (LB broth) (10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 10 g NaCl) with the appropriate antibiotic in a 2-L flask at 37°C, shaken vigorously (225 rpm) in a bacterial orbital incubator-shaker until the OD_{600} of the resulting culture was between 0.4 to 0.6. For cultured bacteria harboring low-copy-number plasmids, amplification of plasmids was carried out by adding chloramphenicol (Sigma, St. Louis, MO) to the culture (final concentration of 170 μg/ml) and incubating the culture for an additional 12-16 h.

The bacteria were lysed by the SDS (sodium dodecyl sulfate) protocol (Sambrook *et al.*, 1982). Bacterial cultures (500 mls) were pelleted at 3750 rpm (4000 X g) in the Beckman Model J-6M centrifuge at 4°C for 10 min. The pellet was washed once with 1X PBS, and resuspended in 10 ml of ice cold 10% sucrose, 50 mM Tris, pH 8.0. This 10 ml solution was split into 4 thick-walled polycarbonate tubes. To each tube, 0.5 ml lysozyme (10 mg/ml, dissolved in 0.25 M Tris, pH 8.0) and 2 ml of 0.25 M EDTA were added, the tubes were gently inverted and then

incubated on ice for 10 min. One ml of 10% SDS, and 1.5 ml of 5 M NaCl were added sequentially to each tube, then the tubes were gently inverted for 20 times. The lysate was incubated on ice for 1 h. The bacterial debris was removed by ultracentrifugation (Beckman Model L8-70M) in a Beckman Ti70.1 fixed angle rotor at 30,000 rpm (83,000 X g) at 4°C for 30 min. The aqueous layer was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and once with chloroform/isoamyl alcohol (24:1) to remove protein. Phases were separated by centrifuging the mixture in the Beckman Model J-6M at 2370 rpm (1600 X g) at 22°C for 10 min. Nucleic acids were precipitated by the addition of 2.5 volumes of ice-cold 95% ethanol (Kodak, New Haven, CT). Nucleic acids were recovered by centrifugation in a Sorvall RC-5B centrifuge (DuPont) using the ss-34 rotor at 4°C for 30 min at 10,000 rpm (10,000 X g). Nucleic acids were dried under vacuum, resuspended in TE, pH 8.0 and treated with RNAse (10 μg/ml) for 1 h at 37°C. This solution was extracted with organic solvents again to remove proteins. Supercoiled plasmid DNA was separated from the linear bacterial chromosomal DNA by equilibrium centrifugation in cesium chloride-ethidium bromide gradients. DNA was centrifuged in 6 M CsCl (BRL, Gaithersburg, MD), 0.8 mg/ml ethidium bromide gradients in Beckman Ti70.1 ultracentrifuge (45,000 rpm) for 48 h at 20°C. After centrifugation, the supercoiled plasmid DNA band was collected using a 18 g syringe needle. Ethidium bromide was removed by

extracting plasmid DNA with isoamyl alcohol 5 times. The plasmid DNA was dialyzed against TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), pH 8.0, and concentrated by ethanol precipitation. The identity of plasmid was always confirmed by its restriction enzyme digestion pattern before and after a large scale preparation.

ii). Small Scale Preparation of Plasmid DNA

To isolate small amounts of plasmid DNA from different bacterial clones, the alkaline lysis miniprep method was employed (Ausubel *et al.*, 1989). Bacteria were grown in 5 ml LB overnight at 37°C, shaken vigorously in a Roto-torque shaker (Cole-Parmer Instrument Company, Chicago, IL). The bacteria were pelleted in a microcentrifuge tube, and resuspended completely in 100 μl TE, pH 8.0. The bacteria were lysed by adding 200 μl solution containing 1% SDS and 0.2 N NaOH, and incubating on ice for 5 min. The mixture was neutralized with 150 μl 3 M potassium acetate, which cause the plasmid DNA to reanneal rapidly. The bacterial debris and chromosomal DNA were pelleted by spinning the sample in a Beckman Model E centrifuge for 5 sec at 12,000 rpm. The supernatant was saved and mixed with 0.9 ml of 100% ethanol at room temperature to precipitate nucleic acids. The nucleic acids were pelleted by centrifugation for 20 sec in Beckman Model E centrifuge. The pellet was washed with 70% ethanol and dried under vacuum. The pellet was resuspended in 20 μl

TE, pH 8.0 and RNAse (10 μg/ml) buffer and subjected to further analysis.

B. Total Cellular RNA Isolation

Total cellular RNA was isolated basically by following the guanidinium isothiocyanate method (Chirgwin *et al.*, 1979). Cells were washed free of media in 1 X ice cold PBS by centrifuging at 1800 rpm (800 X g) for 5 min twice in a Beckman Model TJ-6 table top centrifuge. The cell pellet was lysed by the addition of 3.5 ml guanidinium solution (4 M guanidinium isothiocyanate, 0.02 M sodium acetate, 0.01 M dithiothreitol (DTT), 0.5% Sarkosyl solution). The chromosomal DNA was sheared by passing the cell lysate through a 20 gauge syringe needle 4 times. The lysate was either stored at -85°C or centrifuged immediately. To isolate RNA, the 3.5 ml cell lysate was carefully layered onto a 1.5 ml of 5.7 CsCl, 0.1 mM EDTA cushion in an ultra clear ultracentrifuge tube (Beckman, 14 X 95 mm), and centrifuged at 35,000 rpm in a Beckman Model L8-70M using a SW-40Ti rotor at 18°C for at least 16 h. The supernatant was decanted and the RNA pellet was drained for 5 to 10 min. The RNA pellet was resuspended in 360 μl DEPC-treated H2O, transferred to a clean 1.5 ml microfuge tube, and precipitated with ethanol. The RNA was pelleted in a Beckman Microfuge 11 by spinning at 12,000 X g at 4°C for 30 min. The RNA was finally reconstituted in the appropriate amount of water to reach a concentration of

approximately 5 $\mu g/\mu l$ and stored at -80°C for further analysis.

C. Genomic DNA Isolation

Genomic DNA was isolated from cells following a protocol described previously (Futscher *et al.*, 1992). Cells were pelleted and washed free of media with 1 X ice cold PBS, pH 7.4. The cell pellet was dispersed by gentle vortexing. Cells were then lysed by adding lysis buffer (0.05 M $NaHCO_3/Na_2CO_3$, 1 mM EDTA, 0.5% N-Lauryl Sarcosine (w/v), and 0.3 mg/ml proteinase K, pH 10.4) to 3 X 10^6/ml (10 to 15 X 10^6/ml total). The cell lysate was incubated at 37°C for at least 3 to 4 h with periodic mixing to ensure complete digestion of protein.

The cell lysate was deproteinized by extracting with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) twice, and chloroform/isoamyl alcohol (24:1) once. The cell lysate and organic solvents were mixed until an emulsion was formed. After separation of the aqueous and organic phases by centrifugation in a Beckman J-6M centrifuge at 2370 rpm (1600 X g) at 22°C for 10 min, the genomic DNA was recovered from the aqueous phase by transferring to an Erlenmeyer flask. 0.2 volume of 11 M ammonium acetate and 2.5 volumes of ice cold 95% ethanol were added to precipitate the DNA. The DNA precipitate was spooled on to a glass rod, washed several times by dipping into 70% ethanol, and dissolved in 400 μl

H_2O. The DNA solution was treated with RNase A (Sigma, St. Louis, MO) at a final concentration of 0.1 μg/ml at 37°C for 30 min. Following RNase digestion, the DNA solution was deproteinized again by extraction with phenol/chloroform-/isoamyl alcohol once, and chloroform/isoamyl alcohol once. The extraction and phase separation were carried out in a microfuge tube and centrifuged in a fixed angled rotor in Beckman Microfuge 11 at 500 X g for 5 min. The DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of ice cold 95% ethanol. The DNA precipitate was washed and resuspended in 400 μl TE, pH 8, at 4°C overnight. The DNA solution was ready for quantitation and restriction enzyme digestion.

II. Nucleic Acid Quantitation

Two methods were frequently used to estimate the amount of nucleic acid in a solution. To accurately quantitate RNA or DNA concentrations, a spectrophotometric determination method was used. The concentration of nucleic acid was also estimated by a minigel method when the amount of nucleic acid was minuscule.

A. Spectrophotometric Quantitation of DNA or RNA

A small aliquot of the DNA or RNA sample was quantitated by UV spectrophotometry. The readings at wavelengths of 260 nm and 280 nm were taken. An OD reading of 1.0 corresponds to

approximately 50 μg/ml of double-stranded DNA, or 40 μg/ml single-stranded RNA. The purity of DNA and RNA preparations was estimated by the ratio of the readings at 260 and 280 nm (OD_{260}/OD_{280}). Pure preparations of DNA and RNA have OD_{260}/OD_{280} ratios of 1.8 and 2.0, respectively. Total cellular RNA and genomic DNA used for northern and Southern blot analysis were quantitated by this method.

B. Minigel Method

This method was used to estimate small amounts of DNA in solution. An aliquot of DNA sample was electrophoresed along with a serial dilution of DNA solutions with known concentrations through a 0.8% agarose minigel containing ethidium bromide (0.5 μg/ml). The intensity of fluorescence of the unknown DNA was compared with that of the DNA standard, thus allowing a rough estimation of the quantity of DNA in the sample. This method was frequently used to check DNA quantitation in subcloning of DNA fragments.

III. Purification and Concentration of DNA

DNA fragments eluted from agarose gels or generated from PCR reactions were usually contaminated with agarose gel particles and organic solvents. Purification and concentration were required before further applications. Elutip minicolumns containing a matrix similar to RPC-5 resin were used to absorb up to 100 μg of nucleic acids (size from

50 bp to 50,000 bp) (Schleicher & Schuell, Inc., Keene, NH). The minicolumn was first wetted with low salt buffer (0.2 M NaCl, 20 mM Tris-HCl, pH 7.4, 1.0 mM EDTA). A DNA sample suspended in the low salt solution was applied to the column with a standard Luer-lock syringe. After washing the column with the low salt buffer, the DNA was eluted by high salt buffer (1 M NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA) in 1 ml or less.

IV. Agarose Gel Electrophoresis

A. DNA Agarose Gel Electrophoresis

DNAs from 100 bp to approximately 50 kb in length can be separated on agarose gels of various concentrations (0.5-1.4%, w/v). SeaKem GTG Agarose (FMC, Rockland, ME) was melted in 0.5 X TBE buffer (45 mM Tris-HCl, 45 mM Boric acid, 1 mM EDTA, pH 8.0) by heating in a microwave oven, and cooled to 55°C. Ethidium bromide was added to a final concentration of 0.5 μg/ml and mixed thoroughly before casting agarose solution in an appropriate size gel apparatus (BRL "Babygel" , BRL Horizon 58, or FMC "Resolute"). The gel was allowed to set completely at room temperature, and depending on the agarose concentration, complete solidification occurred in 30 to 60 min. The solidified gel was submerged in 0.5 X TBE buffer (45 mM Tris-borate, 1 mM EDTA) until electrophoresis.

DNA samples were mixed with 6 X loading buffer (0.25% bromophenol blue, 40% sucrose in water) unless otherwise

noted, and loaded into the wells of the submerged gel. One or more marker DNAs of known size were also included in each gel, such as 123 bp ladder, 1 kb ladder, or the supercoiled DNA ladder (BRL, Gaithersburg, MD). The gel was electrophoresed at 1-5 volts/cm (measured as the distance between the electrodes). DNA banding was examined by UV light and the photograph taken by using a Fotodyne UV-440/Polaroid MP-4 photographic transilluminator system (Fotodyne Inc., New Berlin, WI).

B. RNA Agarose Gel Electrophoresis

Agarose gels were prepared in the same way as the gels used for DNA electrophoresis except that agarose was dissolved in RNA running buffer (10 mM Na_2HPO_4/NaH_2PO_4, pH 6.8). Usually 1% agarose gels were used to separate total cellular RNA.

RNA samples were denatured in 50% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO), 1 M glyoxal, 0.01 M Na_2HPO_4/NaH_2PO_4, pH 6.8, at 50°C for 1 h. The denatured RNA samples were chilled on ice and centrifuged for 3 sec to deposit all of the fluid in the bottom of the microfuge tube. The RNA samples were mixed with 1/6 volume of loading dye (6 X, 40% sucrose, 0.25% bromphenol blue), and immediately loaded into the preformed wells. The gel was run at 100 volts for 20 min to allow RNA samples to enter the gel. Then a ministalic pump (Manostat Corp., New York, NY) was turned on to circulate the running

buffer in order to maintain the pH at less than 8. The time needed to separate total cellular RNA was about 3 h.

To size an unknown RNA transcript, either a 0.24-9.5 kb RNA ladder (BRL) was added into outside lanes or 28 and 18 S rRNAs in total RNA were used as markers. RNA markers were cut from the gel and stained with ethidium bromide after completion of gel electrophoresis.

V. Nucleic Acid Blotting

After completion of agarose gel electrophoresis, nucleic acids were transferred to a GeneScreen Plus nylon membrane (DuPont, Boston, MA) by a capillary blotting procedure basically as described by Southern (1975).

To transfer DNA, the gel was immersed in the following solutions sequentially before blotting: 0.25 N HCl for 15 min; 0.5 M NaOH, 0.4 M NaCl for 30 min, and 0.5 M Tris-HCl, pH 7.5 and 3 M NaCl for 45 min. The acid treatment allows depurination of DNA to occur. The depurinated sites are cleaved during the alkali treatment and the smaller DNA fragments transfer more efficiently. The bromphenol blue tracking dye should change from blue to yellow when the acid treatment is complete. The dye should return to blue when the basic denaturation step is complete.

The gel was carefully placed on a wick (two pieces of 3 MM Whatman paper) whose ends were soaked in 10 X SSC buffer (1 X SSC is 0.15 M NaCl, 15 mM trisodium citrate, pH 7.0). A

GeneScreen Plus membrane, which was cut into the same size as the gel and presoaked in 10 X SSC for 15 min, was carefully placed on top of the gel. Air bubbles were then eliminated by cautiously rolling a 5 ml-pipette on the gel. Three pieces of 3 MM Whatman paper, a stack of absorbent towels, and a weight of approximately 500 g were sequentially placed on top of the membrane. The transfer was allowed for at least 18 hours. The absorbent pads were changed whenever necessary. The weight, pads, and 3 MM filter papers were removed by the end of the transfer. The membrane was treated with 0.4 N NaOH for 45 sec, then neutralized in 0.2 M Tris-HCl, pH 7.5, 2 X SSC for one min. The filter was air-dried before hybridization.

The RNA blotting procedure was the same as the DNA transfer with two minor modifications. First, gels were not subjected to the depurination, denaturation, and neutralization steps as in DNA transfer. Second, after the transfer, the membrane was treated with 50 mM NaOH for 15 sec to reverse the glyoxal reaction and neutralized in 0.2 M Tris-HCl, pH 7.5, 1X SSC for 35 sec before air-drying.

VI. Filter Hybridization

The membrane was prehybridized in a solution containing 50% formamide, 10% dextran sulfate, 5 X SSPE (1 X SSPE is 0.15 M NaCl, 10 mM dibasic sodium phosphate, 1 mM EDTA, pH 7.4), 1% SDS, 1 X Denhardt's (0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin Pentax Fraction V),

and 250 μg denatured salmon sperm DNA in a plastic bag for at least 4 h at 42°C in a shaking waterbath. Hybridization was carried out in the same solution, at 42°C for a minimum of 24 h with a ^{32}P labeled probe (10^6 cpm/ml). The probe was denatured by boiling for 5 min and chilled on ice before adding into the hybridization bag. The probe was mixed well with hybridization solution before incubation at 42°C. After hybridization, the blot was washed in 250 ml of 2 X SSPE, 0.5% SDS at room temperature, then washed at 65°C for 6 min in 7 L of 0.1X SSPE and 0.1% SDS in a Disk-Wisk apparatus (Schleicher & Schuell, Inc., Keene, NH). After washing, the membrane was kept damp and sealed in a plastic bag before analysis by autoradiography.

VII. Filter Stripping and Reprobing

The filters were deprobed by boiling in TE, pH 7.5, and 1% SDS solution for 20 to 30 min with constant stirring of the solution. The stripping was usually very successful if the filters were kept damp. Complete deprobing was assured by exposing the filter to an X-ray film for several days. Reprobing conditions were the same as for the fresh filters, except longer prehybridization (24 h) was usually applied to reduce the background.

VIII. Autoradiography

The filter was either sealed in a plastic bag or covered with Saran wrap, then exposed to an X-ray film (X-OMAT AR, Eastman Kodak Co., Rochester, NY or Cronex10, E.I. DuPont de Nemours & Co., Inc., Wilmington, DE) for appropriate time at -80°C with two intensifying screens (Lightning Plus, E.I. DuPont de Nemours & Co., Inc.). The films were developed in an autoprocessor (Kodak, m35A X-OMAT Processor, Eastman Kodak, Rochester, NY).

IX. cDNA Synthesis

Total cellular Colo320HSR RNA (1 μg) was subjected to first strand cDNA synthesis in a solution containing 1 X PCR buffer, 1 mM each of dCTP, dATP, dGTP, and dTTP (Perkin Elmer Cetus Corp., Norwalk, CT), 200 units of Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD), and 20 units of RNasin (Promega, Madison, WI), 0.32 μg of oligo dT (BRL, Gaithersburg, MD), in a total volume of 20 μl. The mixture was set at room temperature for 10 min, and then incubated at 37°C for 1 h, heated to 95°C for 10 min and chilled on ice.

X. DNA Dot Blot

DNA dot blot is a method used to spot gene-specific probes on a nitrocellulose filter. Plasmid DNA containing a specific gene probe was linearized with the appropriate restriction

enzyme. Linearized plasmid DNA was denatured by 0.4 N NaOH and neutralized with equal volume of 2 M NH_4OAc, and put on ice before dotting. A nitrocellulose membrane (9 X 11 cm) (0.2 micron, Schleicher & Schuell, Inc., Keene, NH) was wetted in water and soaked in 1 M NH_4OAc for at least 10 min. The presoaked membrane was sandwiched in a dot blot apparatus (BRL, Gaithersburg, MD). The wells were washed with 1 M NH_4OAc twice, and then 1 μg of the linearized, denatured DNA plasmids was spotted into the corresponding position under slow vacuum. The wells were washed with 1 M NH_4OAc twice. The filter was then labeled and carefully taken off the apparatus, swirled in 6 X SSC briefly, and soaked in 2 X Denhardt's for 15 min. The filter was sliced into strips while it was damp, and sandwiched between 2 pieces of 3 MM Whatman paper. The strips were baked under vacuum at 80°C for 2 h, and then stored at room temperature until hybridization.

XI. DNA Sequencing

DNA sequencing was carried out by using a TaqTrack system (Promega, Madison, WI). To label the primers (T7 and SP6) at their 5' ends, 10 pmol primer was incubated in a solution containing 10 pmol γ-^{32}P-ATP (>3,000 Ci/mmol), 5 units T4 polynucleotide kinase, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine at 37°C for 10 min. The T4 kinase was inactivated by heating the reaction mixture at 90°C for 2 min. Supercoiled plasmid DNA was denatured by a 0.2 M NaOH,

0.2 mM EDTA solution for 5 min at room temperature, and neutralized by adding 0.2 M ammonium acetate, pH 4.6. The denatured DNA were pelleted and air-dried and resuspended in a given volume of deionized water before annealing with ^{32}P-end-labeled primer. After annealing, d/ddNTP mix and Taq DNA polymerase were added into appropriate tubes. The mixture was incubated at 70°C for 15 min, and the reaction stopped by adding appropriate amounts of 10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and heated at 90°C for 5 min. Half of the reaction volume was then loaded on a 6% polyacrylamide sequencing gel (20 ml 5 X TBE, 15 ml 40% acrylamide, 25% ml dH2O, 5 g urea, 1 ml 10% APS, 20 μl TEMED). The gel was run at 55 W for 2 h with 1 X TBE buffer in a BRL sequence apparatus. The second half of the reaction was loaded on the gel for another two h electrophoresis. At the end of electrophoresis, the gel was carefully taken off the plates by adhering to a 3 MM Whatman paper, covered with Saran wrap, dried, and exposed to an X-ray film.

Preparation of DNA probes

I. General Information

The genomic c-*myc* (pHSR-1) and c-*fos* (pc-*fos*-1) clones were purchased from the American Type Culture Collection (Rockville, MD). The human GAPDH probe, a 400 bp fragment of the 5'-flank of the GAPDH gene (-116 to -516 bp), was generously provided by Dr. Maria Alexander-Bridges (Harvard

Medical School, Boston, MA). This probe recognizes only the functional human GAPDH gene on a Southern blot (Ercolani *et al.*, 1988). The rat GAPDH cDNA (pRGAPDH13) (Piechaczyk *et al.*, 1984b; Fort *et al.*, 1985), was supplied by Dr. Hans-Martin Jäck (Loyola University Medical Center, Maywood, IL), and the human ß-actin gene (clone pfhßA-1) (Gunning *et al.*, 1983) by Dr. Richard Morimoto (Northwestern University, Evanston, IL).

The probes used for detecting specific gene expression or gene fragment were generated either by subcloning or PCR amplification. The human c-*myc* exon 2 probe (Figure 3) is a 414 bp PstI fragment subcloned into a pGEM-3Z vector (Promega, Madison, WI). The human c-*myc* exon 1 probe (Figure 3), a 446 bp *Xho*I-*Pvu*II fragment, was subcloned into a pGEM-3Zf(+) vector (Colby *et al.*, 1983; Watt *et al.*, 1983b; Gazin *et al.*, 1984). The human c-*fos* exon 4 probe (Figure 3) was generated by a PCR subcloning method (Scharf, 1990). The histone H3.3 probe was generated by PCR amplification as described in PCR section below. The identity of the subclones was confirmed either by examining the restriction enzyme digestion pattern, or by DNA sequencing using a TaqTrack sequencing system (Promega, Madison, WI).

II. DNA Subcloning

Subcloning was used whenever a specific gene fragment was in need. This technique involves several essential steps,

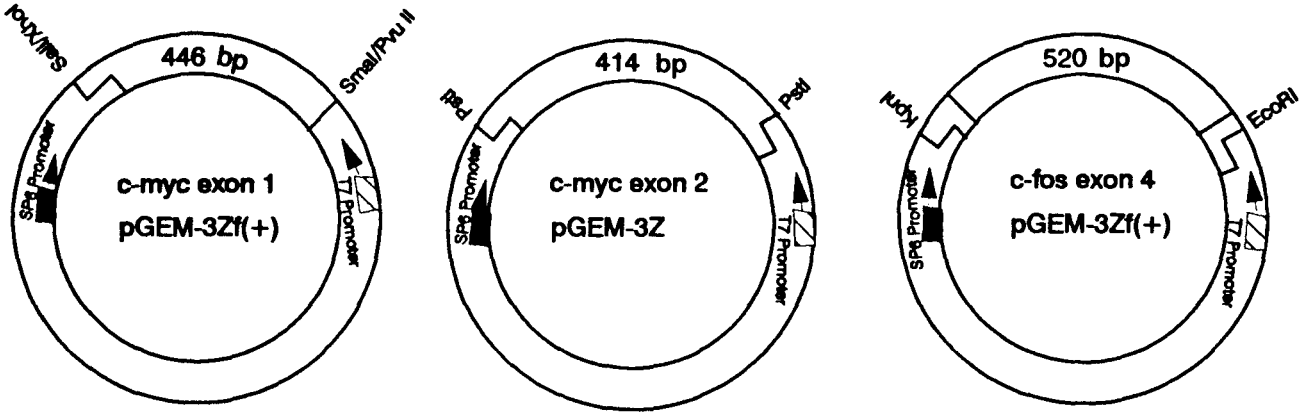


Figure 3. Maps of plasmids containing the subcloned specific gene-fragments.

Experimental details are described in Materials and Methods section.

including restriction enzyme digestion, gel purification of both insert (the desired gene fragment) and vector, BAP reaction, ligation, and transformation. The vector chosen for this study was the pGEM-3Z vector system from Promega (Madison, WI). PCR subcloning was applied as the alternative when there was no convenient restriction site available.

A. Isolation of DNA Fragments from Agarose Gels

Specific DNA fragments and vectors digested with appropriate restriction enzymes were fractionated on an agarose gel. After identifying the correct band, a trough was cut in front of the band, and a clean dialysis membrane (Spectra/Por membrane, Spectrum Medical Industries INC, Terminal Annex, LA) was shaped to fit the trough. With continuous electrophoresis, the DNA fragments of the correct size were trapped against dialysis membrane in the trough which filled with TE, pH 7.5 buffer. The electrodes were reversed briefly (10 sec) to get the DNA fragments back into the trough. The DNA fragments were collected and underwent further purification as described above.

B. BAP reaction

In order to suppress self-ligation of the plasmid cloning vector, the restricted and purified vector was dephosphorylated at its 5' ends by treating with bacterial alkaline phosphatase (BAP) (BRL, Gaithersburg, MD). The

vector was mixed with BAP (70 U/pmol ends) in the presence of 10 mM Tris-HCl, pH 8, heated at 65°C for 60 min. BAP was removed by extracting with a phenol/chloroform/isoamyl alcohol mixture (25:24:1) four times.

C. Ligation

Ends-compatible insert and vector were ligated together by T4 DNA ligase under the following conditions. In a total volume of 20 μl, 150 ng vector was mixed with insert in a 1:3 molar ratio in the presence of 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 5% (w/v) polyethylene glycol-8000, and 1 U T4 DNA ligase (BRL, Gaithersburg, MD). The ligation reaction mixture was incubated at 14-18°C for 12 to 24 h.

D. Transformation

E. coli bacteria were transformed with the ligated new plasmids. Competent *E. coli* DH5α bacteria (BRL, Gaithersburg, MD) were removed from -80°C and thawed on ice for 15 min. The ligation reaction mixture was first diluted five times with TE buffer, then gently added into 50 μl thawed bacteria. After setting on ice for 30 min, the reaction was heated at 37°C for 20 sec, and again chilled on ice for 2 min. Then 0.9 ml sterile LB broth without ampicillin was added and the bacteria allowed to grow at 37°C for 1 h in a shaking incubator (225 rpm). Finally, the bacteria were spread out on an X-

Gal/ampicillin agar plate (15 g Bacto-agar, 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl, 0.8 ml of 50 mg/ml X-gal, 5 ml of 100 mM stock IPTG, and 1 ml of 100 mg/ml ampicillin per liter) and incubated in a 37°C incubator for 14-16 h to allow colony formation. The colonies were picked and analyzed to identify the right subclone.

III. ^{32}P-Labeling of Hybridization Probes

DNA probes were made radioactive by using the random primer labeling method (Feinberg and Vogelstein, 1983). In this method, random oligonucleotides were used to prime DNA synthesis *in vitro* by using denatured linear double-stranded DNA fragments as the template. Over 70% of the precursor triphosphate was routinely incorporated into complementary DNA, and specific activities of over 10^9 dpm/μg of DNA were obtained.

The Prime-a-Gene labeling system (Promega, Madison, WI) was used to label gene-specific probes used in filter hybridization experiments. In the standard 50 μl volume reaction, 25 ng of gel-purified linear DNA fragments was used as the template. The reaction mixture contained 50 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 2 mM DTT, 1 mM HEPES, pH 6.6, 5.2 A_{260} units/ml random hexadeoxyribonucleotides, 20 μM each of unlabeled dGTP, dATP, dTTP, 400 μg/ml acetylated (nuclease-free) BSA, 50 μCi [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham, Arlington Heights, IL), 25 ng heat-denatured DNA fragments,

and 5 units Klenow enzyme. The reaction mixture was gently mixed and incubated at room temperature for at least 2 h or overnight. The unincorporated nucleotides were removed by size exclusion chromatography on Sephadex G-50 spin columns (Boehringer Manheim, Indiapolis, IN). Before the probe was added into the filter hybridization bag, radiolabeled DNA fragments were denatured by heating at 95-100°C for 5 min and subsequently chilling on ice.

IV. Quantitation of ^{32}P Incorporation

Incorporation of ^{32}P into DNA probes or RNA was determined by trichloroacetic acid (TCA) precipitable radioactivity. In general, nucleotides greater that 12 bp are precipitable by this methods. One or two percent of the labeled product was mixed with 25 μg yeast transfer RNA (BRL, Gaithersburg, MD) which served as a carrier to facilitate TCA precipitation in a clear Falcon tube (12 X 75 mm style, Falcon, Becton Dickinson and Company, Lincoln Park, NJ). After addition of 1 ml ice cold 10% TCA, the mixture was vortexed and placed on ice for at least 10 min. The mixture was layered onto a Whatman GF/C glass fiber filter placed in a vacuum manifold which was prewetted with 10% TCA (Millipore, Bedford, MA). The tube was rinsed with cold 2% TCA and also added onto the filter. Lastly, the filter was washed with 95% ethanol, and placed in a scintillation vial. Eight ml of scintillation fluid (Beckman Ready Gel) were added to the vial containing

the filter, and the sample was counted in a Beckman Model LS 5800 liquid scintillation counter. The percent incorporation of ^{32}P in a given sample was calculated as follows:

$$\% \text{ Incorporation} = \frac{\text{counts per aliquot X total volume}}{\text{Total counts}} \text{ X } 100$$

Polymerase Chain Reaction

The histone H3.3 probe was generated by using two primers which cover 215 bp coding region of the histone gene (Figure 4). The 100 μl PCR reaction is comprised of 8 μl cDNA synthesized from Colo320HSR total cellular RNA as described previously, 50 pmol each of the primers, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, and 200 μM each of dCTP, dATP, dGTP, and dTTP, two units of Taq DNA polymerase (Perkin Elmer Cetus, Emeryville, CA). The reaction mixture was added into a Genamp tube (Perkin Elmer Cetus, Norwalk, CT), and covered with 75 μl mineral oil (Sigma, St. Louis, MO) to prevent evaporation and condensation. The tube was placed in a DNA thermocycler (Perkin Elmer Cetus Corp., Emeryville, CA). The PCR reaction mixture was heated at 96°C for 5 min and then subjected to 30 cycles of programmed thermal cycling at 96°C for 30 sec; 55°C for 30 sec; and 72°C for 45 sec. After the completion of PCR cycling, the mineral oil was removed by adding an equal volume of chloroform and the PCR products were

Fos-KI

2785 2802
5′ AAGGTACCGACCTGCCTGCAAGA 3′

Fos-EI

3304 3287
5′ CGAATTCTGAGCGAGTCAGAGGAA 3′

Histone H3.3 1

281 299
5′ CCACTGAACTTCTGATTCGC 3′

Histone H3.3 2

495 478
5′ GCGTGCTAGCTGGATGTCTT 3′

Figure 4. Nucleotide sequence of c-*fos* and histone H3.3 PCR primers.

Numbering corresponds to that of human c-*fos* gene and human histone H3.3 cDNA.

recovered by phenol/chloroform extraction and ethanol precipitation. PCR products were purified further by Elutip minicolumns.

To amplify c-*fos* exon 4, two primers (Figure 4) were designed to amplify 520 bp of exon-specific region which covers more than 80% of the c-*fos* exon 4 (Van Straaten *et al.*, 1983). Fos-KI, a 23 mer (5' AAGGTACCGACCTGCCTGCAAGA 3'), is complementary to the 3' end of the coding strand of the c-*fos* gene (nt 2785-2802). The Fos-KI primer contains five non-*fos*-complementary nt at its 5' end, which encode a recognition sequence for the restriction enzyme *Kpn*I. Fos-EI, a 24 mer (5' CGAATTCTGAGCGAGTCAGAGGAA 3'), is complementary to the 3' end of the non-coding strand of the c-*fos* gene (nt 3304-3287). At its 5' end, the Fos-EI primer has six non-*fos*-complementary nt, which encode a recognition sequence for the restriction enzyme *Eco*RI. Non-*fos*-complementary nucleotides were included in the primers to facilitate subcloning of the c-*fos* PCR product into the pGEM-3Zf(+) vector. The PCR reaction for c-*fos* exon 4 amplification was set up in the same condition as for histone H3.3 probe amplification, except 2 ng linearized pc-*fos*-1 plasmid was used as the template. Also, the reaction mixture was denatured at 96°C for 30 sec; reannealed at 60°C for 30 sec; and extended at 72°C for 1.5 min. The amplified PCR product was digested with *Kpn*I and *Eco*RI, and then subcloned into the pGEM-3Zf(+) vector.

Northern Blot Analysis

Northern blot analysis was used to assess specific gene expression at the steady state mRNA level. Briefly, an equal amount of total cellular RNA isolated from control or drug treated samples was denatured by the DMSO/glyoxal method (Futscher and Erickson, 1990). The denatured RNA was fractionated in a 1% agarose gel in a recirculating buffer system containing 10 mM NaPO4, pH 6.8. After 3 h of electrophoresis at 100 V, the RNA was transferred to a nylon membrane by capillary blotting for 24 h with 10 X SSC. The glyoxal reaction was reversed by immersing the membrane sequentially with 50 mM NaOH (15 sec) and 0.2 M Tris-HCl, pH 7.5, 2 X SSC (35 sec). The membrane was prehybridized and hybridized at 42°C for 24 h with a ^{32}P-labeled probe. After hybridization, the blot was washed and autoradiographed. Subsequently, the filter was stripped and rehybridized with a ^{32}P-labeled histone H3.3 probe to monitor RNA loading equality. Quantitation of mRNA levels was determined by measuring the relative intensity of bands using scanning laser densitometry (LKB Ultrascan XL, LKB, Bromma, Sweden). The intensity of each band was expressed as the area under the curve (AU/mm^2). The percentage of c-*myc* mRNA levels after drug treatment was calculated by comparing the intensity of the band for the drug-treated sample with that of its own time point control. The intensity of the band for c-*myc* mRNA level was normalized with the corresponding histone message. The

normalization was calculated according to the following formula:

$$AA = A + \left(\frac{HC-HP}{HC} \times A\right)$$

AA: adjusted area under the curve of c-*myc* mRNA level;
A: actual measured area under the curve of c-*myc* mRNA level after drug treatment;
HC: area under the curve of histone message in the matched time point control;
HP: corresponding area under the curve of histone message after drug treatment.

Greater than 30% fluctuation in the steady state c-*myc* mRNA level is considered a significant change from baseline, based on the observation that the variation of mRNA levels of histone H3.3 gene were below 25% in northern blot analyses.

DNA Denaturing/Renaturing Gel Electrophoresis and Southern Blot Analysis

This assay was developed by Vos and Hanawalt (1987) and modified in our laboratory (Futscher *et al.*, 1992). The major steps of this assay are diagramed in Figure 5. In brief, after drug treatment and isolation of genomic DNA, the DNA was digested with the appropriate restriction enzyme at 37°C, for 2 h. Digested DNA was resuspended in 0.01 M $NaHPO_4/NaH_2PO_4$, pH 7.0. An equal amount of the DNA sample, either with or without drug treatment, was denatured by adding two volumes of denaturation dye (96% formamide, 1 mM EDTA, 0.1% bromphenol

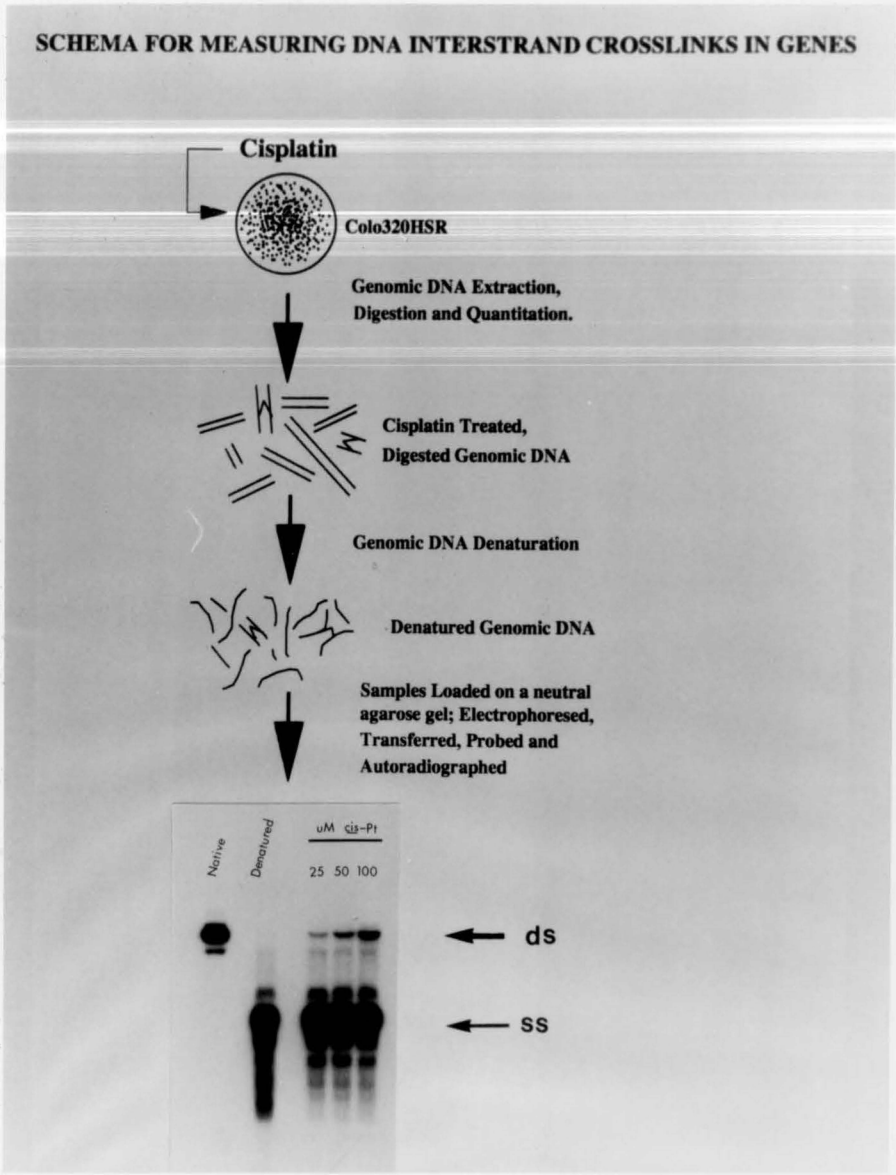


Figure 5. Schema for measuring DNA interstrand crosslinks in genes.

Adapted from Futscher *et al.* (1992).

blue, 0.1% xylene cyanol), and heated at 65°C for 5 min. The denatured samples were quickly chilled on ice-salt slush (1 M $CaCl_2$, -10°C) and loaded onto a 0.5% neutral agarose gel. In addition, a non-denatured DNA sample (in 50% glycerol, 0.1% bromphenol blue) was also loaded on the same gel. Following electrophoresis at 15 volts for 18 h, the gel was treated with acid and alkali solutions sequentially (detailed in nucleic acid blotting section) to facilitate the high molecular weight genomic DNA transfer. After Southern blotting, the membrane was hybridized and washed under the same conditions for northern blot analysis, and then autoradiographed for 24 to 36 h.

The quantitation of band intensities in each lane was determined by Betascope scanning (Betagen Corp., Waltham, MA). The percentage of cross-linked DNA formed in a sample was determined by dividing the amount of radioactivity in the double-stranded DNA band (cpm) by total radioactivity detected in double- and single-stranded DNA bands (cpm).

Nuclear Run-on Analysis

The protocol used for this study was a modified version of Banerji's procedure (1984). The procedure was divided into three major steps: isolation of nuclei; labeling and purification of nascent RNA transcripts; and hybridization of labeled RNA transcripts to cDNA probes dotted onto nitrocellulose.

To isolate nuclei, cells were washed twice with 1 X ice cold PBS and pelleted by centrifuging at 1000 rpm (500 x g) (Beckman J-6M) at 4°C for 5 min. The cells were resuspended in lysis buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4 and 0.5% NP-40) with a wide bore tip. The nuclei were pelleted by spinning briefly (3 sec) in a microfuge, resuspended in an equal volume storage buffer (0.1 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 8 and 40% glycerol), and quickly frozen in ethanol:dry ice bath and stored at -80°C.

Nascent RNA transcripts were labeled by an *in vitro* transcription reaction. An equivalent number of nuclei (~ 2 X 10^7) in 50 μl were mixed with an equal volume of 2 X reaction cocktail (150 mM KCl, 5 mM DTT, 5 mM $MgCl_2$, 50 mM HEPES-KOH, 10% glycerol, 0.7 mM ATP, TTP, GTP, 0.8 μM UTP, and 100 μCi α-[^{32}P] UTP, 3000 Ci/mmol). After incubation for 30 min at room temperature, the reactions were stopped by a 30-min incubation with 10 U RNase-free DNase at room temperature followed by Proteinase K digestion (0.9 mg/ml) in the presence of 1.4% SDS, 4.7 M urea, 235 mM LiCl, 0.67 mM EDTA, 6.7 mM Tris-HCl, pH 8, 1.1 mg/ml tRNA (Sigma, St. Louis, MO) for 1 h at 46°C. The RNA was precipitated by adding ice cold TCA to a final concentration of 10%, and stored on ice for 20 min. The RNA was pelleted in a Hermle microcentrifuge (National Labnet Company, Woodridge, NJ) for 15 min and then washed with 70% ethanol. The RNA pellets were air-dried, and resuspended in TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) containing 0.5% SDS.

The resuspension of RNA was facilitated by heating the solution at 60°C for several hours.

The ^{32}P-labeled RNA was hybridized to the linearized double-stranded cDNA probes immobilized onto nitrocellulose (0.2 micron, Schleicher and Schuell, Inc., Keene, NH). Hybridization was carried out in 4.5 ml (50% formamide, 6 X SSC, 10 X Denhardt's, 0.2% SDS and 50 μg/ml tRNA) for 72 h at 42°C. The membranes were subsequently washed for 30 min in 6 X SSC, 0.2% SDS, twice for 30 min in 2 X SSC, 0.2% SDS, and finally twice for 30 min in 0.2 X SSC, 0.2% SDS at 65°C before autoradiography. The filter was exposed to an X-ray film for 1 to 3 weeks. The overall RNA synthesis rates were determined by the amount of ^{32}P incorporated into *in vitro* RNA transcription reactions measured by TCA precipitable radioactive RNA (Sambrook *et al.*, 1982).

RESULTS

Cytotoxicity Studies

To determine the biologically relevant doses for further studies on interactions between DNA cross-linking agents and gene expression, the cytotoxicities induced by either HN2 or cisplatin in Colo320HSR cells were determined by colony formation assays. As demonstrated in Figure 6, doses that caused 1, 2, and 3 log cell kills are 12.5, 25, and 50 μM for cisplatin after at least three independent experiments. HN2 produced 1, 2, and 3 log cell kills at concentrations of 2.1, 3.25, and 6.25 μM, respectively (Futscher and Erickson, 1990). The plating efficiency of Colo320HSR was 33% ± 10 (mean ± SD, n=7).

DNA Damage and Repair Studies

By using DNA denaturing/renaturing gel electrophoresis and Southern blot analysis, the formation and removal of cisplatin-induced DNA interstrand cross-links were analyzed in a 12.5 kb *Eco*RI fragment that spans the three exons of the entire c-*myc* oncogene. Cisplatin-induced DNA interstrand cross-links formed in the c-*myc* gene were detectable and the levels of cross-links in the gene were produced in a dose-

dependent fashion (Figure 7). The removal of DNA interstrand cross-links in the c-*myc* gene was estimated by monitoring the loss of c-*myc* double-stranded DNA over time after drug treatment. Despite the difference in the initial production of DNA interstrand cross-links, the rate of disappearance of DNA interstrand cross-links induced by cisplatin in the c-*myc* gene was similar for different doses (Figures 8 and 9). Maximal DNA interstrand cross-links were observed immediately after cisplatin exposure, then gradually decreased over time. Detectable amounts of interstrand cross-links were still present 24 and 36 h after drug treatment.

Effects of DNA Cross-linking Agents on Gene Expression Studies

To study the effects of DNA cross-linking agents on gene expression after either cisplatin or HN2 treatment, the relative transcription rates, and steady state mRNA levels of several genes were examined. By using nuclear run-on analysis, the relative transcription rates of c-*myc*, GAPDH, and ß-actin genes were measured. Northern blot analysis was chosen to detect the steady state mRNA levels of c-*myc* and histone H3.3 genes. The c-*myc* oncogene is the main focus of the study because how an activated oncogene responds to DNA cross-linking agents is of interest. The GAPDH, histone H3.3, and ß-actin genes served as controls mainly for the validity of the assay.

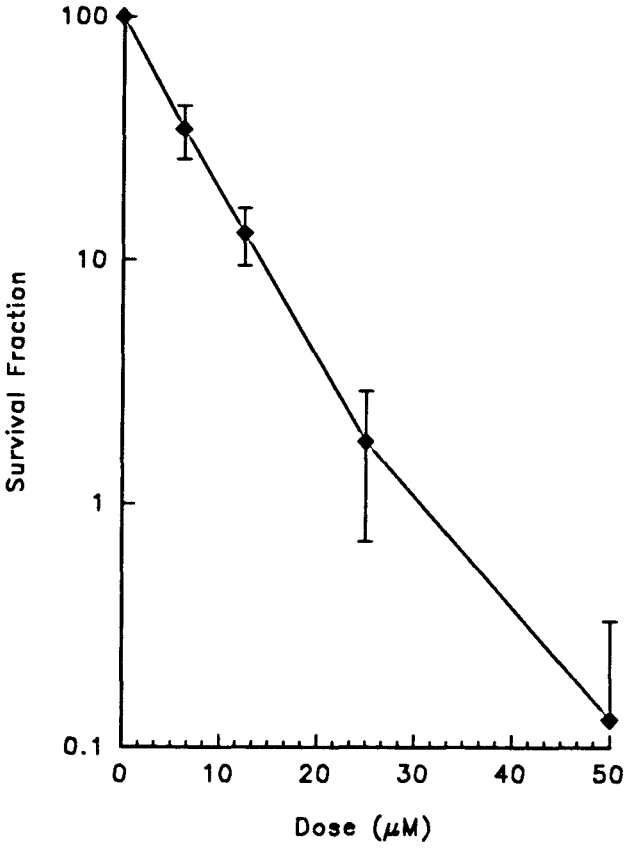


Figure 6. Survival of Colo320HSR cells following exposure to various concentrations of cisplatin for 1 h as determined by inhibition of colony formation.

Following drug exposure, cells were seeded into tubes containing soft agar, and colonies were counted 14 days later. The percentage of survival was determined by dividing the number of colonies formed by drug-treated cells by that formed by control cells. Points represent the mean of at least 3 independent experiments, and bars indicate the standard deviation.

Figure 7. Detection of cisplatin-induced DNA interstrand cross-links in the c-*myc* oncogene immediately after 1 h drug exposure.

*Eco*RI-digested DNA isolated from control or cisplatin-treated Colo320HSR cells was loaded on a 0.5% neutral agarose gel as either native or formamide-denatured samples. After electrophoresis, Southern blotting, and hybridization with the ^{32}P-labeled c-*myc* probe, the membrane was washed and autoradiographed for 24 to 36 h. Drug doses and positions of double-stranded (ds) and single-stranded (ss) DNA are indicated. Each lane contained 10 μg DNA except the native control lane which was loaded with 5 μg of DNA.

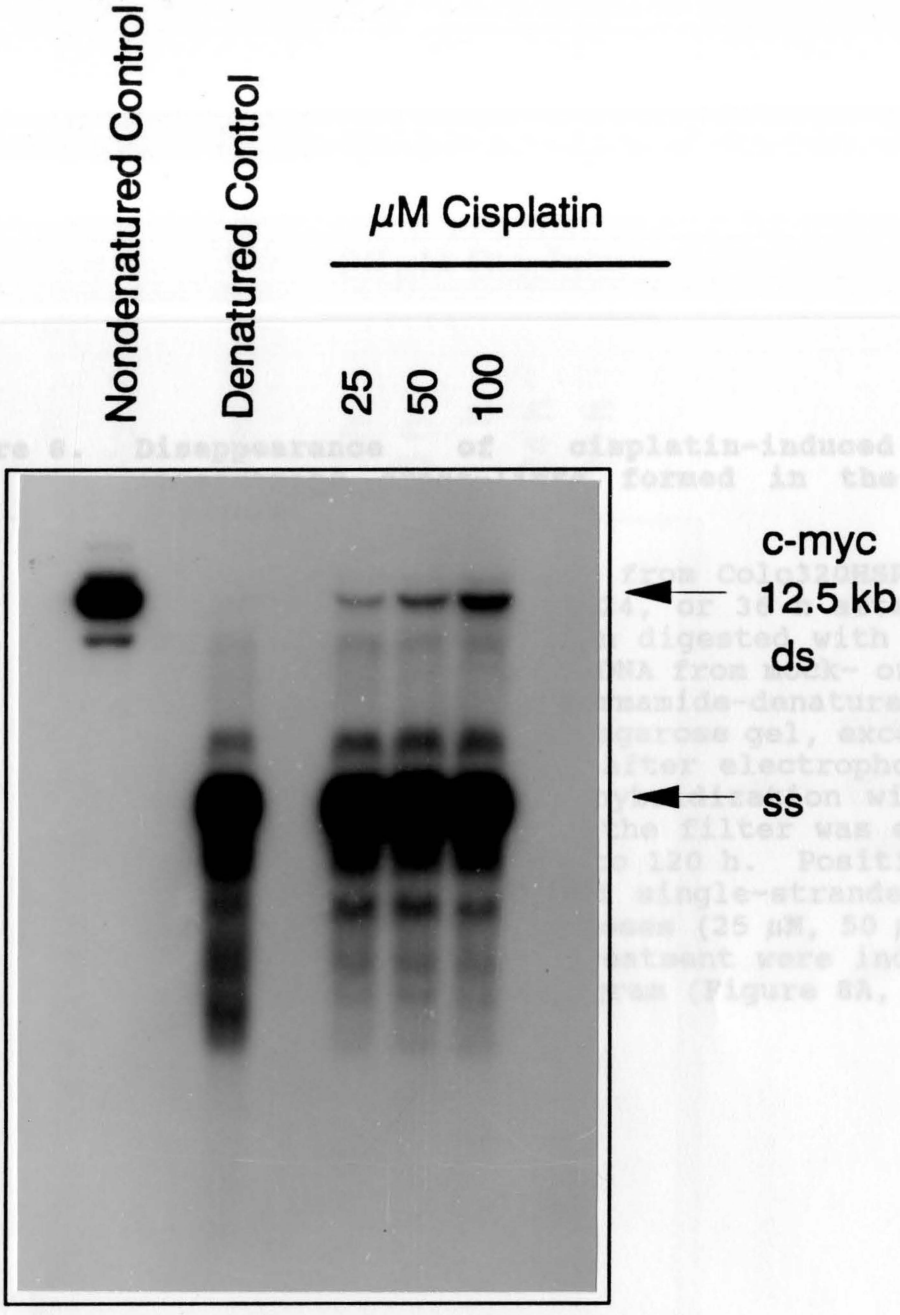

Nondenatured Control
Denatured Control
μM Cisplatin
25
50
100
c-myc
12.5 kb
ds
ss

Figure 7.

Figure 8. Disappearance of cisplatin-induced DNA interstrand cross-links formed in the c-*myc* oncogene.

Genomic DNA was isolated from Colo320HSR cells immediately (0), 6, 12, 24, or 36 h after drug removal, then restriction digested with *Eco*RI. Equal amounts (10 μg) of DNA from mock- or drug-treated samples were formamide-denatured, and loaded on a 0.5% neutral agarose gel, except the native control (5 μg). After electrophoresis, Southern transfer, and hybridization with the ^{32}P-labeled c-*myc* probe, the filter was exposed to an x-ray film for 36 to 120 h. Positions of double-stranded (ds) and single-stranded (ss) DNA are indicated. The doses (25 μM, 50 μM, and 100 μM) used for drug treatment were indicated on top of each autoradiogram (Figure 8A, Figure 8B, and Figure 8C).

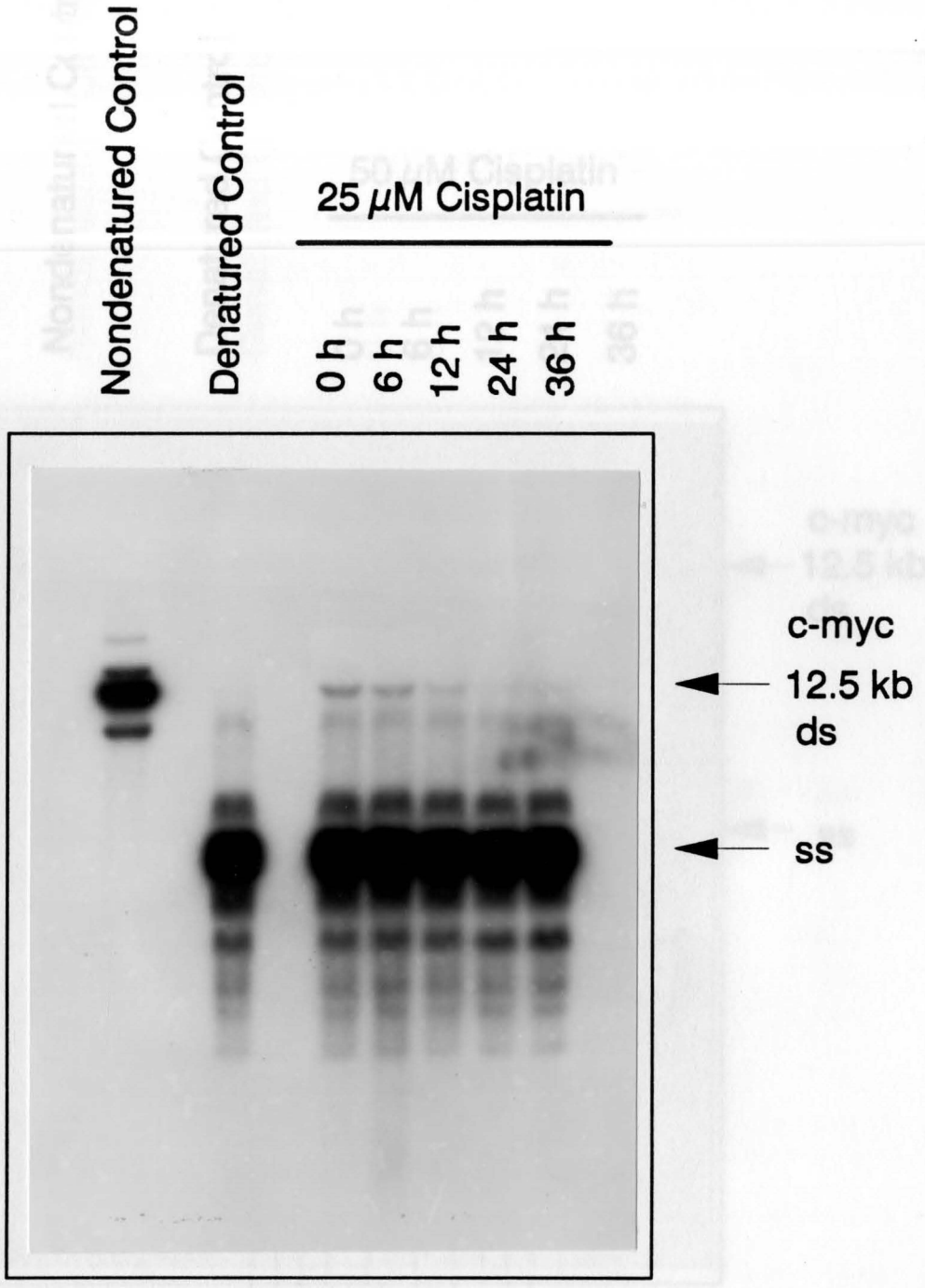

Nondenatured Control
Denatured Control
25 μM Cisplatin
0 h
6 h
12 h
24 h
36 h
c-myc
12.5 kb
ds
ss

Figure 8A.

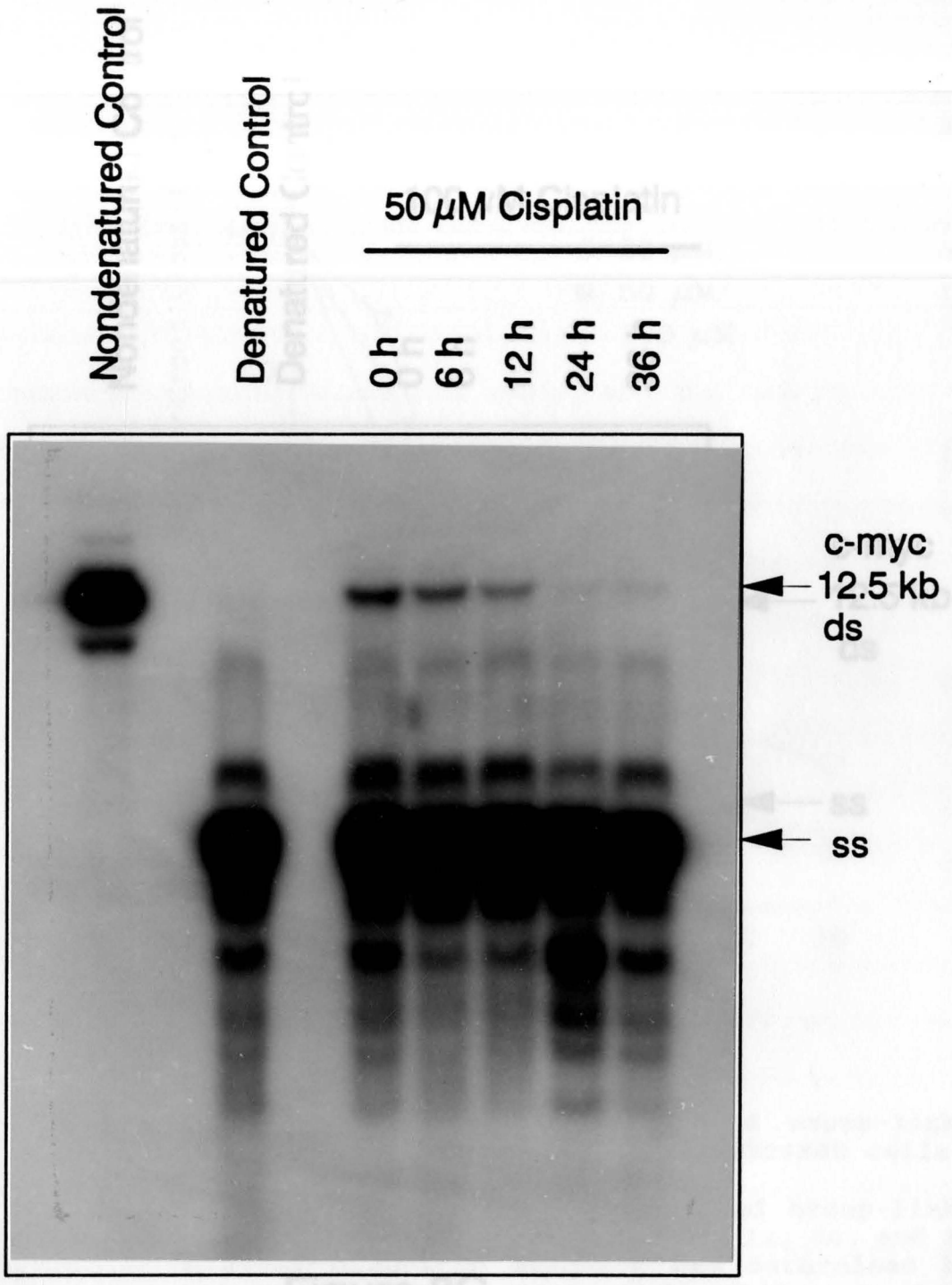

Nondenatured Control
Denatured Control
50 μM Cisplatin
0 h
6 h
12 h
24 h
36 h
c-myc
12.5 kb
ds
ss

Figure 8B.

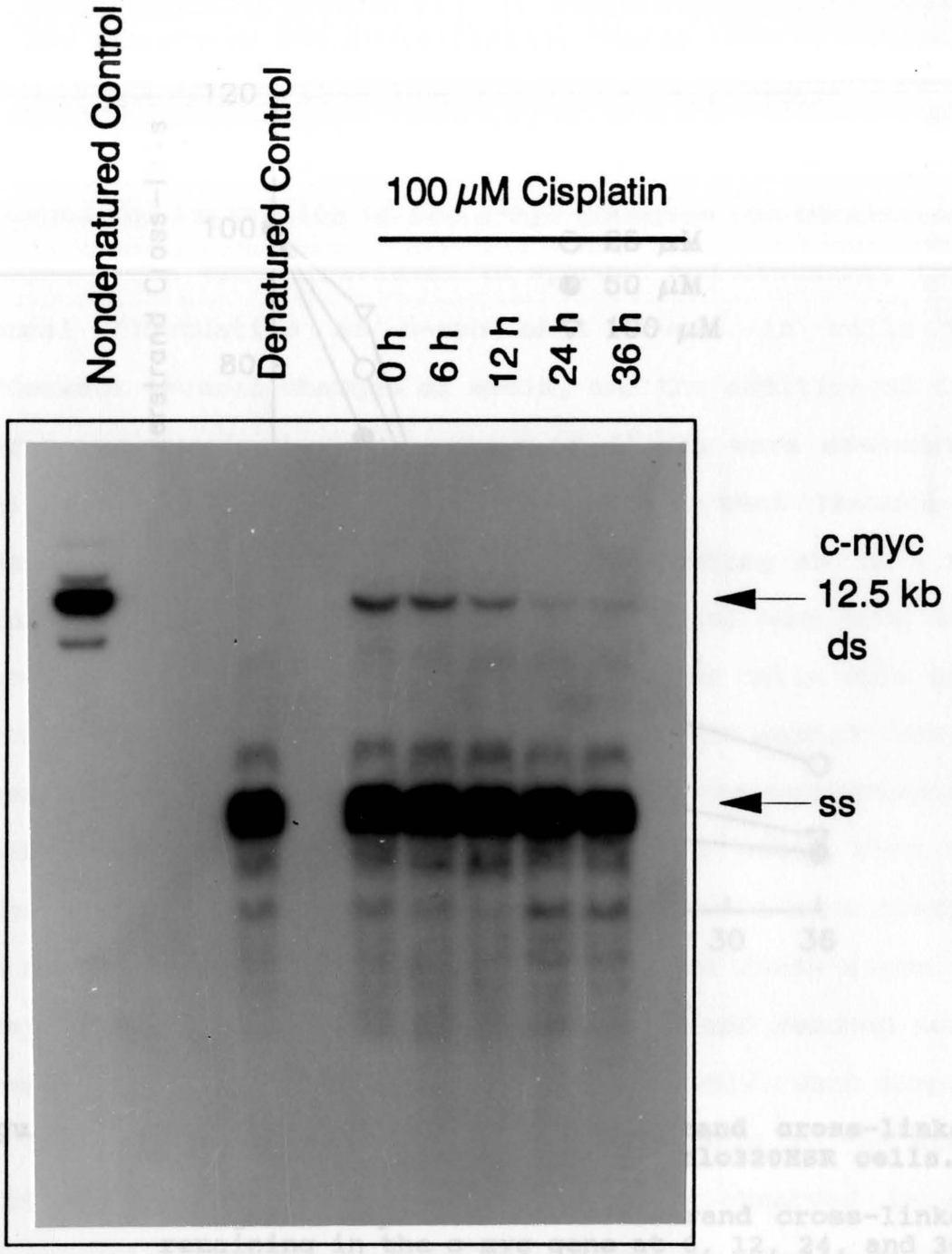

Nondenatured Control
Denatured Control
100 μM Cisplatin
0 h
6 h
12 h
24 h
36 h
c-myc
12.5 kb
ds
ss

Figure 8C.

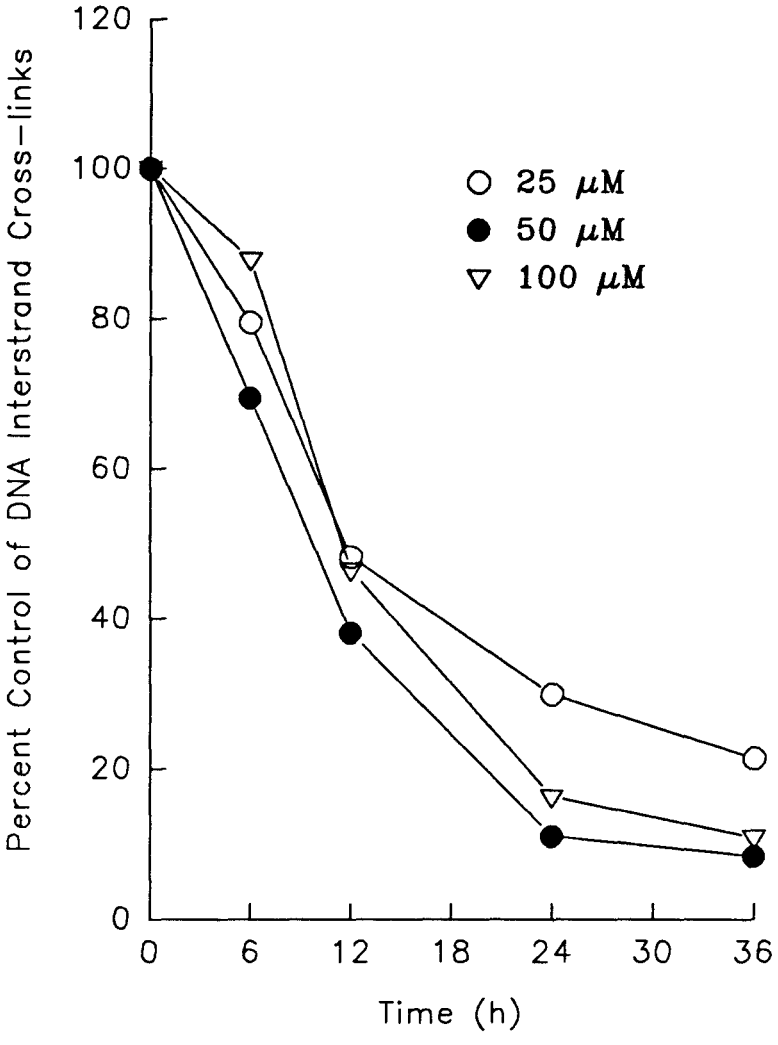


Figure 9. Disappearance of DNA interstrand cross-links after cisplatin treatment in Colo320HSR cells.

The percentage of DNA interstrand cross-links remaining in the c-*myc* gene at 6, 12, 24, and 36 h after cisplatin exposure was determined by using initial cross-links (0 h) of the same dose as 100% control. The above graph is a representative result of three independent experiments.

Normal Fluctuation of c-*myc* mRNA

The effects of DNA cross-linking agents (HN2 or cisplatin) on gene expression were examined in the Colo320HSR cell line which harbors an amplified and overexpressed c-*myc* oncogene. Because the expression of the c-*myc* oncogene can be altered by adding fresh serum (reviewed in Spencer and Groudine, 1991), normal fluctuation of c-*myc* mRNA levels in cells that underwent several changes of media, and the addition of fresh serum was examined. Two groups of flasks were seeded with cells. One group (selected at random) went through the identical steps of centrifugation and washing as in a drug treatment experiment. Another group (sham) was spun down, resuspended in the same medium in which the cells were grown (conditioned medium). Sham samples were not washed, nor was fresh medium added. Total cellular RNA was harvested from both groups at times 0, 6, 12, or 24 h after mock drug treatment. The steady state c-*myc* mRNA levels were constant in sham samples. In cells with addition of fresh serum, the c-*myc* mRNA levels decreased at 6 and 12 h and reached normal levels or doubled at 24 and 36 h, respectively, when compared to the sham samples (Figure 10). This change in the steady state c-myc mRNA level was consistently observed in each experiment. However, no fluctuations were observed in the steady state levels of histone H3.3 gene in both conditions.

Effects of Cisplatin and HN2 on Gene Expression at the mRNA Level

The effects of cisplatin or HN2 on steady state c-*myc* mRNA levels were examined by northern blot analysis. By comparing the steady state c-*myc* mRNA levels after drug treatment with a matched time point control, no dramatic changes were observed immediately after cisplatin exposure (0 h) in spite of different doses applied (Figure 11). The decrease in steady state c-*myc* mRNA levels was noted at 6, 12, 24, and 36 h after 1 h cisplatin exposure, with a maximal decrease (~60%) occurring at 24 to 36 h. Steady state c-*myc* mRNA levels in Colo320HSR cells following 25, 50, or 100 μM cisplatin treatment are summarized in Table 1.

Following HN2 treatment, the steady state c-*myc* mRNA levels were not changed immediately (0 h), but decreased at 6 and 12 h, reaching a nadir (~70% decrease) at 24 and 36 h (Figure 12). Steady state c-*myc* mRNA levels after 6.25 or 12.5 μM HN2 treatment is presented in Table 2. The decrease of c-*myc* transcripts after either cisplatin or HN2 treatment was not an immediate, but rather a delayed response of the cells to DNA cross-linking agents.

The variability in steady state mRNA levels of histone H3.3 after either cisplatin or HN2 treatment was less than 25% as measured by northern blot analysis, which served as a constitutively expressed control gene for equal RNA loading.

As shown in Figures 11 and 12, histone H3.3 gene expression was not affected by either drug.

Effects of Cisplatin and HN2 on Gene Expression at the Transcription Level

By nuclear run-on assays, the relative transcription rates of the c-*myc*, GAPDH, and ß-actin genes after either cisplatin or HN2 treatment were measured. Due to the transcription rate difference in exon 1 and 2 of the c-*myc* oncogene (Bentley and Groudine, 1986), both exon 1 and exon 2 were spotted on the filter as targets. No dramatic differences in c-*myc* transcription were observed immediately after cisplatin treatment (Figure 13). By comparing the level of transcription with the matched time point control, the c-*myc* transcription rates decreased in both exon 1 and exon 2 regions of the gene at 6, 12, and 24 h. A similar result was observed in cells treated with HN2 (Figure 14).

By comparing the rate of decrease in c-*myc* transcription and mRNA level after equitoxic doses of either cisplatin or HN2 treatment, the suppression in c-*myc* transcription paralleled the reduced steady state levels of c-*myc* mRNA detected by northern blot analysis (Figures 15 and 16). By 24 h after HN2 removal, c-*myc* transcription rates tended to approach normal levels as compared with those in cisplatin-treated cells. These results suggest that the decrease in steady state c-*myc* mRNA levels produced by either HN2 or

cisplatin appears to be mediated, at least in part, at the transcriptional level. In addition, after either HN2 or cisplatin exposure, the reduction observed in exon 2 was consistently greater than that occurring at the exon 1. In contrast, the relative transcription rates in the GAPDH and ß-actin genes did not change dramatically after exposure to either drug (Figures 13 and 14).

Total RNA Synthesis and Cell Proliferation

Total RNA synthesis after drug treatment was estimated by UTP incorporation *in vitro*. As shown in Tables 3 and 4, the overall UTP incorporation did not change dramatically after either HN2 or cisplatin treatment, as compared to the matched time point control.

Cell proliferation was monitored by electronically counting cells before harvesting for nuclei or nucleic acids preparation. In drug-treated samples, the cell concentrations did not change over time (Tables 5 and 6). However, cell concentrations in controls doubled between 24 and 36 h, in agreement with the doubling time of this cell line (about 24 h).

Studies on Accessibility of Individual Genes to DNA Damage Induced by HN2.

Early studies demonstrated that some DNA damaging agents interact with DNA in a heterogeneous fashion (reviewed in Bohr

Figure 10. Changes of the steady state c-*myc* mRNA levels in a mock experiment assessed by northern blot analysis.

Total cellular RNA was isolated immediately (0 h), 6, 12, 24 and 36 h after cells were resuspended in fresh serum. Sham samples were harvested at each corresponding time. Equal amounts of denatured total RNA (10 μg) were loaded in each lane. After electrophoresis and capillary transfer, the filter was hybridized with ^{32}P-labeled c-*myc* exon 2 probe (top panel), autoradiographed, then the filter was stripped and reprobed with a ^{32}P-labeled histone H3.3 probe and autoradiographed (bottom panel). The northern blot autoradiogram shown above is a representative of three independent experiments.

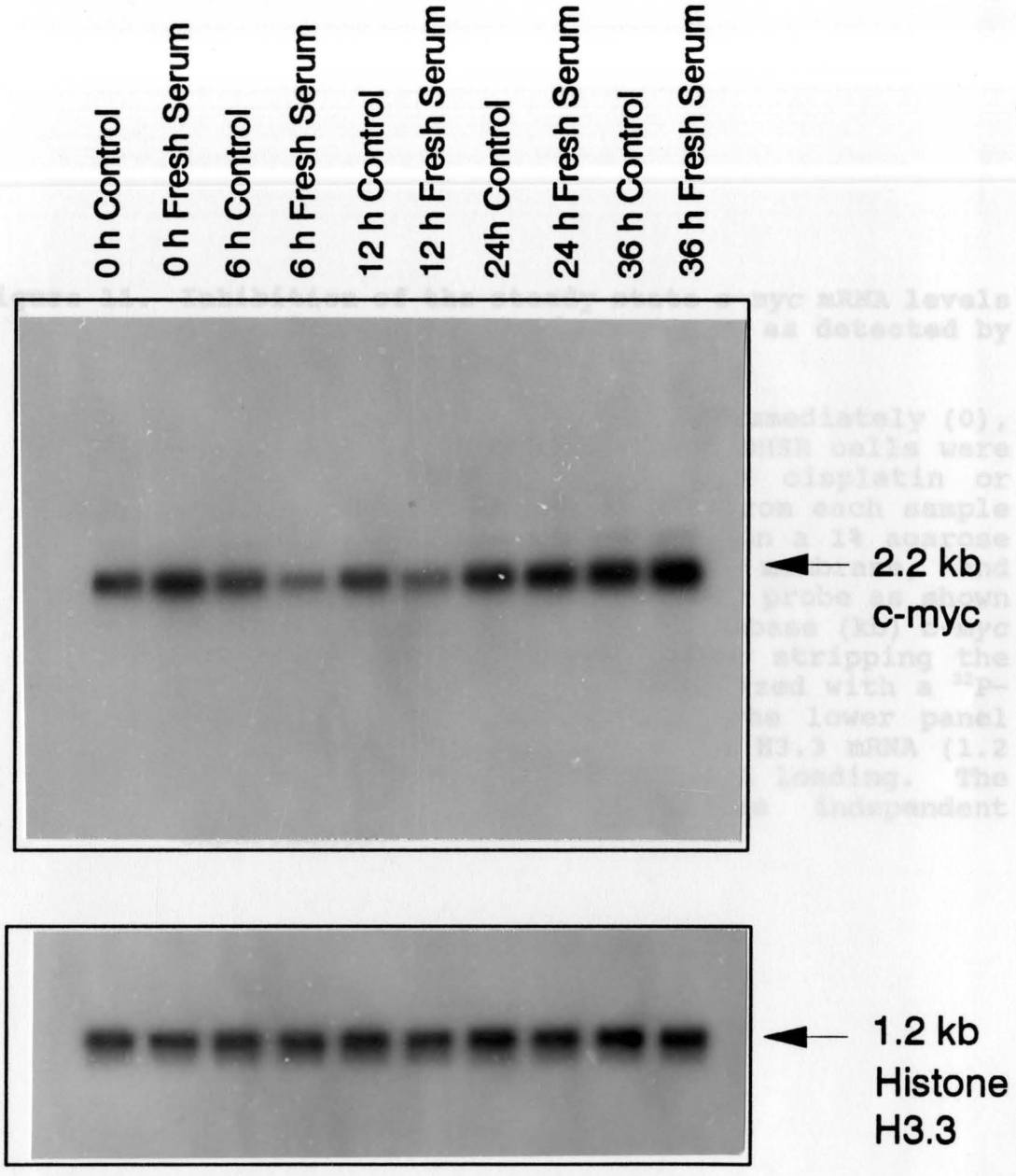

0 h Control
0 h Fresh Serum
6 h Control
6 h Fresh Serum
12 h Control
12 h Fresh Serum
24h Control
24 h Fresh Serum
36 h Control
36 h Fresh Serum
2.2 kb
c-myc
1.2 kb
Histone
H3.3

Figure 10.

Figure 11. Inhibition of the steady state c-*myc* mRNA levels following 1 h cisplatin exposure as detected by northern blot analysis.

Total cellular RNA was isolated immediately (0), 6, 12, 24, or 36 h after Colo320HSR cells were exposed to 25, 50, or 100 μM cisplatin or vehicle (control). 10 μg RNA from each sample was denatured, electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled c-*myc* probe as shown in the upper panel. The 2.2-kilobase (kb) c-*myc* transcript is indicated. After stripping the filter, the membrane was hybridized with a ^{32}P-labeled histone H3.3 probe. The lower panel shows the corresponding histone H3.3 mRNA (1.2 kb in size) indicating equal RNA loading. The above figure represents three independent experiments.

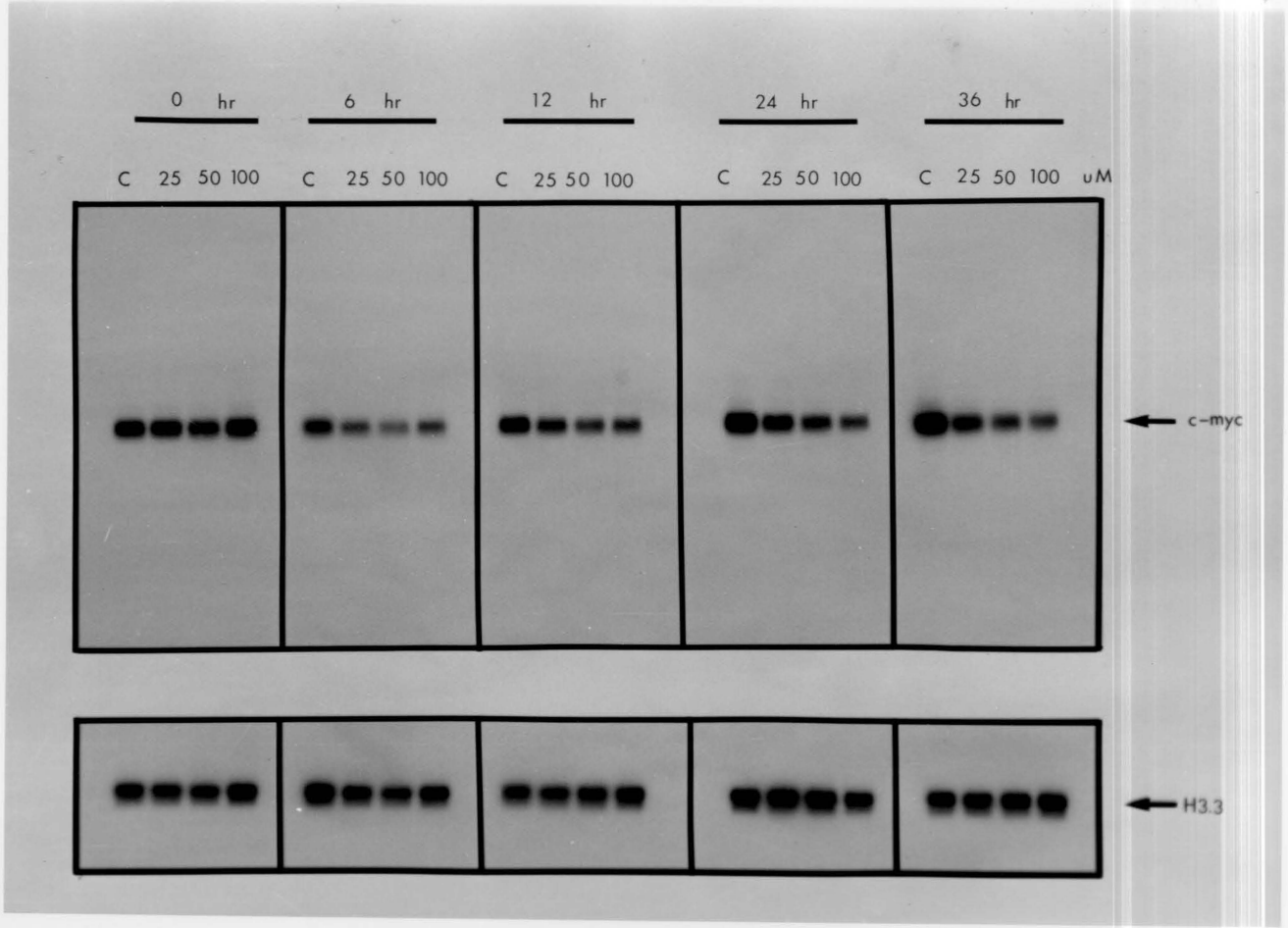

0 hr
6 hr
12 hr
24 hr
36 hr
C 25 50 100
C 25 50 100
C 25 50 100
C 25 50 100
C 25 50 100 uM
c-myc
H3.3

Figure 12. Inhibition of the steady state c-*myc* mRNA levels after 1 h HN2 treatment measured by northern blot analysis.

The upper panel shows the decrease of the steady state levels of c-*myc* mRNA over time after exposing Colo320HSR cells to either vehicle or HN2 (6.25 or 12.5 μM). The bottom panel shows corresponding histone H3.3 mRNA levels indicating equal RNA loading. Conditions were the same as described in Figure 11.

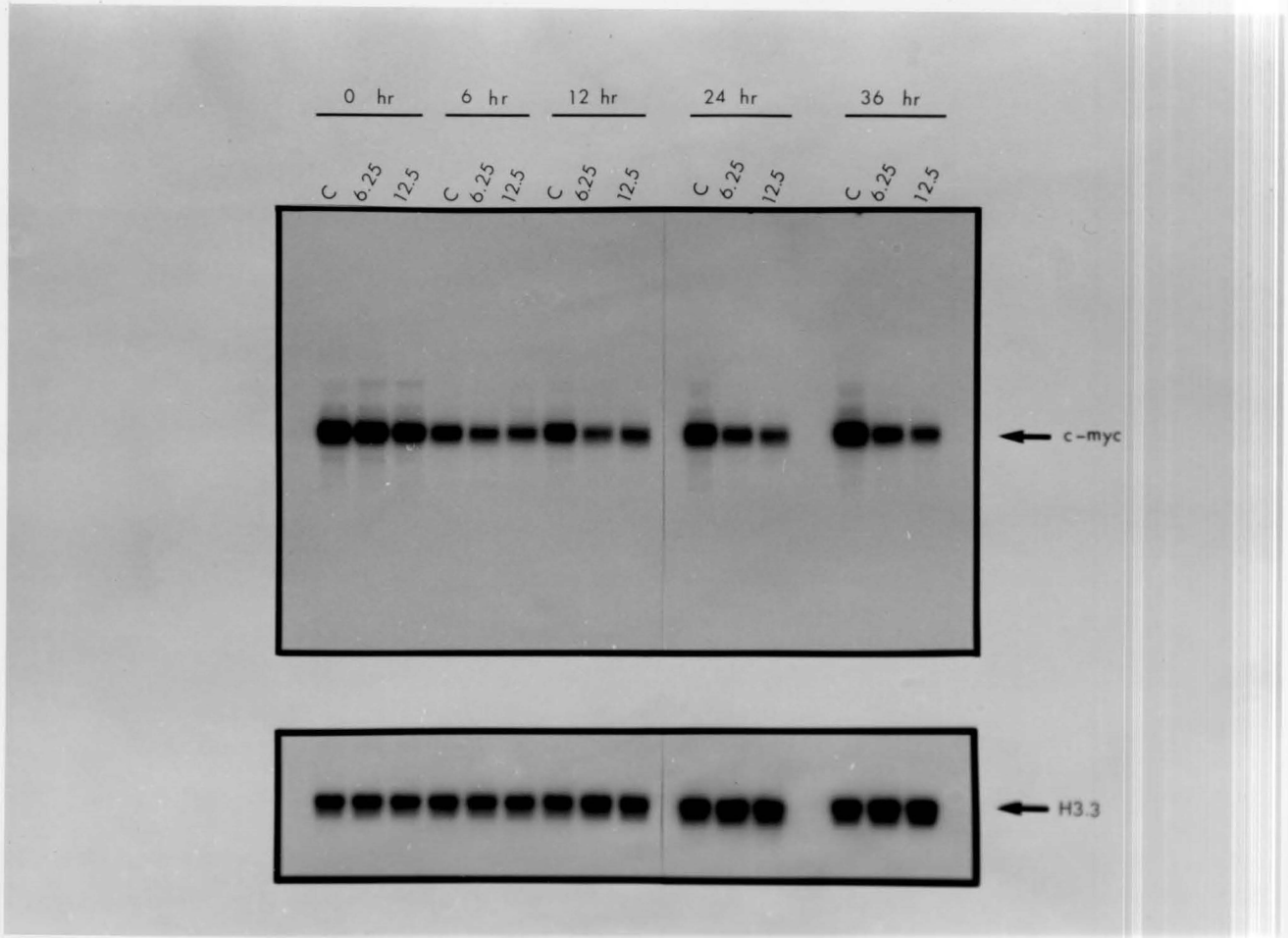

0 hr
6 hr
12 hr
24 hr
36 hr
C
6.25
12.5
c-myc
H3.3

Table 1

Effects of Cisplatin on c-myc mRNA Levels

Time (h)	0		6		12		24		36	
Dose (μM)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
25	104	± 19	91	± 29	84	± 10	60	± 11	60	± 16
50	82	± 15	71	± 29	65	± 11	53	± 12	47	± 15
100	96	± 18	66	± 21	58	± 11	41	± 13	39	± 32

The change in steady state c-myc mRNA levels after cisplatin treatment (immediately (0), 6, 12, 24, and 36 h) is expressed as a percentage of non-drug-treated control (details see **Materials and Methods**). Mean and standard deviation of three or more experiments are presented.

Table 2

Effects of HN2 on c-myc mRNA Levels

Time (h)	0		6		12		24		36	
Dose (μM)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
6.25	89	± 3	63	± 8	36	± 4	41	± 7	33	± 9
12.50	90	± 9	65	± 12	54	± 13	32	± 9	29	± 3

The change in steady state c-myc mRNA levels after HN2 treatment (immediately (0), 6, 12, 24, and 36 h) is expressed as a percentage of non-drug-treated control (details see **Materials and Methods**). Mean and standard deviation of three experiments are presented.

Figure 13. Nuclear run-on analysis of *in vitro* transcription in isolated Colo320HSR nuclei following 1 h cisplatin exposure.

Nascent transcripts were ^{32}P-labeled in nuclei isolated from cells immediately (0), 6, 12, or 24 h after 50 or 100 μM cisplatin exposure, and hybridized to DNA probes immobilized on nitrocellulose. An equivalent number of nuclei was used in each *in vitro* transcription reaction. pBR322 vectors and pGEM-3Zf were included as negative controls. The filters were washed and autoradiographed for 1 to 3 weeks with an intensifying screen.

0 h
6 h
12 h
24 h
C 50 100 C 50 100 C 50 100 C 50 100
pBR 322
pGEM-3Zf
c-myc
Exon I
Exon II
GAPDH
ß-actin

Figure 14. Nuclear run-on analysis of *in vitro* transcription in isolated Colo320HSR nuclei following 1 h HN2 treatment.

Nascent transcripts were ^{32}P-labeled in nuclei isolated from cells immediately (0), 6, 12, or 24 h after 6.25 or 12.5 μM HN2 treatment, and hybridized to DNA probes immobilized on nitrocellulose. Conditions were the same as in Figure 13.

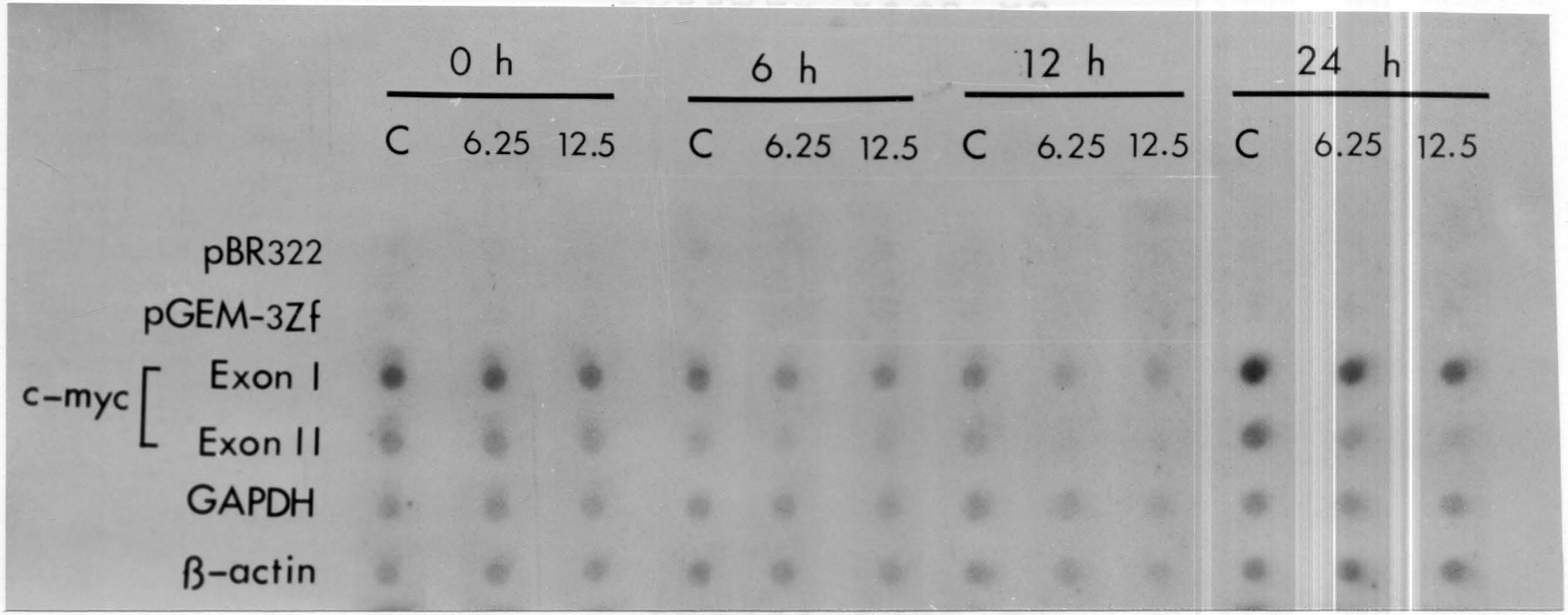
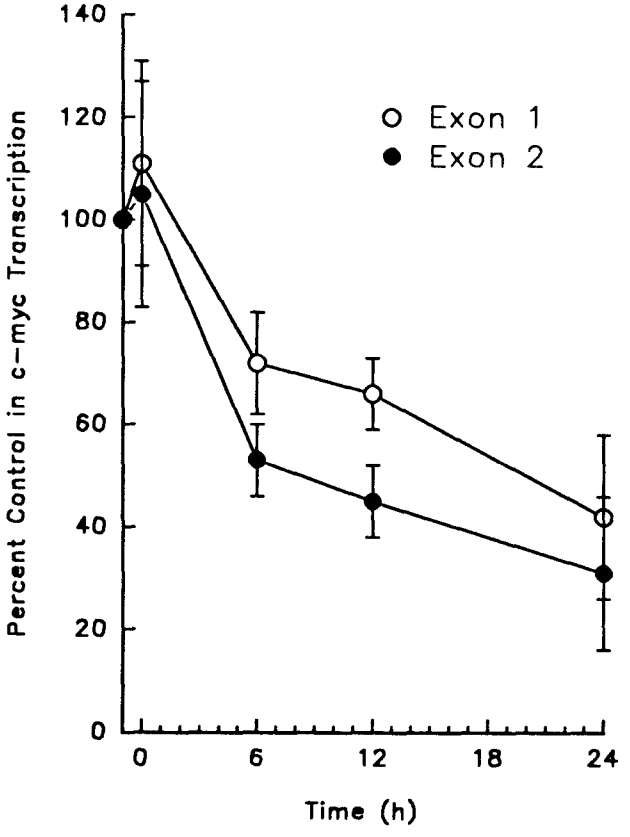

0 h
6 h
12 h
24 h
C
6.25
12.5
pBR322
pGEM-3Zf
c-myc
Exon I
Exon II
GAPDH
β-actin

Figure 15. Comparison of c-*myc* transcription rates and mRNA levels after 1 h cisplatin treatment.

C-*myc* transcription rate relative to the matched time point control following 50 μM cisplatin exposure was measured by the standard nuclear run-on analysis, and expressed as a percentage (left panel). Steady state c-*myc* mRNA levels following 50 μM cisplatin exposure was measured by northern blot analysis, and expressed as a percent control by comparing the level of c-*myc* mRNA in drug-treated sample with that of the matched time point control (right panel). Points: mean of 3 independent experiments; Bars: SD.


Percent Control in c-myc Transcription
140
120
100
80
60
40
20
0
0
6
12
18
24
Time (h)
Exon 1
Exon 2

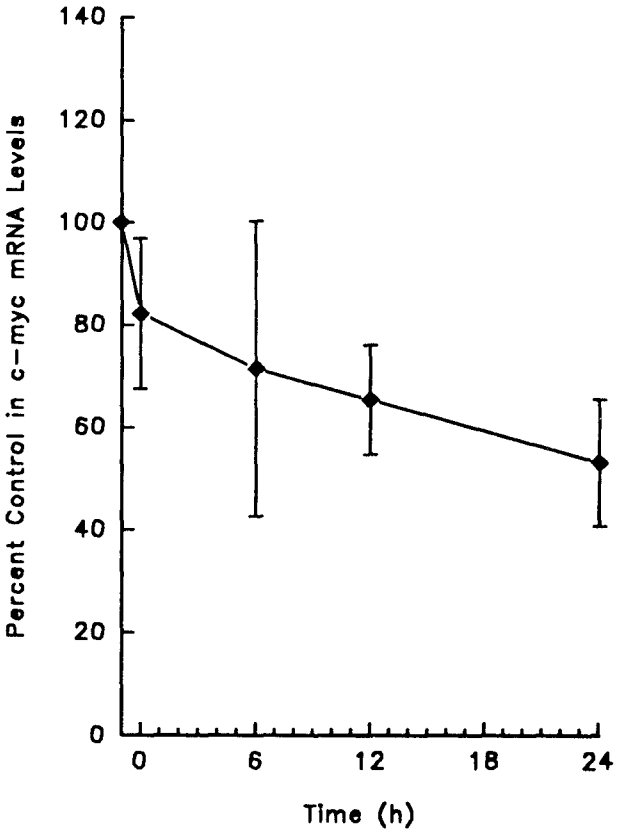
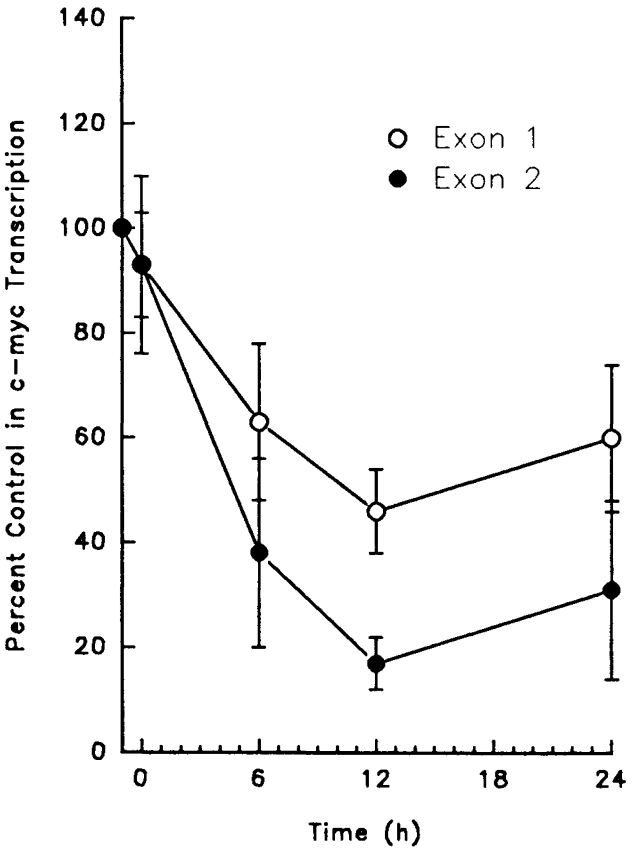

Percent Control in c-myc mRNA Levels
140
120
100
80
60
40
20
0
0
6
12
18
24
Time (h)

Figure 16. Comparison of c-*myc* transcription rates and mRNA levels after HN2 exposure.

Quantitation of c-*myc* transcription rates and mRNA levels following 6.25 μM HN2 treatment was measured by scanning densitometry, and expressed as a percentage as described in Figure 15.


Percent Control in c-myc Transcription
140
120
100
80
60
40
20
0
Exon 1
Exon 2
0
6
12
18
24
Time (h)

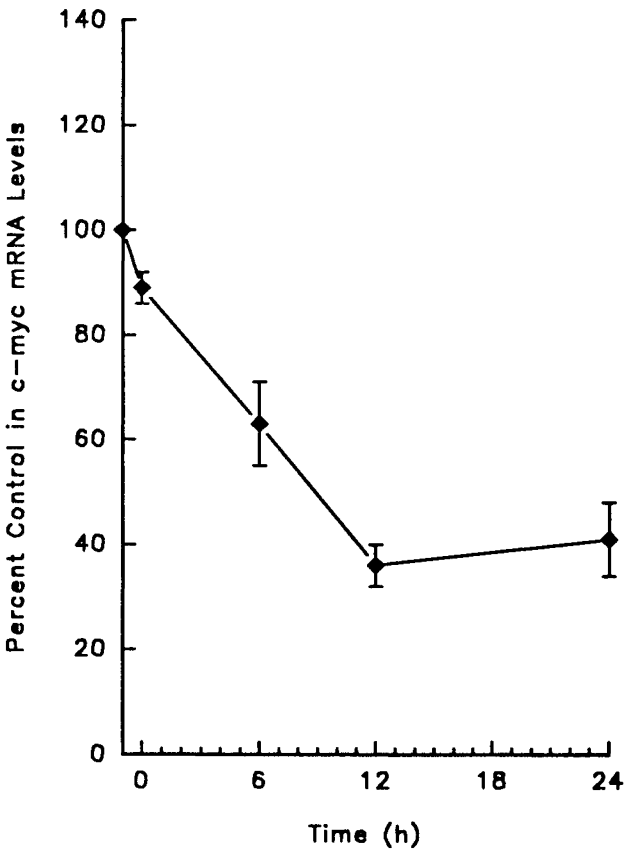

Percent Control in c-myc mRNA Levels
140
120
100
80
60
40
20
0
0
6
12
18
24
Time (h)

Table 3

Incorporation of α-^{32}P-UTP after Cisplatin Exposure

Time (h) / Dose (μM)	0	6	12	24
	Mean SD	Mean SD	Mean SD	Mean SD
Control	42 ± 5	60 ± 15	60 ± 9	52 ± 7
50	42 ± 17	55 ± 9	46 ± 10	43 ± 9
100	41 ± 15	48 ± 12	51 ± 6	37 ± 7

The amount of α-^{32}P-UTP incorporated into nuclei with or without cisplatin treatment is expressed as 10^5 cpm per 10^7 nuclei. Mean and standard deviation of three independent experiments are presented here. Paired-t tests show that there are no differences between control and drug treated samples ($P>0.05$).

Table 4

Incorporation of α-^{32}P-UTP after HN2 Exposure

Time (h)	0	6	12	24
Dose (μM)	Mean SD	Mean SD	Mean SD	Mean SD
Control	38 ± 10	58 ± 37	37 ± 6	28 ± 7
6.25	27 ± 10	32 ± 7	34 ± 3	27 ± 5
12.50	23 ± 4	29 ± 18	31 ± 18	27 ± 6

The amount of α-^{32}P-UTP incorporated into nuclei with or without HN2 treatment is expressed as 10^5 cpm per 10^7 nuclei. Mean and standard deviation of three independent experiments are presented. Paired-t tests show that there are no differences between control and drug treated samples ($P>0.05$).

Table 5

Colo320HSR Cell Concentration after Cisplatin Exposure

Time (h)	0	6	12	24	36
Dose (μm)	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD
Control	4.04 ± 0.73	4.43 ± 0.67	4.70 ± 1.60	6.36 ± 0.88	8.28 ± 1.38
25	3.16 ± 0.48	3.92 ± 1.04	4.07 ± 1.20	3.81 ± 0.58	4.74 ± 0.96
50	3.70 ± 0.55	3.61 ± 1.09	3.63 ± 1.32	3.38 ± 0.47	3.62 ± 0.39
100	3.69 ± 1.00	3.64 ± 0.90	3.62 ± 1.18	2.64 ± 3.17	3.72 ± 0.42

Cell concentration (X 10^5/ml) was monitored over time after cisplatin treament. Mean and standard deviation of six experiments are presented.

Table 6

Colo320HSR Cell Concentration after HN2 Treatment

Time (h)	0	6	12	24	36
Dose (μm)	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD
Control	2.5 ± 0.7	2.7 ± 0.5	3.3 ± 0.8	4.8 ± 1.2	5.7 ± 1.2
6.25	2.6 ± 0.9	2.8 ± 0.4	2.4 ± 0.3	1.9 ± 0.5	1.9 ± 0.7
12.5	2.3 ± 0.8	2.4 ± 0.5	2.6 ± 0.3	2.3 ± 0.7	2.6 ± 0.8

Cell concentration (X 10^5/ml) was monitored over time after HN2 treament. Mean and standard deviation of six experiments are presented.

et al., 1987). The accessibility of a gene to HN2 may be correlated with the transcriptional state of the gene by some indirect evidence (Wassermann *et al.*, 1990; Futscher *et al.*, 1992). In this study, the formation of HN2-induced DNA interstrand cross-links in genes with different transcriptional states was evaluated. Figure 17 is the schema for the following groups of experiments.

Changes in mRNA Levels of the c-*myc*, c-*fos* and GAPDH Genes in Colo320HSR Cells after Serum Starvation

In search of a model to study formation of DNA interstrand cross-links in a gene with different transcriptional states, an attempt was made to up-regulate c-*myc* expression by serum-starving and serum-stimulating Colo320HSR cells (Kelley *et al.*, 1983; Dean *et al.*, 1986). To our surprise, the steady state c-*myc* mRNA levels in Colo320HSR cells substantially decreased after addition of fresh medium, regardless of growth state. In exponentially growing cells, the c-*myc* mRNA levels decreased about 50-60% after fresh medium addition (Figure 10). When shifted into fresh medium, stationary phase cells exhibited a deeper decrease in the c-*myc* mRNA levels in comparison with logarithmically growing cells. Figure 18 shows a gradual decrease in the c-*myc* mRNA levels when cells underwent serum starvation for three days (D1-D3). Cells kept in the stationary phase for 3 days had only 60% of the c-*myc* mRNA as compared to the exponentially growing controls. After

addition of fresh medium, the c-*myc* mRNA levels continuously decreased as much as 80% within several hours (H1-H8). However, 24 h after fresh medium addition, the c-*myc* mRNA levels returned to control levels (H24). The c-*fos* mRNA level was not detectable in exponentially growing cells, but increased to a detectable level 1 h after fresh medium addition, and then gradually diminished by hour H5. The GAPDH mRNA levels also decreased after serum starvation and addition.

The Relative Transcription Rates of Genes in Colo320HSR Cells

In exponentially growing cells, the relative transcription of the c-*myc*, GAPDH, and ß-actin genes were detectable by standard nuclear run-on analysis as shown in the control lane of Figure 19. Transcription of the c-*fos* gene was too low to be detected under the same conditions. Using an equivalent number of nuclei isolated from cells in logarithmically growing phase, or cells in stationary phase with 3-h conditioned or fresh medium addition, the relative transcription of c-*myc* and GAPDH decreased in cells having undergone serum starvation as compared to exponentially growing cells. When serum-starved cells were incubated in the conditioned medium, the relative c-*myc* transcription rates decreased by more than 50% of the exponentially growing control (47 ± 19, mean ± SD, in exon 1 region; 35 ± 20, mean ± SD, in exon 2 region; n=3); whereas cells that were

incubated in fresh medium, c-*myc* transcription rates decreased to approximately 20% of the control (23 ± 1, mean ± SD, in exon 1 region; 10 ± 4, mean ± SD, in exon 2 region, n=3). These results suggest that the decrease in c-*myc* mRNA levels was mediated, at least in part, at the transcriptional level. Surprisingly, the relative transcription rates in the constitutively expressed GAPDH gene, also decreased in both serum-starved populations. The ß-actin transcription rates remained unchanged in cells with or without serum starvation.

Formation of DNA Interstrand Cross-links at the Gene Level in Exponentially Growing Cells

To measure the formation of DNA interstrand cross-links in individual genes with different transcription states, exponentially growing Colo320HSR cells were exposed to a 3 log cell kill dose of HN2 (6.25 μM) for 1 h (Futscher, 1990). By using DNA denaturing/renaturing gel electrophoresis and Southern blot analysis, the formation of DNA interstrand cross-links formed in c-myc, GAPDH, and c-fos gene was assessed (Figure 19). In the c-*myc* gene, approximately 15% of the gene copies were cross-linked following 1 h drug exposure. In the GAPDH gene, a single copy and highly transcribed gene, approximately 10% of the DNA was cross-linked after the drug treatment. However, in the transcriptionally silent c-fos gene, less than 1% of the DNA was found as cross-linked DNA. Thus, DNA interstrand cross-links were formed to a greater

extent in the two highly transcribed genes, whereas the cross-links were minimal in a gene with a low transcription rate.

The GC dinucleotide is a potentially cross-linkable site for DNA interstrand cross-links induced by HN2 (Colvin, 1982). Using the GCG program (Genetics Computer Group, Madison, WI), the frequencies of GC dinucleotides in these three genes were determined. 587 GC dinucleotides were found in an 8 kb fragment of the c-*myc* gene, 424 in a 6.2 kb fragment of the c-*fos* gene, and 449 in a 5.4 kb fragment of the GAPDH gene. These three regions cover all exons of each gene, and are included in the restriction fragments in which HN2-induced DNA interstrand cross-links were measured. Overall, based on the GC dinucleotide content, the potentially cross-linkable sites are not dramatically different from one gene to another.

Formation of DNA Interstrand Cross-links at the Gene Level in Cells in Stationary Phase

As revealed by nuclear run-on analysis, c-*myc* transcription decreased in cells that underwent serum starvation and addition (Figure 20). After exposing cell populations with different c-*myc* transcription rates to a 3 log cell kill dose of HN2 (6.25 μM) for 1 h, the intensity of double-stranded DNA was reduced in the c-*myc* gene with a decreased transcription rate (Figure 21). Production of DNA interstrand cross-links in the c-*myc* gene decreased by 40% and 80%, respectively, in cells with c-*myc* transcription rates reduced to a similar

extent (Figure 22). This finding suggests that the decrease in formation of DNA interstrand cross-links in the c-*myc* gene paralleled the decrease in c-*myc* transcription rates. Similar findings were noted in the GAPDH gene and are presented in Figure 23. The amount of DNA interstrand cross-links formed in the GAPDH gene also correlated with its transcription state. DNA interstrand cross-links formed in the c-*fos* gene were minimal in cells with or without serum starvation.

Formation of DNA Interstrand Cross-links at the Gene Level in Cells Treated with Hydroxyurea

Hydroxyurea was used to down-regulate c-*myc* expression. When Colo320HSR cells were exposed to hydroxyurea for 24 h, c-*myc* mRNA levels also decreased by 40% and 60% after either 1 or 10 mM exposure, respectively (Figure 24). As demonstrated by nuclear run-on analysis, this decrease also was mediated at the transcriptional level (Figure 25). However, the amounts of DNA interstrand cross-links formed in the c-*myc* gene were the same in both control and HU-treated samples (Figure 26). The expression of the GAPDH gene did not change dramatically in both transcriptional and post-transcriptional levels (Figures 24 and 26). No changes in the formation of DNA interstrand-cross links in the GAPDH gene were observed.

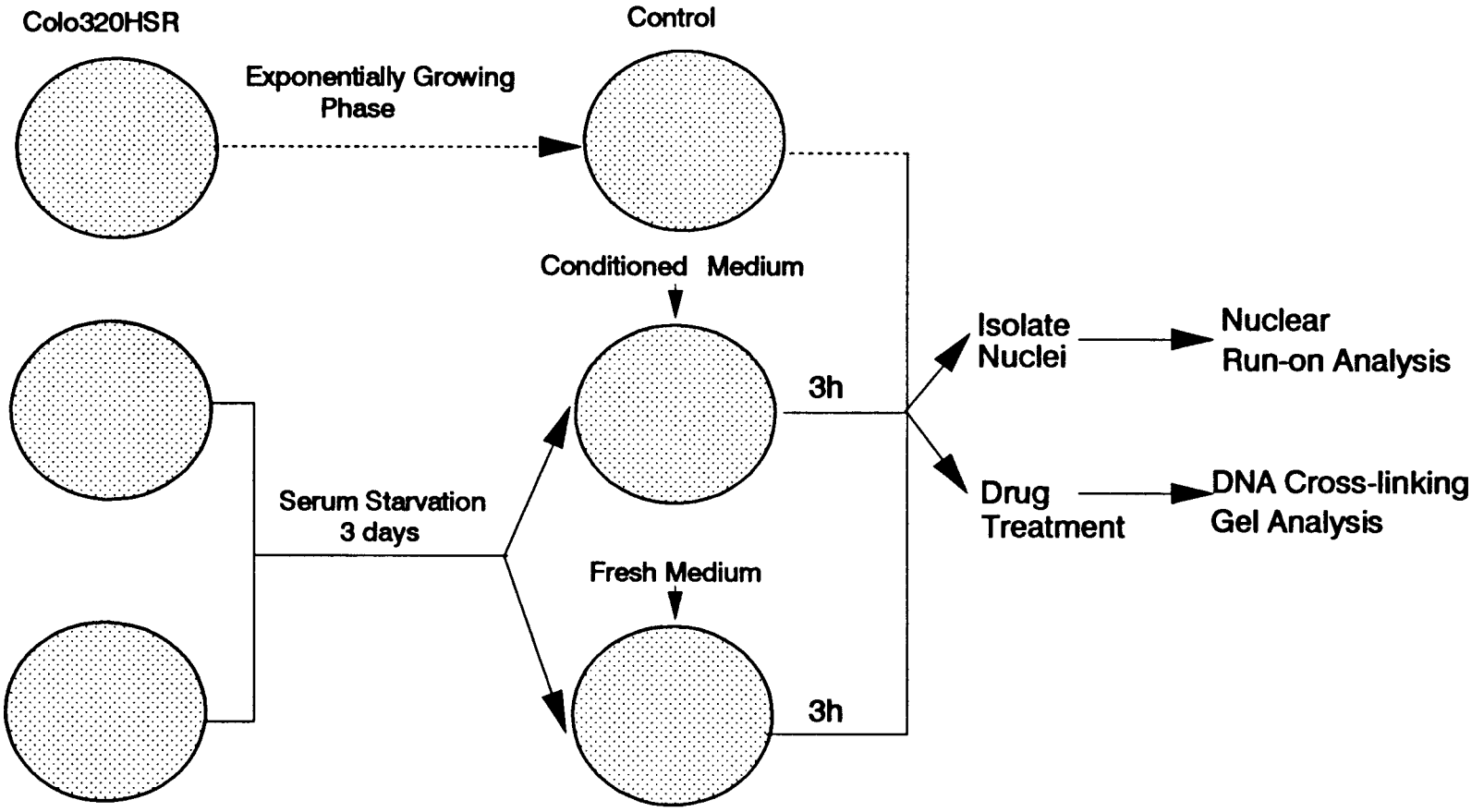
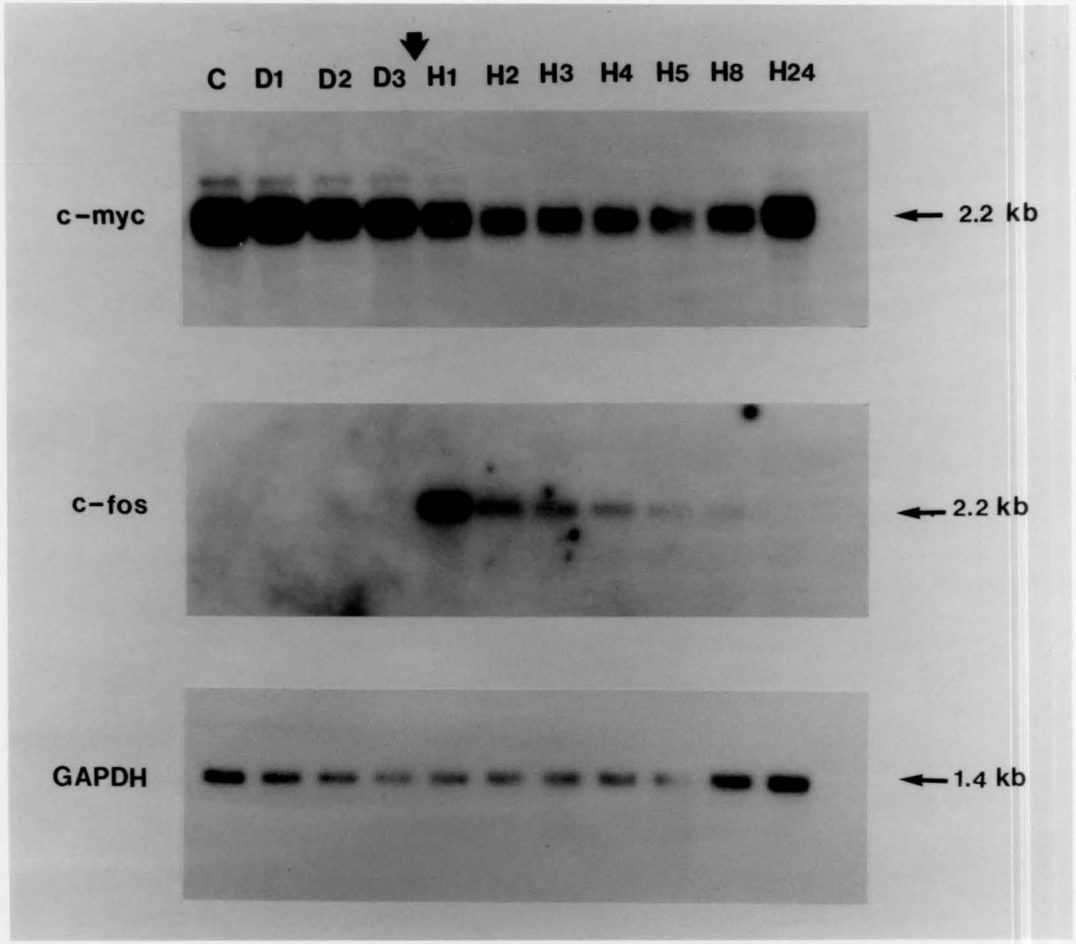


Figure 17. Experimental design for studies on accessibility of a gene to DNA damage induced by HN2.

Figure 18. Northern blot analysis of changes in c-*myc*, c-*fos*, and GAPDH mRNA levels after serum starvation in Colo320HSR.

Total RNA was isolated from cells in exponential growth phase(C), cells in stationary phase for 1 to 3 days (designated as D1, D2, and D3), and stationary cells resuspended in fresh serum for various times (1 to 24 h, defined as H1, H2, H3, H4, H5, H8, and H24). 10 μg total RNA from each sample was denatured and fractionated on a 1% agarose gel. After northern blotting, the filter was first hybridized with a ^{32}P-labeled c-*myc* exon 2 probe, then stripped and hybridized with ^{32}P-labeled c-*fos* exon 4 and mouse GAPDH cDNA probes sequentially. The exposure time was 6 h for the c-*myc* blot , 3 days for the c-*fos* blot, and 2 days for the GAPDH blot. The sizes of the transcripts are indicated at the right side of each autoradiogram. The above figure shows the result of a single experiment.


C
D1
D2
D3
H1
H2
H3
H4
H5
H8
H24
c-myc
2.2 kb
c-fos
2.2 kb
GAPDH
1.4 kb

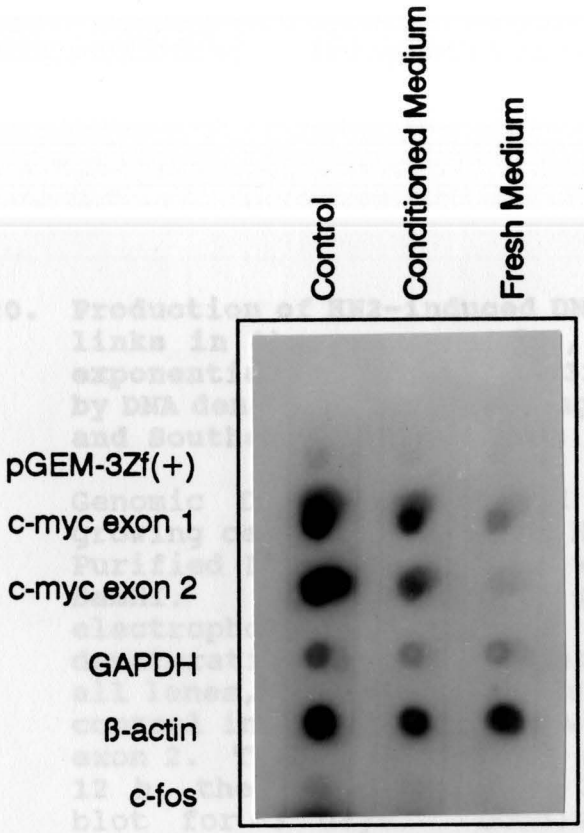


Figure 19. Nuclear run-on analysis of *in vitro* transcription in isolated Colo320HSR nuclei after serum starvation.

Nuclei were isolated from cells in exponential growth phase (control), or cells in stationary phase for 3 days resuspended for 3 h in either conditioned medium or fresh medium. An equivalent number of nuclei were used for each *in vitro* transcription reaction. Nascent ^{32}P-labeled RNA was purified and hybridized to the probes immobilized on a nitrocellulose membrane. The filters were exposed to x-ray film for 1 week.

Figure 20. Production of HN2-induced DNA interstrand cross-links in the c-*myc*, c-*fos*, and GAPDH genes in exponentially growing Colo320HSR cells detected by DNA denaturing/renaturing gel electrophoresis and Southern blot analysis.

Genomic DNA was isolated from exponentially growing cells with 6.25 μM HN2 exposure for 1 h. Purified DNA was digested with either *Eco*RI or *Bam*HI. Restricted genomic DNA was electrophoresed with or without formamide denaturation. 10 μg of genomic DNA were used in all lanes, except 1 μg was used for nondenatured control in the blot probed with ^{32}P-labeled c-*myc* exon 2. The c-*myc* blot was autoradiographed for 12 h, the GAPDH blot for 24 h, and the c-*fos* blot for 3 days. Double-stranded (ds) and single-stranded (ss) DNA are indicated by arrows. The size of ds DNA of the c-*myc* gene is 12.5 kb (*Eco*RI digestion), the GAPDH gene 12 kb (*Bam*HI digestion), the c-*fos* gene 9 kb (*Eco*RI digestion).

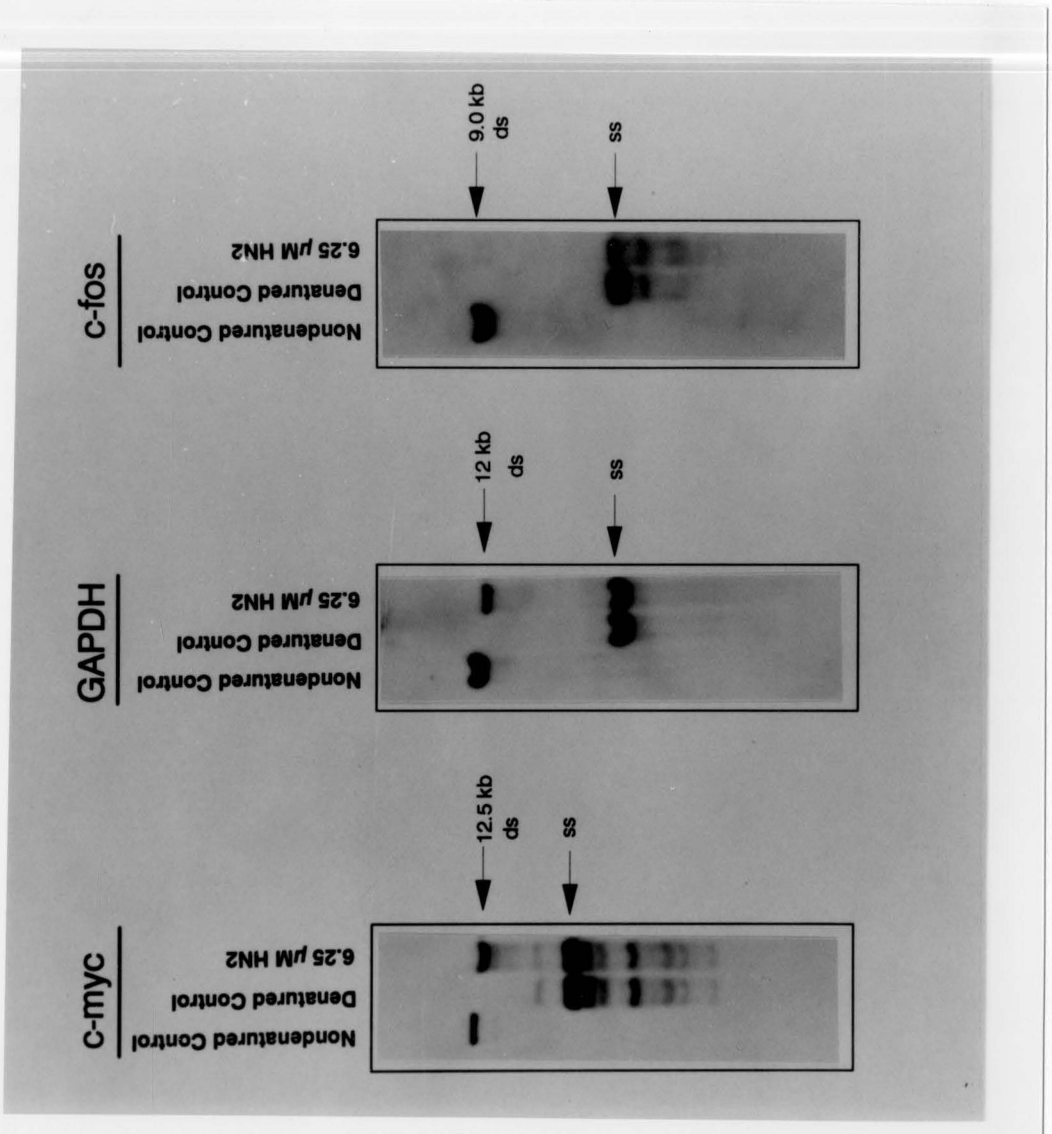

c-myc
Nondenatured Control
Denatured Control
6.25 μM HN2
12.5 kb
ds
ss
GAPDH
Nondenatured Control
Denatured Control
6.25 μM HN2
12 kb
ds
ss
c-fos
Nondenatured Control
Denatured Control
6.25 μM HN2
9.0 kb
ds
ss

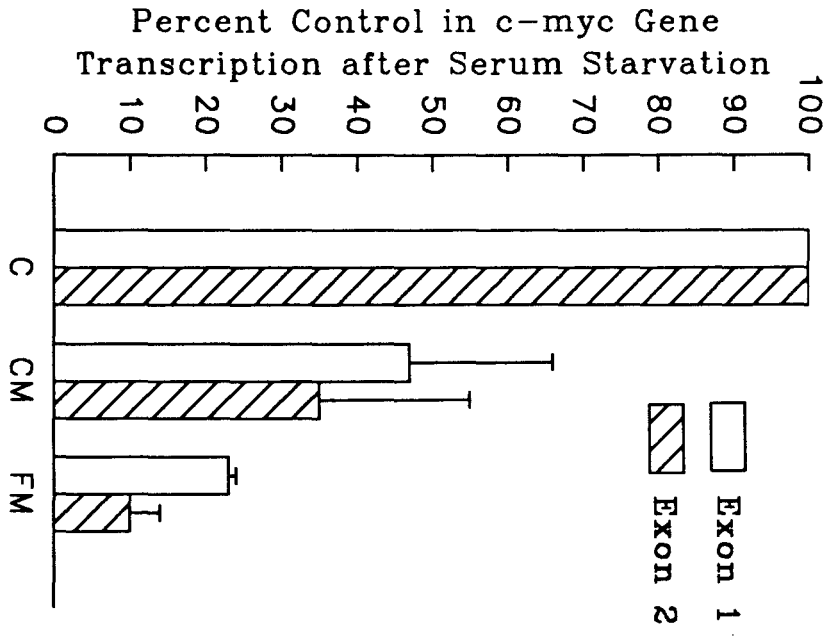
Figure 21. Formation of HN2-induced DNA interstrand cross-links in the c-*myc*, GAPDH, c-*fos* genes after serum starvation measured by DNA denaturing/renaturing gel electrophoresis and Southern blot analysis.

Cells in logarithmically growing phase, or cells in stationary phase for 3 days with 3-h conditioned or fresh medium addition, were subjected to 6.25 μM HN2 treatment for 1 h. Genomic DNA was isolated immediately after drug removal, and digested with either *Eco*RI or *Bam*HI. 10 μg genomic DNA were loaded in each lane, except 1 μg in nondenatured control for the c-*myc* blot. Exposure times were 12, 24, and 72 h for the c-*myc*, GAPDH, and c-*fos* blots, respectively. The probes used for each blot were indicated on the top of each autoradiogram. Double-stranded DNA (ds) and single- stranded (ss) DNA are as indicated by arrows. The size of ds DNA for each gene detected is as described in Figure 20.

Nondenatured Control
Denatured Control
Control
Conditioned Medium
Fresh Medium
c-myc
ss
ds
12.5 kb
Nondenatured Control
Denatured Control
Control
Conditioned Medium
Fresh Medium
GAPDH
ss
ds
12 kb
Nondenatured Control
Denatured Control
Control
Conditioned Medium
Fresh Medium
c-fos
ss
ds
9.0 kb

Figure 22. Comparison of changes in c-*myc* transcription and DNA interstrand cross-link formation in the c-*myc* gene induced by HN2 in Colo320HSR cells.

Left panel shows the relative percentage of c-*myc* transcription rates in exon 1 (open bars) and exon 2 (hatched bars) regions of the gene after serum starvation in Colo320HSR cells by comparison to the c-*myc* transcription in exponentially growing cells (C). CM stands for cells resuspended in conditioned medium, and FM for fresh medium. Right panel shows the relative percentage of DNA interstrand cross-links in the c-*myc* gene in cells with different growth states as determined by using the percent formation of DNA interstrand cross-links in the exponentially growing cells as the reference. Histograms are the average of three independent experiments, and the standard deviation of each group is indicated by an error bar. The percent cross-links formed in the exponentially growing cells after 6.25 μM HN2 treatment is from 7.5% to 15%.


Percent Control in c-myc Gene
Transcription after Serum Starvation
0
10
20
30
40
50
60
70
80
90
100
C
CM
FM
Exon 2
Exon 1

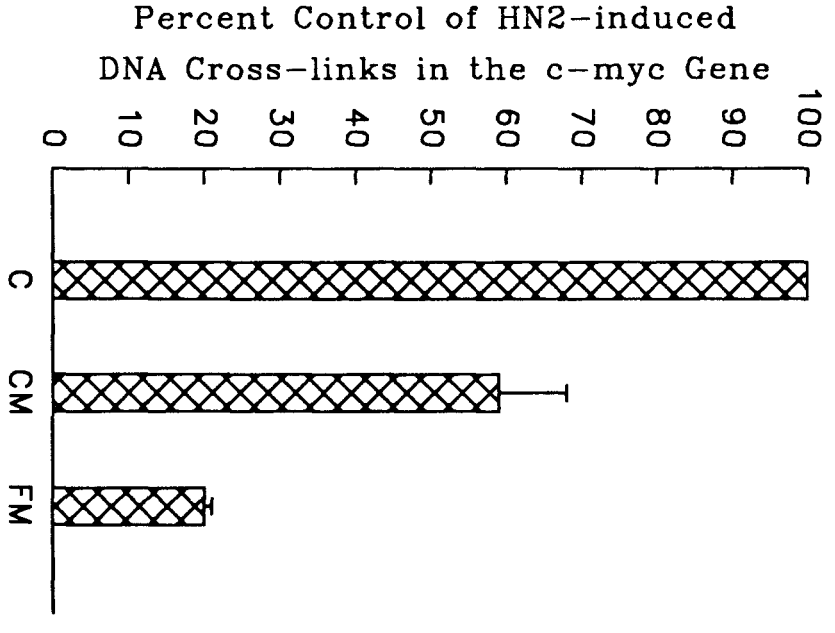
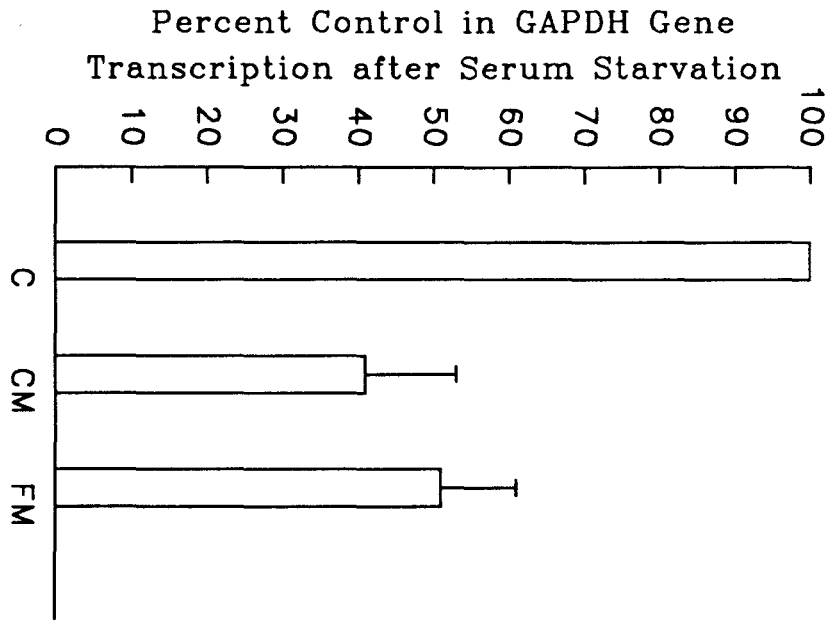

Percent Control of HN2-induced
DNA Cross-links in the c-myc Gene
0
10
20
30
40
50
60
70
80
90
100
C
CM
FM

Figure 23. Comparison of changes in GAPDH transcription and DNA interstrand cross-link formation in the gene induced by HN2 in Colo320HSR cells.

GAPDH transcription in cells after serum starvation and resuspension in either conditioned medium (CM) or fresh medium (FM) was determined by comparison to GAPDH transcription in exponentially growing cells (C) (left panel). The relative percentage of DNA interstrand cross-links in the GAPDH gene in cells after serum starvation was determined by comparison to the percent formation of DNA interstrand cross-links in the exponentially growing cells as shown in right panel. The mean of experiments is indicated by the histogram, and the standard deviation of each group is indicated by an error bar. Three experiments were performed to determine GAPDH transcription rate, two experiments were done to determine HN2-induced DNA interstrand cross-links formed in the GAPDH gene. The percent formation of DNA interstrand cross-links in exponentially growing cells was from 9.4% to 11.8%.


Percent Control in GAPDH Gene
Transcription after Serum Starvation
0
10
20
30
40
50
60
70
80
90
100
C
CM
FM

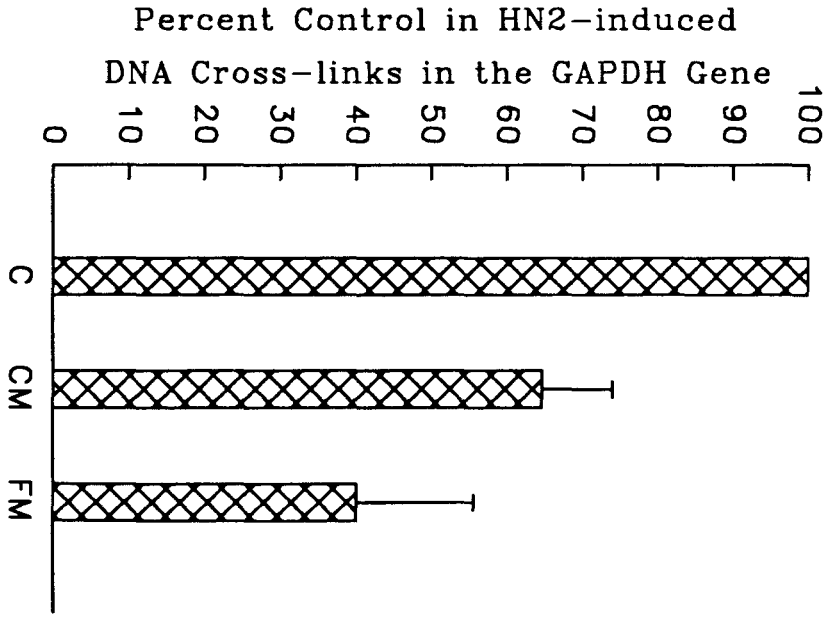
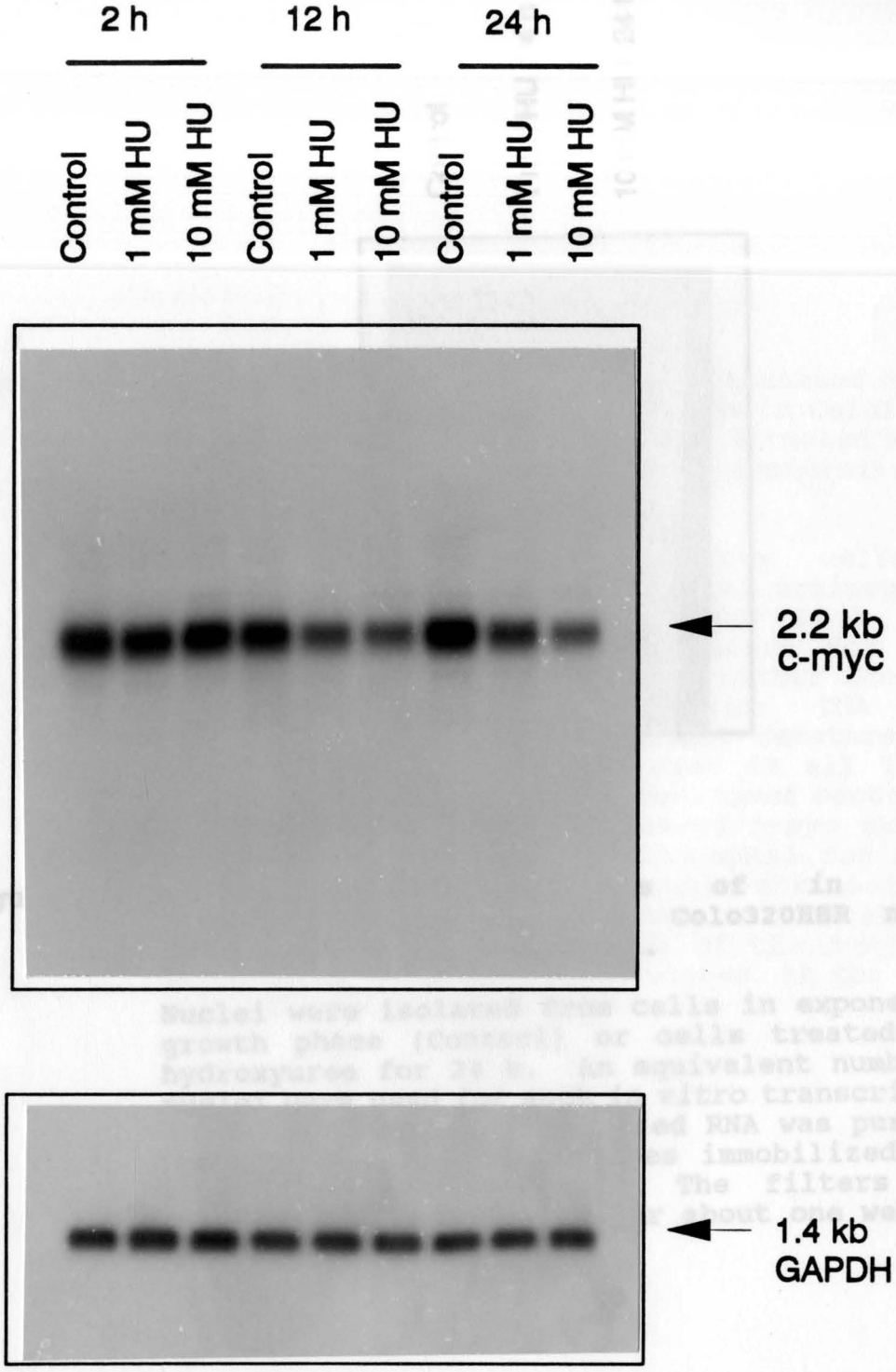

Percent Control in HN2-induced
DNA Cross-links in the GAPDH Gene
0
10
20
30
40
50
60
70
80
90
100
C
CM
FM

Figure 24. Northern blot analysis of changes in the steady state c-*myc* mRNA levels after hydroxyurea treatment.

Total cellular RNA was isolated from cells treated with 1 or 10 mM hydroxyurea for 2, 12, or 24 h. Sham samples were set up for each corresponding time point. 10 μg total RNA from each sample was denatured and fractionated on a 1% agarose gel. After capillary transfer, the membrane was hybridized with a ^{32}P-labeled c-*myc* exon 2 probe. The autoradiogram is shown in the top panel. The same membrane was stripped and hybridized with a ^{32}P-labeled GAPDH probe, indicating equal loading.


2 h
12 h
24 h
Control
1 mM HU
10 mM HU
Control
1 mM HU
10 mM HU
Control
1 mM HU
10 mM HU
2.2 kb
c-myc
1.4 kb
GAPDH

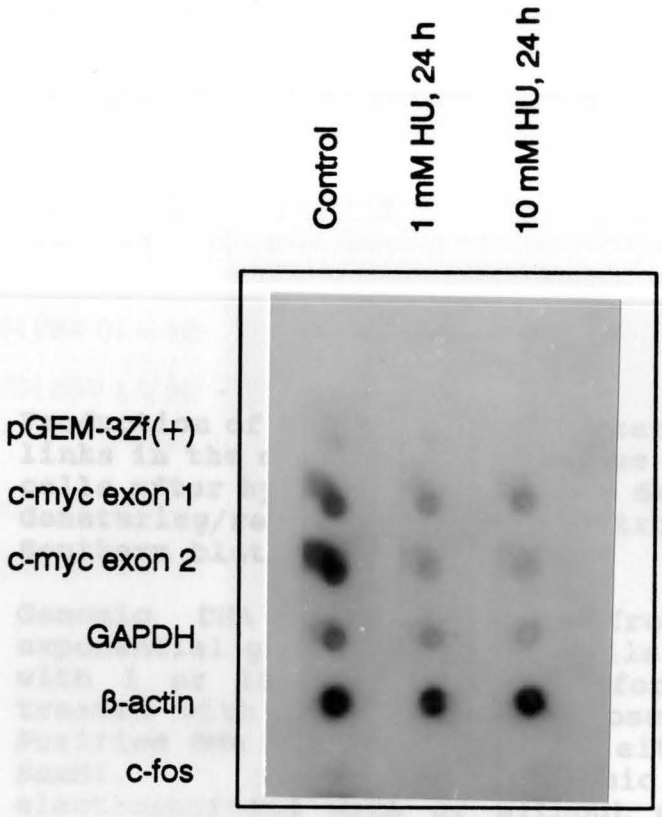


Figure 25. Nuclear run-on analysis of *in vitro* transcription in isolated Colo320HSR nuclei after hydroxyurea treatment.

Nuclei were isolated from cells in exponential growth phase (Control) or cells treated with hydroxyurea for 24 h. An equivalent number of nuclei were used for each *in vitro* transcription reaction. Nascent ^{32}P-labeled RNA was purified and hybridized to the probes immobilized on a nitrocellulose membrane. The filters were exposed to an x-ray film for about one week.

Figure 26. Production of HN2-induced DNA interstrand cross-links in the c-*myc* and GAPDH genes in Colo320HSR cells after hydroxyurea exposure detected by DNA denaturing/renaturing gel electrophoresis and Southern blot analysis.

Genomic DNA was isolated from cells in exponential growth phase, or cells preincubated with 1 or 10 mM hydroxyurea for 24 h, then treated with 6.25 μM HN2 exposure for 1 h. Purified DNA was digested with either *Eco*RI or *Bam*HI. Restricted genomic DNA was electrophoresed with or without denaturation. 10 μg of genomic DNA were used in all lanes, except 1 μg was used for nondenatured control in the blot probed with ^{32}P-labeled c-*myc* exon 2. The c-*myc* blot was autoradiographed for 12 h, the GAPDH blot for 24 h. Double-stranded (ds) and single-stranded (ss) DNA are indicated by arrows. The sizes of ds DNA of the c-*myc* and GAPDH genes detected are indicated at the right side of each autoradiogram.

Nondenatured Control
Denatured Control
Control
24 h 1 mM HU
24 h 10 mM HU
c-myc
ss
ds
12.5 kb
Nondenatured Control
Denatured Control
Control
24 h 1 mM HU
24 h 10 mM HU
GAPDH
ss
ds
12 kb

DISCUSSION

This dissertation has studied the relationship between DNA damage and repair in individual genes, and alterations of gene expression in response to DNA damage induced by DNA cross-linking agents (HN2 and cisplatin) in the human tumor cell line Colo320HSR. These two DNA cross-linking agents were found capable of not only producing DNA interstrand cross-links in the c-*myc* oncogene, but also decreasing steady state c-*myc* mRNA levels at the transcriptional level. In addition, some evidence indicates that the accessibility of a gene to HN2-induced DNA interstrand cross-links may be determined by the transcriptional state of the gene.

DNA Interstrand Cross-link Formation and Disappearance in Individual Genes

Previously, studies of DNA cross-linking produced by HN2 and cisplatin were only possible at the genomic level. With the advent of molecular biologic techniques, it became possible to study DNA interstrand cross-links at the gene level (Vos and Hanawalt, 1987). Cisplatin-induced DNA interstrand cross-links in the genome were detectable by treating cells with a dose as low as 1 μM (Zwelling *et al.*,

1978). In order to detect cisplatin-induced DNA interstrand cross-links in specific genomic regions, a dose as high as 200 μM was used in a cell line in which only 9.5 μM of cisplatin was needed to kill 50% of the cells (Jones *et al.*, 1991). Using a modification of the original method (Futscher *et al.*, 1992), DNA interstrand cross-links produced by cisplatin were detectable in the c-*myc* oncogene when Colo320HSR cells were exposed to a dose as low as 25 μM (produces a 2 log cell kill) in this study. This result indicates that cisplatin-induced interstrand cross-links can be detected at the gene level at biologically relevant doses.

The kinetics of formation and disappearance of cisplatin-induced interstrand cross-links at the gene level are similar to those noted in the overall genome with the exception that maximal cross-links occourred immediately (0 h) after one hour drug treatment. Using the alkaline elution technique, DNA interstrand cross-links were maximal at 6 to 12 h after cisplatin treatment in the whole genome, and the majority disappeared by 24 h (Zwelling *et al.*, 1978, 1979 and 1981). As shown in Figure 8, interstrand cross-links induced by cisplatin in the c-*myc* gene were maximal immediately (0 h) after drug treatment despite the dose difference. DNA interstrand cross-links disappeared over time with the majority of them removed by 24 hours as depicted in Figure 9. It is possible that cisplatin-induced DNA interstrand cross-links in the c-*myc* oncogene formed much more rapidly than

those in the overall genome. However, the time needed for isolation of DNA from drug treated cells was about 16 to 18 hours in the DNA denaturing/renaturing gel electrophoresis assay used for detecting interstrand cross-links in a specific genomic region. This situation is similar to the conditions used in the experiments done by D'Incalci *et al.* (1985), who showed that cisplatin induced cross-links continued to form up to 24 h after lysis of cells on a membrane filter which was incubated at pH 10, and 37°C. It seems more likely that continued formation of cisplatin cross-links in the process of DNA isolation contributes to the maximal cross-links formed at 0 h.

Disappearance of cisplatin cross-links in the c-*myc* gene occurred as early as 6 h after drug treatment. 12 h after drug treatment, about 50% of the cross-links were removed. These data are in contrast to those noted in the overall genome, in which cisplatin cross-links peaked at 12 h after drug treatment (Zwelling *et al.*, 1978 and 1979). This finding suggests that cisplatin-induced DNA interstrand cross-links may be preferentially repaired from the actively transcribed c-*myc* oncogene, which is consistent with the notion that DNA damage is repaired faster in actively transcribed genes as compared to DNA repair in the overall genome (Bohr *et al.*, 1985).

In similar experiments, HN2-induced DNA interstrand cross-links disappeared by 6 h after drug treatment in the c-*myc*

gene in Colo320HSR cells (Futscher *et al.*, 1992). These data suggest that similar types of DNA lesions may pose a different challenge to cells, triggering different cellular repair processes.

Suppression of c-*myc* mRNA Expression Mediated at the Transcriptional Level

C-*myc* mRNA levels decreased substantially in Colo320HSR after either HN2 or cisplatin exposure, while overall RNA synthesis was not changed. The steady state c-*myc* mRNA level was not reduced immediately after exposing cells to either drug, but decreased at 6 h and gradually reached its nadir at 24 and 36 h. In addition, nuclear run-on analysis revealed that the decrease in c-*myc* mRNA levels was mediated, at least in part, at the transcriptional level. The delayed decrease in c-*myc* expression suggests that the suppression is a secondary response to either drug rather than a direct consequence of DNA lesions formed in the gene.

The inhibition of c-*myc* transcription may be caused by several mechanisms. Maximal DNA interstrand cross-links produced by either drug were observed immediately after drug treatment, while the inhibition of c-*myc* transcription at this point was minimal. This observation suggests that the inhibition of c-*myc* expression is most likely an indirect response to DNA damage produced by these drugs. It is possible that the decrease in c-*myc* transcription was caused

by a specific cellular process triggered by DNA lesions produced by the drug. Lippard and colleagues have reported recently that cisplatin adducts could bind to the high-mobility group protein, HMG1 (Pil and Lippard, 1992). HMG1 has been identified as a possible general transcription factor (Singh and Dixon, 1990). The suppression of c-myc expression, therefore, may be a consequence of inhibition of transcription factors. In addition, extensive evidence has indicated that the constantly high levels of c-*myc* expression in proliferating cells would decrease when cells escape from the cell cycle and undergo terminal differentiation (Kelly *et al.*, 1983; Campisi *et al.*, 1984; Simpson *et al.*, 1987; Siebenlist *et al.*, 1988). The suppression of c-*myc* transcription, alternatively, may be caused by a general response to inhibition of cell multiplication.

The possibility that the inhibition of c-*myc* transcription is partly due to a direct consequence of DNA lesions produced by either drug on the DNA template, however, cannot be completely excluded. As revealed by nuclear run-on analysis, the extent of decrease of c-*myc* transcription at exon 2 was greater than that at exon 1. This finding implies that transcription elongation may be blocked by the damage on the DNA template, or the termination of c-*myc* transcripts at the end of exon 1 may be increased after drug treatment. Suppression of c-*myc* expression at the mRNA level may be caused by inhibition of c-*myc* transcription initiation,

increased termination of c-*myc* transcripts, and/or blockage of transcription elongation by DNA adducts.

A recent study has shown that expression of c-*myc* protein is required to induce apoptosis (a genetically programmed form of cell death) in fibroblasts when cells were grown in low serum (Evan *et al.* 1992). Perturbation in c-*myc* expression may initiate a cell death program. It would be worthwhile to find out the expression level of c-*myc* protein in cells after cisplatin or HN2 exposure. Anti-tumor agents, like cisplatin and HN2, may trigger the cell death pathway by disturbing c-*myc* protein expression in cells.

Studies on the Accessibility of a Gene to HN2-induced DNA Interstrand Cross-linking Damage

Consistent with previous findings that HN2-induced DNA alkylation adducts and DNA interstrand cross-links are heterogeneously distributed in different sub-genomic regions (Wassermann *et al.*, 1990; Futscher *et al.*, 1992), HN2-induced DNA interstrand cross-links formed preferentially in highly transcribed genes in Colo320HSR cells. It is generally accepted that actively transcribed genes that are temporarily dissociated from nucleosome particles are more accessible to DNA damage induced by various agents than inactive DNA (Bohr *et al.*, 1987). This study presents direct evidence which correlates the transcriptional activity of a gene with its accessibility to HN2-induced DNA damage. In the c-*fos* gene,

a transcriptionally inactive gene, the formation of DNA interstrand cross-links was minimal, and could be a result of transient activation of the c-*fos* gene induced by HN2 (Futscher and Erickson, 1990; Hollander and Fornace, 1989). However, in the transcriptionally active c-*myc* and GAPDH genes, whose transcription rates were high enough to be detected by standard nuclear run-on assays, substantial amounts of DNA interstrand cross-links were found in each gene. Moreover, when c-*myc* gene expression was down regulated by serum starvation and addition at the transcriptional level, the production of HN2-induced DNA interstrand cross-links in the gene decreased parallel to c-*myc* suppression. The same also was true with the GAPDH gene. When GAPDH gene transcription was decreased, the amounts of DNA interstrand cross-links formed in the gene were also reduced. These results are in agreement with the notion that transcriptionally active genes have a more open conformation than transcriptionally inactive genes and are more susceptible to DNA damage.

Unfortunately, complete nucleotide sequences of the entire DNA fragments probed are not available for analyzing potentially cross-linkable sites. No substantial differences, however, have been found when comparing GC dinucleotide frequencies existing in the regions that span all exons in each gene. The possibility that the differences in production of DNA interstrand cross-links are due to the differences in

potentially cross-linkable sites in these genes is very unlikely.

In contrast, no changes in the amounts of DNA interstrand cross-linking produced in the c-*myc* gene were observed when the gene was down-regulated at the transcriptional level by hydroxyurea. This result is puzzling because presumably when a gene is not actively transcribed, it exists in a condensed form associated with basic histone proteins. A gene in the condensed chromatin would be less accessible to DNA damage induced by drugs. As we know, hydroxyurea inhibits DNA synthesis by depleting the pool of deoxyribonucleoside triphosphate (Bianchi *et al.*, 1986; Collins and Oates, 1987). It is possible that the chromatin structure is decondensed in the genome after hydroxyurea treatment. If this is the case, the c-*myc* gene may remain accessible to DNA damage induced by nitrogen mustard, even though the c-*myc* expression is suppressed.

Regulation of c-*myc* and GAPDH Gene Expression

Regulation of c-*myc* gene expression is a complicated cellular process as revealed by numerous studies (Spencer and Groudine, 1991; Cole, 1986; Kelly, 1986). C-*myc* proto-oncogene expression is regulated by stimuli that control cell growth. Growth-promoting agents generally induce c-*myc* expression, whereas growth-inhibiting agents often result in a decrease of c-*myc* expression. For instance, growth

stimulation of quiescent cells by serum elevates expression of the c-*myc* proto-oncogene up to 10 to 40 fold in Balb/c3T3 (A31) cells (Campisi *et al.*, 1984). In two chemically transformed A31 derivatives, however, c-*myc* expression is constitutive (Campisi *et al.*, 1984). C-*myc* expression in Colo320HSR cells did not increase after serum addition as expected, which may be a cell-specific phenomenon. Furthermore, an aberrant response to growth stimuli in a cell line, which harbors an amplified and overexpressed c-*myc* oncogene is not totally unexpected (Alitalo *et al.*, 1983; Schwab *et al.*, 1986).

The GAPDH gene has been widely used as a constitutively expressed control in gene expression studies (Blanchard *et al.*, 1985; Laird-Offringa *et al.*, 1990; Pei and Calame, 1988; Gringnani *et al.*, 1990; Penn *et al.*, 1990). GAPDH was chosen in this study as the constitutive gene, but it failed to serve as a constitutively expressed control in the serum starvation and addition experiments. This is not totally unexpected for enhanced expression of the GAPDH gene has been found in some tumor cell lines (Tokunaga *et al.*, 1987; Schek *et al.*, 1988; Persons *et al.*, 1989), and its expression can be altered by many stimuli, such as insulin, interleukin 2, and a calcium ionophore (Alexander *et al.*, 1985 and 1988; Sabath *et al.*, 1990; Chao *et al.*, 1990). Furthermore, in quiescent rat fibroblasts, GAPDH mRNA levels increase as a result of stimulation with EGF or serum (Matrisian *et al.*, 1985). After

all, there is probably no universal constitutively expressed gene.

In summary, this study has attempted to elucidate the relationship between DNA cross-linking agents and gene expression. HN2 and cisplatin produced DNA interstrand cross-links in the c-*myc* gene and inhibited c-*myc* gene expression. However, HN2-induced DNA interstrand cross-links were found in the GAPDH gene, but no dramatic decrease in GAPDH gene transcription were observed. In addition, when the production of HN2-induced DNA interstrand cross-links in the same gene or different genes of different transcriptional states were measured, the amounts of cross-links formed in the gene were dependent on the rate of transcription, indicating that the accessibility of a gene to HN2-induced DNA interstrand cross-links is correlated with its transcriptional state.

Overall, this study shows that DNA interstrand cross-linking agents like HN2 and cisplatin can produce cross-links in highly transcribed genes, but DNA interstrand cross-links may not necessarily suppress gene expression. This finding indicates that inhibition of certain gene expression by DNA cross-linking agents is not a direct consequence of DNA lesions themselves, but rather represents an overall cellular response to these agents. Inhibition of critical gene expression like that of c-*myc* may prevent cells from proceeding through the cell cycle or may trigger cell death

pathways, thereby contributing to the cytotoxicity induced by these agents.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

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