THE UNIVERSITY OF MANITOBA

MOLECULAR MECHANISMS OF DRUG RESISTANCE AND TUMOR PROMOTION INVOLVING MAMMALIAN RIBONUCLEOTIDE REDUCTASE

by

BOB B.K. CHOY

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REDUCTASE

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A thesis submitted to the Faculty of Graduate Studies of
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Abstract

Mammalian ribonucleotide reductase is a highly regulated, rate - limiting activity responsible for converting ribonucleoside diphosphates to the deoxyribonucleotide precursors of DNA. The enzyme consists of two nonidentical proteins often called M1 and M2, both of which are required for activity. Hydroxyurea is an antitumor agent which inhibits ribonucletide reductase by interacting with the M2 component specifically at a unique tyrosyl free radical. Studies of a series of drug resistant mouse cell lines, selected by a step-wise procedure for increasing levels of resistance to the cytotoxic effects of hydroxyurea. Each successive drug selection step leading to the isolation of highly resistant cells was accompanied by stable elevations in cellular resistance and ribonucleotide reductase activity. The drug resistant cell lines exhibited gene amplification of the M2 gene, elevated M2 mRNA, and M2 protein. Analysis of wild type, moderately resistant, and highly resistant cells indicated that, in addition to M2 gene amplification, posttranscriptional modulation also occurred during the drug selection. Studies of the biosynthesis rates with exogenously added iron also suggests a role for iron in regulating the level of M2 protein when cells are cultured in the presence of hydroxyurea. The hydroxyurea-inactivated ribonucleotide reductase protein M2 has a destabilized iron centre, which readily releases iron. In addition, altered expression of the iron storage protein, ferritin, appears to be required for the development of hydroxyurea resistance in mammalian cells. The results show an interesting relationship between the expressions of two highly regulated activities, ribonucleotide reductase and ferritin. These results illustrate the complexity of the drug-resistant phenotype and provide further information about the molecular processes that lead to the development of cells resistant to low, intermediate, and high concentrations of hydroxyurea. The phorbol ester tumor promoter, TPA, is also able to alter the expression of M2. TPA was able to induce M2 mRNA levels transiently up to 18-fold within ½ hour. This rapid and large elevation of ribonucleotide reductase suggests that the enzyme may play a role in tumor promotion. Studies of the M2 promoter region were undertaken in order to better understand the mechanism of TPA induction of M2.

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DON'T PANIC - Hitchhiker's Guide to the Galaxy

給我父母。

不必再担憂

我即將完成

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ABBREVIATION

AP	Alkaline phosphatase
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
CAT	Chloramphenicol transferase
cDNA	Complementary DNA
CDP	Cytidine 5'-triphosphate
cpm	Counts per minute
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
CsCl	Cesium chloride
dATP	2'-Deoxyadenosine 5'-triphosphate
dCTP	2'-Deoxycytidine 5'-triphosphate
dGDP	2'-Deoxyguanosine 5'-diphosphate
dGTP	2'-Deoxyguanosine 5'-triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dTTP	2'-Deoxythymidine 5'-triphosphate
dUTP	2'-Deoxyuridine 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid disodium
EGTA	Ethylene-bis(oxyethylenenitrilo)tetraacetic acid
EMSA	Electromobility shift assay

EPR	Electron paramagnetic resonance
FBS	Fetal bovine serum
$FeCl_2$	Ferric chloride
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GITC	Guanidinium isothiocyanate
HCl	Hydrogen chloride
Hepes	*N*-(2-Hydroxyethyl)piperazine-*N'*-(2-ethane sulfonic acid)
hMT-II_A	Human metallothionein II_A
IgG	Immunoglobulin G
Kb	Kilobase pair
KD	Kilodalton
KCl	Potassium chloride
KOH	Potassium hydroxide
LB	Luria-Bertani medium
α-MEM	Alpha modified minimal essential medium
$MgCl_2$	Magnesium chloride
MOPS	3-(*N*-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NP-40	Nonidet P-40
OD	Optical density

ODC	Ornithine decarboxylase
PBS	Phosphate buffer saline
PMSF	Phenylmethylsulfonyl fluoride
POMC	Proopiomelanocortin
RPE	Relative plating efficiency
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel
TCA	Trichloroacetic acid
TfR	Transferrin receptor
TPA	12-*o*-tetradecanoylphorbol-13-acetate
TREs	TPA responsive element
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UDP	Uridine 5'-diphosphate

Introduction

1. RIBONUCLEOTIDE REDUCTASE

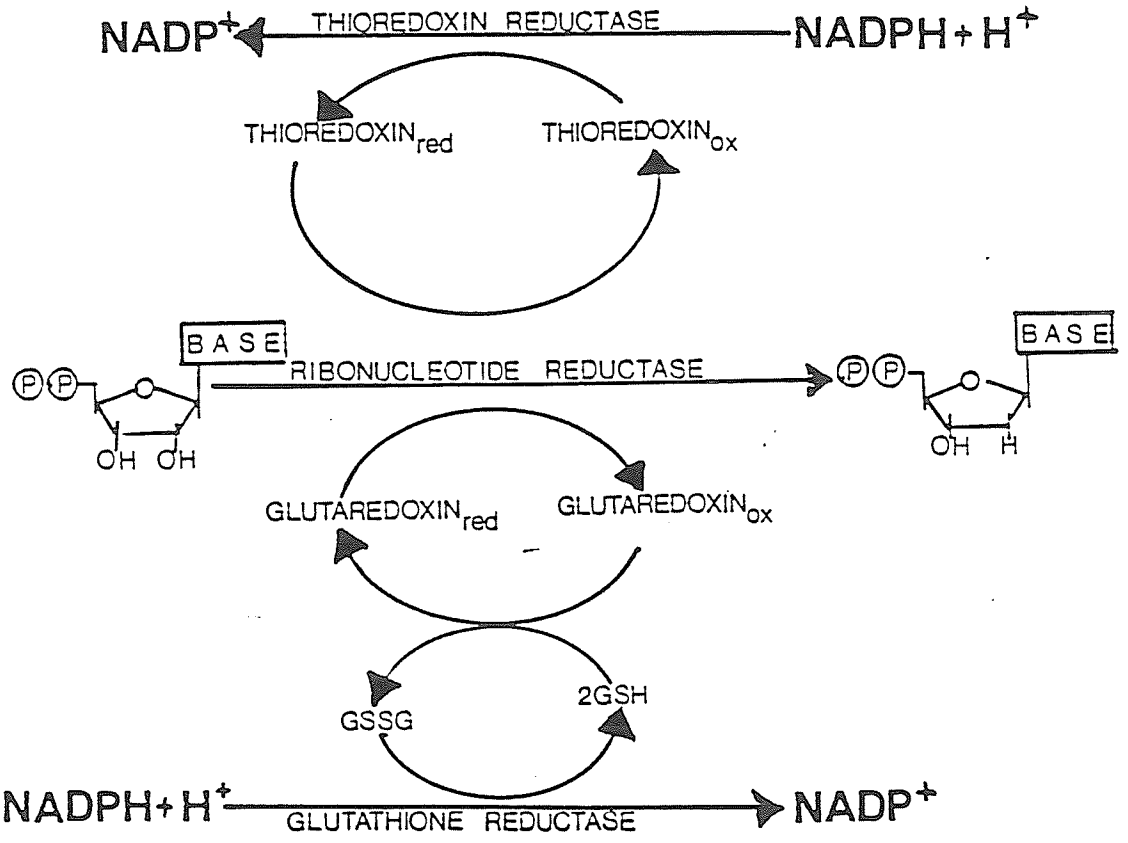
1.1. Ribonucleotide Reductase and DNA synthesis: Ribonucleotide reductase is a highly regulated enzyme that is responsible for the conversion of ribonucleotides to their corresponding deoxyribonucleotides, the precursors of DNA synthesis (Thelander and Reichard, 1979; Wright, 1989; Wright *et al.*, 1990). In both prokaryotes and eukaryotes, the formation of deoxyribonucleotides occurs by the direct reduction at the 2′ position of the corresponding ribonucleotides.

The importance of this enzyme for cell growth is demonstrated by its key role in DNA synthesis. While cells do possess measurable levels of deoxyribonucleoside triphosphates, those cells in S-phase have sufficient levels to maintain DNA synthesis for only a very short time. The smallest of the deoxynucleoside triphosphate pools, dGTP, was estimated to be present in amounts capable of sustaining DNA replication for only 15 to 30 seconds (Skoog and Nordenskjold, 1971). Thus S-phase cells must be constantly supplied with deoxyribonucleoside triphosphates. While there are enzymatic salvage pathways for recycling deoxyribonucleotides, the amounts required for DNA replication places a heavy dependence on *de novo* synthesis. To date, ribonucleotide reductase is the only enzyme identified which is capable of supplying sufficient quantities of deoxyribonucleotides. In bacterial cells, the isolation of a temperature sensitive mutant of DNA synthesis (*DNA*F) demonstrated the essential nature of ribonucleotide reductase. The mutant was found to contain a lesion in the gene coding for ribonucleotide reductase (Fuchs *et al.*, 1972).

Measurable levels of ribonucleotide reductase activity were found to be correlated with the fraction of cell populations in which DNA synthesis was occurring (Turner *et al.*, 1968). Cell sychronization studies involving mammalian cells also illustrated the critical association of ribonucleotide reductase activity with cell growth. The pool sizes of all four deoxyribonucleoside triphosphates increase immediately prior to the initiation of DNA synthesis. The pools remain elevated during S-phase, and afterwards decrease to the low basal levels found in G1 cells (Walters *et al.*, 1973; Bray and Brent, 1972). The increase in deoxyribonucleotide pools correlates with dramatic increase in ribonucleotide reductase activity just prior to DNA synthesis, and a return to low basal levels near the end of S-phase through to G1 (Murphree *et al.*, 1969; Peterson and Moore, 1974; Lewis *et al.*, 1978).

1.2. Ribonucleotide Reductase Reaction: The reactions performed by this universal enzyme is a direct reduction of the ribose moiety in a ribonucleotide to the deoxyribose of a deoxyribonucleotide (Fig. 1). The reducing system requires the presence of a molecule containing sulfhydryl groups which, in the reaction, are oxidized to the disulfide. Two small protein molecules, thioredoxin and glutaredoxin can serve as the intermediate hydrogen carriers (Laurent *et al.*, 1964; Engstrom, N.E. *et al.*, 1974; Luthman *et al.*, 1979). These proteins function through the thioredoxin reductase system (Moore *et al.*, 1964; Larsson, 1973), and glutaredoxin via glutathione and the glutathione reductase system (Holmgren, 1976; Luthman and Holmgren, 1982). The reduced form of

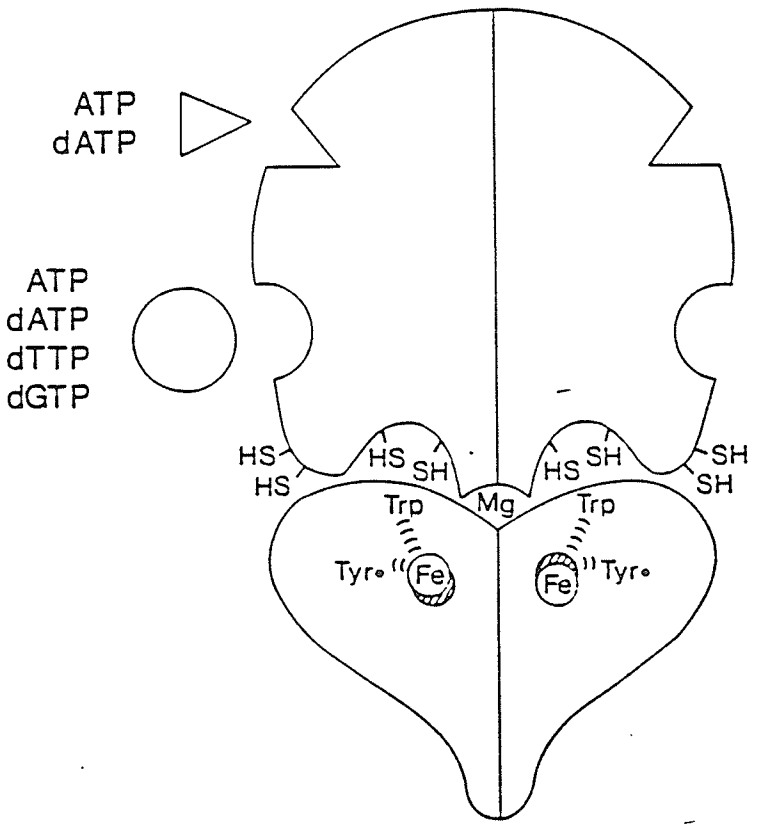
Figure 1. Reaction performed by mammalian ribonucleotide reductase.


NADP+
THIOREDOXIN REDUCTASE
NADPH + H+
THIOREDOXIN red
THIOREDOXIN ox
BASE
RIBONUCLEOTIDE REDUCTASE
BASE
OH OH
OH H
GLUTAREDOXIN red
GLUTAREDOXIN ox
2GSH
GSSG
NADPH + H+
GLUTATHIONE REDUCTASE
NADP+

nicotinamide adenine dinucleotide phosphate (NADPH) provides the ultimate reduction potential for both systems.

1.2.1. Classes of Ribonucleotide Reductases: The ribonucleotide reductases characterized to date are of several different types (Thelander and Reichard, 1979; Wright *et al.*, 1981; Lammers and Follman, 1983; McFarlan and Hogenkamp, 1989) which are divided into four classes of the enzyme (Eliasson *et al.*, 1990). The enzyme isolated from aerobically grown *E. coli* is the prototype for Class I ribonucleotide reductases, but higher animal ribonucleotide reductases and plant ribonucleotide reductases are also members of the Class I enzyme (Fig. 2). The *E. coli* ribonucleotide reductase consists of two different proteins, called B1 and B2. Each of these proteins is a homodimer and the structure of the active complex is $\alpha_2\beta_2$. Protein B1 contains redox-active dithiol groups and protein B2 contains dinuclear ferric iron centres and a stable tyrosyl free radical. Both the redox-diols and the radical are essential for enzyme activity. The Class II enzymes are found in bacteria. They are poorly characterized, but seem to be similar to the Class I enzymes, the difference being that they contain manganese instead of iron. The ribonucleotide reductases assigned to Class III are found in microorganisms, consist of a protein monomer and are dependent on adenosyl-cobalamin (vitamin B_{12}) as a radical generator during catalysis. The most recently discovered Class IV is found in anaerobically grown *E. coli* (Fontecave *et al.*, 1989). This anaerobic *E. coli* enzyme was found to be immunochemically related to protein B1 of the aerobic reductase, and requires S-adenosylmethionine as cofactor (Eliasson *et al.*, 1990). This

Figure 2. Model of a Class I (*E. coli*) ribonucleotide reductase holoenzyme. Protein B1 forms an α_2 dimer. There are two independent regulatory domains in the protein B1 subunit. One of these ( ) is responsible for regulating overall activity through the binding of ATP (activator) or dATP (inhibitor). The other domain regulates substrate specificity through the binding of ATP, dTTP, dGTP, and dATP. Protein B2 forms a heart shaped dimer. The B2 (β_2) dimer contains four non-heme iron centres which help stabilize the tyrosyl free radical. Adapted from Nordlund *et al.*, 1990.


ATP
dATP
ATP
dATP
dTTP
dGTP
HS
HS
HS
SH
HS
SH
SH
SH
Trp
Mg
Trp
Tyr•
Fe
Fe
Tyr•
B1 (α2)
2 x 85.7 kDa
B2 (β2)
2 x 43.4 kDa

type of reductase is also suggested to work via a radical mechanism.

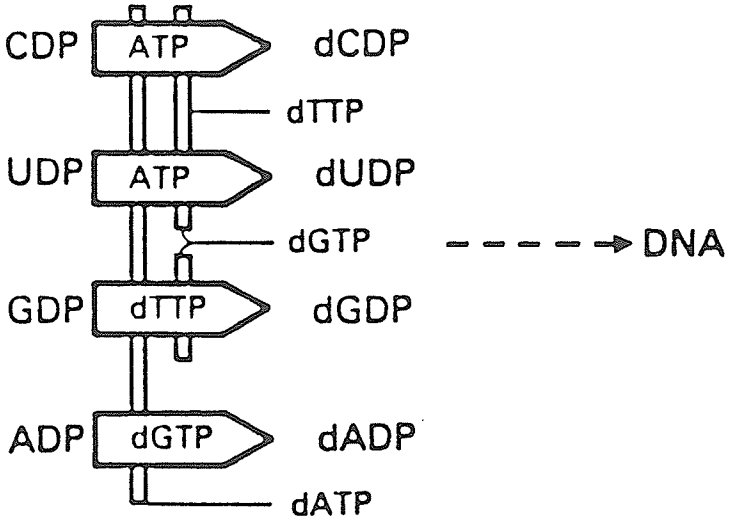
Thus, despite their differences, it seems possible that all the different ribonucleotide reductases function via a similar mechanism involving a radical (Lammers and Follman, 1983; Ashley and Stubbe, 1989; Eliasson *et al.*, 1990). Apparently, one of the most efficient ways to chemically reduce ribonucleosides to the corresponding deoxyribonucleosides also involves a free radical generator (Robins *et al.*, 1983).

1.2.2. Mammalian Riboncleotide Reductase: The structure of mammalian ribonucleotide reductase is similar to that of *E. coli*. The holoenzyme has an $\alpha_2\beta_2$ structure composed of two separable protein dimers which are frequently referred to as M1 and M2 (Wright *et al.*, 1990; Moore, 1977; Cory *et al.*, 1978; Chang and Cheng, 1979). Substrates and effectors bind to protein M1, which has a dimer molecular weight of 170,000 (Thelander *et al.*, 1980; Eriksson *et al.*, 1982). The M2 protein is also a dimer, with a molecular weight of 88,000 (Thelander *et al.*, 1985; McClarty *et al.*, 1987) and contains the non-heme iron and tyrosyl free radical. The presence of this unique tyrosyl free radical as a part of the functional M2 component allows the determination of M2 protein expression in whole cells by measuring, with the use of electron paramagnetic resonance spectroscopy, the characteristic asymmetric doublet of the tyrosyl free radical (Graslund *et al.*, 1982; Wright *et al.*, 1987; McClarty *et al.*, 1987; Choy *et al.*, 1988).

1.3. Allosteric Regulation of Mammalian Ribonucleotide Reductase: Since excess deoxyribonucleoside triphosphates are cytotoxic to cells and

because pool sizes and ratios of each of the four deoxyribonucleotides must be strictly maintained for efficient DNA synthesis, ribonucleotide reductase must have rigid cellular control of its activity. The mammalian enzyme is regulated in a complex fashion by nucleoside triphosphate effectors (Thelander and Reichard, 1979; Wright, 1983, 1989). A schematic representation of this regulation is shown in Fig. 3. The reduction of CDP to dCDP and UDP to dUDP requires the presence of ATP activated enzyme. The reduction of GDP to dGDP requires activation by dTTP, and the reduction of ADP to dADP requires the activation by dGTP. Reduction of all four ribonucleoside diphosphates substrates is inhibited by dATP. The scheme for the allosteric regulation of mammalian ribonucleotide reductase suggests that deoxyribonucleoside diphosphate formation begins with reduction of CDP and UDP. dUDP is consequently converted to dTTP which then stimulates GDP reduction and dGTP stimulates ADP reduction. Accumulation of dATP (*e.g.*, in the absence or slowing down of DNA synthesis) eventually leads to the complete inhibition of ribonucleotide reductase. In addition to the above, dTTP is a good inhibitor of pyrimidine (CDP, UDP) reductions and dGTP is a negative feedback inhibitor of GDP reduction and pyrimidine reduction. This overall scheme is a simplified version, as some studies of intracellular regulation of mammalian ribonucleotide reductase may be more complex than described above (*e.g.* Hards and Wright, 1984; Fox, 1985). This general model of regulation of ribonucleotide reductase has proven to be very useful in investigations of this enzyme (Wright, 1989; Wright *et al.*, 1989).

Figure 3. Allosteric regulation of mammalian ribonucleotide reduction. The nucleotides shown in the figure and the arrow blocks represent the positive effectors, the open bars represent inhibition. Figure adapted from Wright, 1989a; Wright *et al.*, 1990.


CDP
ATP
dCDP
dTTP
UDP
ATP
dUDP
dGTP
DNA
GDP
dTTP
dGDP
ADP
dGTP
dADP
dATP

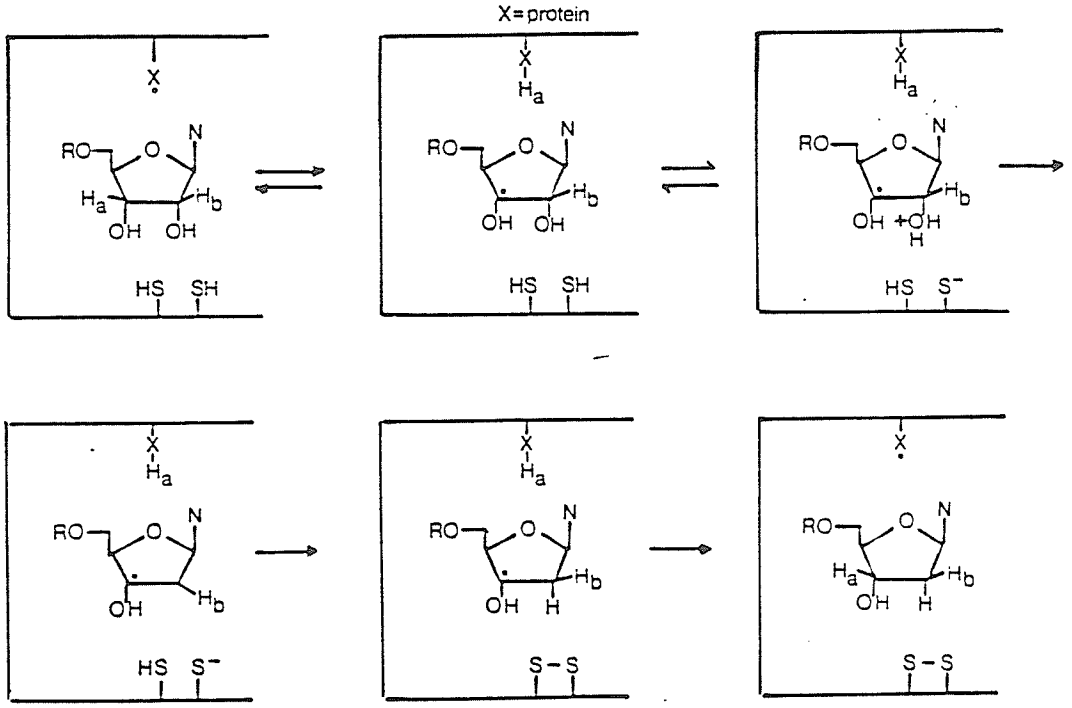
1.4. The Reaction Mechanism: Despite extensive studies, primarily by Joanne Stubbe and coworkers (Ashley and Stubbe, 1989; Stubbe, 1990), there is not much known about the reaction mechanism for ribonucleotide reductase. The work has been done with Class I (*E. coli*) and Class III (*Lactobacillus leichmannii*) ribonucleotide reductases, but the results can probably be extended to the other classes as well (Ashley and Stubbe, 1989). The proposed model for the mechanism of 2′ carbon-hydroxyl bond cleavage is initiated by Fenton's Reagent [Fe (II)/H_2O_2] as shown in Fig. 4 (Ashley and Stubbe, 1985).

Radical involvement was suggested from the strict dependence of the activity of the *E. coli* enzyme on the presence of the radical (Ehrenberg and Reichard, 1972). Further evidence was obtained from the use of substrate analogues substituted at the 2′ position with azide (Sjoberg *et al.*, 1983; Ator *et al.*, 1984). These analogues are suicide inhibitors and, after a single turnover, protein B2 has lost the radical. During this reaction a substrate radical is formed.

The reaction mechanism proposed by Stubbe involves the initial formation of a substrate radical at the 3′ position of the substrate (Fig. 4). Exchange experiments have shown that the 3′ hydrogen is transferred to an exchangeable position in the protein during turnover and that the same hydrogen atom is returned to the 3′ position when the product is formed (Ashley and Stubbe, 1989).

Two thiol groups are directly involved in substrate reduction and become oxidized in the process (Thelander, 1974). These thiols are redox-active cysteine residues and were recognized at an early date as being

Figure 4. Proposed reaction mechanism for Class I ribonucleotide reductase (according to Ashley and Stubbe, 1989).


X=protein
RO
N
O
H_a
H_b
OH
HS
SH
S^-
S–S

part of the active site of ribonucleotide reductase. A recent suggestion, based on x-ray crystallography data (Nordlund *et al.*, 1990), is that a third cysteine is directly involved in the reaction mechanism, as a thiyl radical (Stubbe, 1990). This would indicate an electron transfer from the radical-harbouring tyrosine to this cysteine.

1.5. **Regulation of Enzyme Synthesis**: As discussed above, ribonucleotide reductase activity levels are a function of the cell cycle with activity peaking in S phase and then declining to a low level in G1 cells (Murphree *et al.*, 1969; Peterson and Moore, 1974; Lewis *et al.*, 1978). It would seem that the observed increases were due to *de novo* synthesis of ribonucleotide reductase. When cycloheximide, an inhibitor of protein synthesis in mammalian cells, was added to either logarithmically growing or synchronized mouse L cells in S phase, at a concentration of 1 μg/ml in growth medium, ribonucleotide reductase activity was found to decay exponentially with a half-life of approximately 2 hours (Turner *et al.*, 1968). At the time of this study, this half-life represented one of the shortest measured in mammalian tissue (Schimke and Doyle, 1970). Untreated exponentially growing cells maintained an unaltered level of activity. Cycloheximide did not act directly upon the reductase as cell free extracts assayed in the presence of up to 100 μg of drug/ml showed no inhibition. Also, the addition of actinomycin D, an inhibitor of RNA synthesis, at concentrations of either 1 or 10 μg per ml of culture medium to synchronized Chinese hamster fibroblasts during the G1 phase, blocked the increase in ribonucleotide reductase activity usually observed as the cells passed through S phase (Murphree *et al.*, 1969). These results

suggest that both RNA and protein synthesis are required for the cell cycle dependent increase of ribonucleotide reductase.

1.6. **Proliferation and Ribonucleotide Reductase**: Enhanced levels of ribonucleotide reductase are characterstic of rapidly proliferating tissues such as regenerating liver cells (Larsson, 1969) and embryonic organs such as liver (Elford, 1972) and brain (Millard, 1972). New born rats (5 to 6 days old) show a peak of reductase activity in spleen, thymus (Elford, 1972) and brain (Eels and Spector, 1982) which declines as the animals mature. In all of these organs the peak of enzyme activity corresponds to a time of high mitotic activity within the particular organ (*ie.*, production of white blood cells in the spleen or neuronal proliferation in the brain).

Some work suggests that ribonucleotide reductase may be involved in mechanisms of transformation (Tagger *et al.*, 1989; Wright *et al.*, 1989; Choy *et al.*, 1989). For example, a direct relationship between elevated levels of ribonucleotide reductase activity and the growth rates of a series of rat hepatomas has been shown to exist (Elford, 1972; Takeda and Weber, 1981; Weber *et al.*, 1981). Although the reductase activity is high in rapidly growing normal cells, it appears that activity increases even further in neoplastic cells with similar growth rates (Takeda and Weber, 1981; Weber *et al.*, 1981). Studies on the deoxyribonucleotide pools in hepatoma cells showed all four deoxribonucleotide concentrations were significantly higher in these cells than in newborn and regenerating rat liver which have similar growth rates to the hepatoma cells (Weber *et al.*, 1981). Thus it seems that commitment to neoplastic replication in this

system is accompanied by a marked increase in ribonucleotide reductase activity, and in the concentration of the accompanying deoxyribonucleotide pools.

1.7. Ribonucleotide Reductase and Iron: The effects of various combinations of hydroxyurea with iron-chelating agents and with other inhibitors of ribonucleotide reductase on partially purified enzyme and with cells in cultured have been studied (Brockman *et al.*, 1972; Sato and Cory, 1981; reviewed in Cory and Chiba, 1989). They found that combination with the iron-chelating agents EDTA, desferrioxamine (Desferal^{R}), or 8-hydroxyquinoline increased inhibition of the enzyme by hydroxyurea. This result can now be explained by the requirement for iron in the regeneration process of the tyrosyl free radical (McClarty *et al.*, 1990). The iron chelators reduced the available iron and thus reduced the ability of ribonucleotide reductase to regenerate active M2. Although the iron chelators work synergistically with hydroxyurea, they do not inhibit strongly by themselves.

McClarty *et al.* (1986b) reported that ribonucleotide reductase from a hydroxyurea resistant mouse L cell line, which overproduced the subunit M2 when grown in the presence of 5 mM hydroxyurea, became unusually sensitive to inhibition to bleomycin. In this study, 8 μM bleomycin inhibited CDP reduction *in vitro* by 20% with enzyme from wild type or mutant cells grown in the absence of hydroxyurea, but enzyme from mutant cells grown in the presence of hydroxyurea was inhibited by 70%. However, this effect was not specific to bleomycin or to mutant cells. The wild type cells after exposure to hydroxyurea for 1 hour also gave sensitive

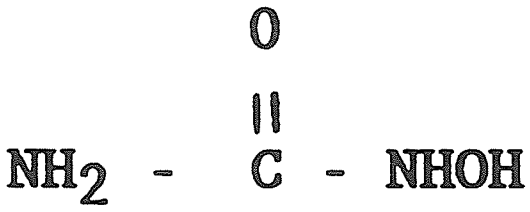
enzyme preparations. Also, wild type enzyme could be converted to the sensitive state by exposure of enzyme preparations *in vitro* to 10 mM hydroxyurea followed by gel filtration to remove the hydroxyurea. The same inhibition by 8 μM bleomycin was obtained with 15 μM EDTA, and inhibition by either could be reversed by the addition of 6 μM $FeCl_2$.

2. COMMENTS ON HYDROXYUREA

2.1. Ribonucleotide Reductase as a Site of Drug Action: Because of the key role ribonucleotide reductase plays in the process of cell growth, there is a great deal of interest in drugs that can control its intracellular activity. Such drugs are of particular interest in the field of cancer chemotherapy due the correlation between levels of the enzyme activity and the rate of malignant cell growth (Elford, 1972; Weber *et al.*, 1981).

2.2. Hydroxyurea: Hydroxyurea is one of the most widely used drugs to control ribonucleotide reductase activity (Fig. 5). Although it was first synthesized in 1869 (Dresler and Stein, 1869), current interest in the drug arose from a 1960 animal screening study that indicated hydroxyurea possessed anti-tumor activity in sarcomas (Stock *et al.*, 1960). Since then, the drug has been shown to be selectively toxic for rapidly proliferating mammalian tissues and has been used clinically to treat a wide variety of solid tumors as well as acute and chronic leukemia (Bergsagel *et al.*, 1964; Bolton *et al.*, 1964; Fishbein *et al.*, 1964; Bolin

Figure 5. Structure of hydroxyurea.



et al., 1982; Donovan *et al.*, 1984). The first clinical trials suggested that the drug interfered with DNA synthesis (Bersagel *et al.*, 1964), and subsequent studies confirmed this observation in a variety of organisms including bacteria (Rosenkranz and Levy, 1965; Gale *et al.*, 1964), HeLa cells (Young and Hodas, 1964), ascites tumor cells (Gale 1964; Yarbro *et al.*, 1965a), regenerating liver (Yarbro *et al.*, 1965b), hamster cells (Mohler, 1964; Lewis and Wright, 1974) and mouse jejunal crypts (Allison and Wright, 1979). Ribonucleotide reductase was identified as the site of action for the drug in *E. coli* (Krakoff *et al.*, 1968; Elford, 1968), bacteriophage (Yeh and Tessman, 1978; Berglund and Sjoberg, 1979), and mammalian cells including Novikoff hepatoma rat tumor (Elford, 1968), mouse L cells (Adams and Lindsay, 1967; Kuzik and Wright, 1980; Koropatnick and Wright, 1980), Ehrlich ascites cells (Turner *et al.*, 1966), human bone marrow cells (Frenkel *et al.*,1964), normal human diploid fibroblasts (Dick and Wright, 1980), and Chinese hamster ovary cells (Lewis and Wright, 1974, 1979; Hards and Wright, 1981).

Ehrenberg and Reichard (1972) first reported that the free radical was destroyed by hydroxyurea, and this phenomenon (measured by electron paramagnetic resonance, EPR) has been used in subsequent investigation of the nature of the free radical in ribonucleotide reductase from various species, its correlation with enzyme activity, and its participation in the reaction (*eg.* McClarty *et al.*, 1987; Choy *et al.*, 1988; Wright *et al.*, 1987; McClarty *et al.*, 1990). As further evidence of this mechanism, Kjoller-Larsen *et al.* (1982) compared a series of hydroxyurea analogs with regard to both inhibition of the *E. coli* reductase and free-radical scavenging ability. Those compounds that reacted very rapidly with the

model free-radical salt (potassium nitrosodisulfonate) included all of the most active enzyme inhibitors and some moderately active inhibitors. The compounds that reacted more slowly with the model free-radical were moderate to poor enzyme inhibitors, and those that did not react at all were inactive as enzyme inhibitors.

2.3. Mechanism for Development of Cellular Resistance to Hydroxyurea: Resistance to hydroxyurea in cultured cell lines correlated with production of an excess of ribonucleotide reductase, and/or with a reductase having altered sensitivity. The first report of hydroxyurea resistant mammalian cell lines was by Wright and Lewis (1974), and the first report that cellular extracts from hydroxyurea-resistant Chinese hamster ovary (CHO) cells and mouse L cells were less sensitive to hydroxyurea than control extracts was by Lewis and Wright (1974). Substantial studies on resistant mutants of CHO cells have been carried out (*e.g.*, Wright and Lewis, 1974; Lewis and Wright, 1979; Hards and Wright, 1981, 1983; Koropatnick and Wright, 1980; Wright *et al.*, 1987; Tagger and Wright, 1988; Wright, 1989). Increased enzyme activity exhibiting wild type or drug resistant characteristics have been observed (Dick and Wright, 1984; Wright, 1989; Wright *et al.*,1989). Lankinen (1980) and Akerblom *et al.* (1981) also have reported increased reductase activity in hydroxyurea resistant mouse cells. In one cell line they found a 30-fold increase in M2 activity and 10-fold increase in overall activity, but no increase in M1 protein. The EPR signal due to the free radical, measured in cells packed into an EPR tube went from undetectable

to quite visible, a more than 20-fold increase. The *in vitro* sensitivity of the enzyme to hydroxyurea was unchanged.

3. THESIS SCOPE

3.1 Thesis Scope

The critical role that ribonucleotide reductase plays in DNA synthesis, and therefore cell proliferation, makes ribonucleotide reductase a very attractive target for cancer chemotherapy. However, the toxic drugs used almost never destroys 100% of the cancerous cells. A single cell or a small population of cells can develop resistance to the cytotoxic effects of the drug. This single cell or small population eventually becomes dominant, the chemotherapy benefits become nil, and another choice of antineoplastic drug or strategy is mandated. Development of drug resistance is therefore one of the major problems in cancer treatment. This thesis examines the complex molecular mechanisms involved in the development of resistance to hydroxyurea. Also the affect of a tumor promoter on the regulation of ribonucleotide reductase was examined since alterations in ribonucleotide reductase activity have been detected in some neoplastic cells.

Materials and Methods

1. Cell Lines and Culture Conditions

1.1. Culture Conditions

All cell lines were routinely maintained at 37°C on the surface of plastic tissue culture plates (Lux Scientific Ltd.) in alpha-minimal essential medium (α-MEM; Flow Laboratories) supplemented with antibiotics, penicillin G (100 units/ml) (Sigma Chemical Co.), and streptomycin sulfate (100 μg/ml) (Sigma Chemical Co.) and 10% (v/v) fetal calf serum (FCS, Gibco, Ltd.). The formulation of α-MEM has been published by Stanners *et al.* (1971). Cultures were incubated at 37°C in a 5% CO_2 atmosphere in a humidity controlled incubator.

1.2. Cell lines

Wild type mouse L cells: The mouse L cells used in this study were originally isolated by Earle (1943). This immortal mouse fibroblast has been studied extensively and used successfully to isolate a variety of mutant phenotypes (*e.g.*, Dubbs and Kit, 1964; Thompson *et al.*, 1971; Kuzik and Wright, 1980; Wright *et al.*, 1983; McClarty *et al.*, 1986a, 1987). The mouse L cells grow well in culture and exhibit a doubling time of about 18 hours.

A series of hydroxyurea resistant mouse L cell lines with gradually increasing drug resistance properties were isolated from the wild type population by culturing non-mutagenized mouse L cells in the presence of

increasing concentrations of drug (McClarty *et al.*, 1986a; Choy *et al.*, 1988). Starting with the wild type population, the following drug concentrations were used in the selection of hydroxyurea resistant lines: Wild type cells ⇒ 0.35 mM (H^R-0.35) ⇒ 1.3 mM (H^R-1.3) ⇒ 1.5 mM (H^R-1.5) ⇒ 2.0 mM (H^R-2.0) ⇒ 3.0 mM (H^R-3.0) ⇒ 4.0 mM (H^R-4.0) ⇒ 5.0 mM (LHF). The cell line resistant to 5.0 mM hydroxyurea has been called LHF in published studies (McClarty *et al.*, 1986a; McClarty *et al.*, 1986b; McClarty *et al.*, 1987). For the sake of clarity, the LHF cell line has been (and in this thesis will be) referred to as H^R-5.0 (Choy *et al.*, 1988). At each selection step between 1.3 mM and 5.0 mM hydroxyurea, cells were frozen in the presence of growth medium containing 5% dimethyl sulfoxide and stored at -70°C. Cells selected in 0.35 mM drug were reisolated from the wild type population at a later date and also stored at -70°C. From the cell line H^R-5.0 several subclones were isolated and screened for the presence of high ribonucleotide reductase activity. One such subclone isolated was SC2 (McClarty *et al.*, 1986a, 1986b). To carry out the present study, the cell lines were thawed, put into cell culture, and maintained in the absence of hydroxyurea as described above. The drug resistance properties of the various lines were stable and did not change during approximately 1 year of continuous culture in the absence of a selective agent.

Balb/c 3T3 cells: This cell line is one of several lines developed by Aronson and Todaro (1968a) from disaggregated 14 - 17 day old Balb/c mouse embryos. This immortal cell line is non-tumorigenic, extremely sensitive to contact inhibition, grows at a high dilution, exhibits a low

saturation density, and is highly susceptible to transformation in tissue culture by the DNA virus SV40 and murine sarcoma virus (Aronson and Todaro, 1969). The Balb/c 3T3 cells have been used in studies relating to *in vitro* properties associated with tumorigenicity (Aronson and Todaro, 1968b), and contact inhibition and viral transformation (Jainchill and Todaro, 1970). The cells were cultured in 10% FBS αMEM as noted above.

Gat$^-$ CHO cells: The gat$^-$ CHO cell line was isolated from Chinese hamster ovary (CHO) cells and was auxotrophic for glycine, adenosine, and thymidine (McBurney and Whitmore, 1974). From this cell line an hydroxyurea resistant mutant, gat$^-$ CHO H^R-12SC8, was isolated for the ability to grow in 0.33 mM hydroxyurea (Lewis and Wright, 1979). The gat$^-$ cell lines were cultured in α-MEM supplemented with 10 μg/ml thymidine, 10 μg/ml adenosine and 10% FBS.

Rat L6 myoblasts: The rat L_6 myoblasts used in this study are a subclone of the original L_6 myogenic line previously described by Yaffe (1968). The myoblasts are able to fuse to form multinucleated and striated muscle fibres. The L6 myoblasts retain the capacity to differentiate even after long periods of proliferation in culture. The cells were maintained as subconfluent monolayers as previously reported (Creasey and Wright, 1984) in order to avoid myoblast fusion. The rat L_6 H^R-1 line was selected in the presence of 1 mM hydroxyurea (Creasey and Wright, 1984).

HeLa cells: A HeLa S3 cell line was used in a stepwise selection of increasing drug concentrations to isolate a human cell line (HeLa H^R-1) resistant to 2 mM hydroxyurea (Wright *et al.*, 1987). The HeLa S3 cell line is a clonal derivative of the parental HeLa line (Puck *et al.*, 1956).

1.3. Routine Culture Procedures

1.3.1. Cell Removal with Trypsin Solution

Phosphate buffered saline (PBS):	137 mM sodium chloride 2.68 mM potassium chloride 1.62 mM potassium phosphate monobasic 8.10 mM sodium phosphate dibasic pH 7.3

Sterile 0.3% trypsin (Sigma Chemical Co.; Difco Laboratories) was prepared in phosphate buffered saline (PBS) and 2 mM ethylenediaminetetra-acetate (EDTA) stored at 4°C. To remove cells from the surface of tissue culture plates, the growth media was aspirated and the plates washed once with PBS. One to two ml of trypsin was then added to the plates. After a period of incubation, varying from 30 seconds for less tightly adhering cells and up to 5 minutes for more tightly adhering cells, 2 to 3 ml of media containing 10% FCS was added to the plates to inhibit the trypsin and remove the cells. The cell suspension was then centrifuged, resuspended in growth medium, and dispensed as required.

1.3.2. Subculture

Cell cultures that approached confluence, about 2 to 3 $\times$ 10^6 cells per 100 mm plate, were subcultured. The cells were detached from the surface of tissue culture plates with the aid of trypsin solution as described above. The density of the cell suspensions was determined and an aliquot of 1 $\times$ 10^5 cells was transferred to a fresh 100 mm plate containing 10 ml of fresh growth media.

1.3.3. Long-term Storage of Cells

For long-term storage, all cells were suspended between 10^6-10^7 cells in 1.0 ml α-MEM containing 10% FCS and 10% dimethylsufoxide (DMSO). The cell suspensions were placed in 1 ml cyrotube vials (Nunc) and frozen slowly down to -80°C. To recover cells, the vial was rapidly thawed in a 37°C water bath and the suspension placed in 3 ml of growth media in a sterile 10 ml Falcon plastic tube, and then centrifuged at 500 $\times$ g for 5 minutes in order to pellet the cells. The cell pellet was then resuspended in normal growth medium and dispensed onto tissue culture plates.

1.3.4. Cell Counting

Aliquots of cells removed with trypsin solution as described above were diluted with saline, usually 100 μl of cell suspension was added to 40 ml saline, and counted with the aid of a Coulter Particle Counter (Coulter Electronics Ltd., Florida) in order to determine cell densities. Optionally, cell suspensions were counted using a hemocytometer (American Optic), averaging at least 4 seperate counts.

2. Determination of Colony-forming Ability

To determine the colony forming ability, exponentially growing cells were harvested with the aid of trypsin solution and counted as described above. A pre-determined number of cells ranging from 100 to 1000 were added to 100 mm culture plates with 10 ml of αMEM plus 10% FBS. After an incubation period of about 8 to 10 days at 37°C, the cells were stained with a filtered 50% solution of ethanol saturated with methylene blue (Sigma Chemical Co.) at room temperature for about 15 minutes. Colonies consisting of more than 40 cells were counted. Plating efficiency was defined as the ratio of colonies relative to the number of cells plated.

The effect of various drugs on the growth of cells can be studied by determining the relative plating efficiency (RPE). The RPE is defined as the plating efficiency in the presence of drug divided by the plating efficiency in the absence of drug. The RPE was determined by plating a pre-determined number of cells in culture plates containing medium with

increasing concentrations of drug, and in the absence of drug as a control (Hards and Wright, 1981).

3. Determination of Protein Concentrations

The concentration of protein in cell free preparations was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories), Technical Bulletin 1051. Protein concentrations were determined by using the Standard Assay Procedure and Micro Assay Procedure described in the kit literature. Purified bovine serum albumin fraction V (Sigma Chemical Co.) was used to generate a protein standard curve.

4. Preparation of Dowex-1-borate resin

The ion exchange resin was purchased from Bio-Rad Laboratories Ltd. as Dowex-1-chloride, 200-400 mesh and the chloride ions were replaced with borate ions. Approximately 450 g of resin was resuspended in 4 litres of saturated sodium borate solution and stirred overnight at room temperature. The resin was collected by gravity filtration and then resuspended in a another 4 litres of saturated sodium borate solution. Again after overnight stirring, the resin was collected by filtration and washed with 16 litres of deionized water. Finally, the Dowex-1-borate resin was resuspended in about 500 ml of water to make a thick slurry and stored at 4°C.

5. Preparation and Assay of Ribonucleotide Reductase.

Reagents used in the assay were:

CDP assay mix:	50 mM Hepes pH 7.2 2 mM ATP (Sigma Chemical Co.) 6 mM Dithiothreitol (DTT) 8 mM $MgCl_2$ (Fisher Scientific) 0.4 mM ^{14}C-CDP (5,000 dpm/nmole) (Amersham Ltd.)

Enzyme preparations used for ribonucleotide reductase assays were obtained from wild type cells and the various drug-selected cell lines (Hards and Wright, 1984; McClarty *et al.*, 1986a; McClarty *et al.*, 1986b; Choy *et al.*, 1988). Exponentially growing cells were harvested and washed twice with PBS, pH 7.2. The cells were resuspended at approximately 8×10^6 cells per 200 μl of 1 mM DTT in 20 mM Tris-HCl pH 7.2. The cell suspension was disrupted with three 10 second pulses of sonication at 30% power and then centrifuged at 12,000 $\times$ g (Micro Centaur, MSA; Eppendorf 5145) at 4°C for 10 minutes to remove cellular debris. Preparations containing 1 to 3 mg of protein/ml were used to measure CDP reductase activity by the method of Steeper and Stuart (1970). An aliquot of the supernatant was added to 25 μl of CDP reductase assay mixture and made up to a final volume of 150 μl with 1 mM DTT, 20 mM Tris-HCl pH 7.2. Reactions were incubated for 30 min at 37°C, and terminated by boiling for 4 min. The nucleotides were converted to nucleosides by the addition of 50 μl (1 mg) of *Croalus atox* venom (Sigma Chemical Co.) with 10 mM $MgCl_2$ in 0.1 M Hepes pH 8.0. After incubation for 1 hour at 37°C the reaction

was terminated by boiling for 4 min and the addition of 500 μl of distilled deionized water. The tubes were centrifuged to remove the heat precipitable material. The supernatant was passed through a Dowex-1-borate column 5 x 80 mm (Bio-Rad Laboratories) (Steeper and Stuart, 1970). The non-reduced cytosine is bound through interaction of its cis-diol to the borate ions. The deoxycytidine was eluted from the column with 5 ml distilled deionized water into scintillation vials. The eluate was mixed with 10 ml of Scintiverse II (Fisher Scientific) and counted with a liquid scintillation counter. Enzyme activity was expressed as nmoles of dCDP formed/3 $\times$ 10^6 cells/hour.

6. Southern and Northern Blot Analysis

6.1. DNA Isolation

TE buffer:	10 mM Tris-HCl, pH 8.0 1 mM EDTA
TES buffer:	25 mM Tris-HCl, pH 8.0 10 mM NaCl 10 mM EDTA
DNA isolation buffer:	0.5% sarcosyl 0.15 M NaCl 0.1 M EDTA
Chloroform:	note: references to chloroform means chloroform:isoamyl alcohol, 24:1 unless stated otherwise.

Genomic DNA was isolated from cells according to the procedure of Blin and Stafford (1976). Cells were harvested from 3 to 5 subconfluent 150 mm plates, centrifuged, washed once with PBS and resuspended in ice-cold TE at a concentration of approximately 10^8 cells/ml. To the suspension, 10 volumes of DNA isolation buffer and 100 μg/ml proteinase K was added. Following incubation at 50°C for 3 hours, the DNA was extracted 3 times with an equal volume of phenol. The DNA was then dialyzed overnight against 4 litres of TES buffer, allowing room in the dialysis tubing for the sample to expand about 3 fold in volume. The sample was then treated with 100 μg/ml of DNase free RNase (Sigma Chemical Co.) at 37°C for 3 hours, extracted twice with an equal volume of phenol/chloroform (1:1) and then once with an equal volume of chloroform. The DNA sample was then concentrated with 1 to 3 volumes of sec-butyl-alcohol and dialyzed extensively against TE. A couple of drops of chloroform was added to the final solution of DNA for preservation and stored at 4°C.

The concentration of DNA was determined by usually diluting 50 μl of DNA in 950 μl deionized water and measuring the absorbance at both 260 and 280 nm. Only those preparations having $OD_{260\lambda}/OD_{280\lambda}$ ratios of 1.75-1.80 were used. Using the formula: 1 $OD_{260\lambda}$ = 50 μg/ml DNA, when the 260λ/280λ ratio is 1.8 (Maniatis *et al.*, 1982), allows for calculation of the DNA concentration.

6.2. Southern Blot Analysis

6× Gel Loading Buffer:	0.25% bromphenol blue 0.25% xylene cyanol 30% glycerol
1× TBE buffer:	90 mM Tris-HCl 90 mM boric acid 20 mM EDTA
20× SSC:	3 M NaCl 0.3 M sodium citrate pH 7.0
1× SSC:	0.15 M NaCl 0.015 M sodium citrate pH 7.0
50× Denhardt's reagent:	5 g Ficoll, Type 400 5 g polyvinyl pyrrolidone 5 g BSA dissolved in 500 ml water
Hybridization solution:	50% (v/v) formamide 6× SSC 5× Denhart's reagent 0.5% SDS

For Southern blots, 20 μg of DNA was digested to completion with 3 to 4 units/μg DNA of the desired restriction endonuclease at 37°C from 3 hours to overnight. The sample was then evaporated down to a volume of 40μl by using a speed-vac concentrator (Savant). To the sample, 1/5 volume of the 6× gel loading buffer was added, and the sample loaded onto a 0.7% agarose 1× (or ½×) TBE gel containg 0.5 μg/ml ethidium bromide. The gel was electrophoresed overnight at 25 volts in the same concentration of TBE buffer. Molecular weight markers were loaded onto separate lanes in solutions containing the same salt concentration. Gels

were viewed under ultraviolet light to evaluate the digestion, to ensure that equivalent amounts of DNA were loaded, and to measure the distance between the well and each band of the molecular weight markers. Gels were then treated in order that they may be transferred to either nitrocellulose or nylon membranes using the capillary blotting method (Southern, 1975; Maniatis *et al.*, 1982).

To facilitate efficient transfer of high molecular weight DNA to the membranes, the DNA was partially acid depurinated in 0.25 N HCl with slight agitation for 15 minutes or until the bromphenol blue tracking dye changed colour to a light yellow. The gel was then removed from the acid and rinsed with water. For transfer onto nylon membranes (Zeta-probe, Bio-Rad Laboratories Inc.; Nytran, Schleicher and Schuell Inc.; Gene Screen Plus, New England Nuclear Research Products Inc.), the gel was equilibrated in the transfer buffer, 0.4 N NaOH, with slight agitation for 20 minutes and blotted onto the nylon membrane with the transfer buffer for at least 4 hours. For transfer onto nitrocellulose membranes (Bio-Rad Laboratories; Schleicher and Schuell), the gel was treated with 0.5 M NaOH, 1.5 M NaCl twice for 20 min. and neutralized with 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl twice for 20 min. and finally blotted onto the membrane overnight with 10× SSC.

After transfer, the membrane was removed from the gel, rinsed in 2 - 6× SSC, air dried, and then baked at 80°C for 2 hours in a vacuum gel drier to fix the DNA to the membrane. The blots were prehybridized for 3 hours to overnight at 42°C in hybridization buffer supplemented with 10% (v/v) dextran sulfate (Sigma Chemical Co.) and 100 μg/ml boiled single stranded salmon sperm DNA (Sigma Chemical Co.). Hybridizations were

performed in the same solution for 16 hours with 10^6 counts per minute (cpm)/ml of ^{32}P-labeled probe. The blots were washed twice in 2× SSC and 0.1% SDS at room temperature for 20 min. each, and then washed twice with 0.1 to 0.5× SSC and 0.1% SDS for 30 min. each. The membranes were then autoradiographed at -70°C using Kodak X-Omat AR film and Cronex Lightning Plus intensifying screens (DuPont).

6.3. RNA Isolation

6.3.1. Isolation of Total RNA by GITC

Guanidinium lysis buffer:	4 M guanidine isothiocyanate (GITC) 25 mM sodium citrate (pH 7.5) 0.5% N-laurylsarosine 0.1% Anti-foam A (Sigma Chemical Co.) 0.007% β-mercaptoethanol (added immediately before use)
CsCl buffer:	5.7 M CsCl 0.1 M EDTA (pH 7.0)
RNA water:	0.01% diethyl pyrocarbonate (DEPC) in deionized water and autoclaved 15 min, 120°C.

Total cellular RNA was extracted from logarithmically growing cells using the guanidinium isothiocyanate/cesium chloride method (Chergwin *et al.*, 1979). Cells were harvested from 6 to 8, 150mm culture plates, washed once with PBS and centrifuged. The pellet of approximately 10^8 cells was then resuspended in 2 ml of guanidinium lysis buffer. The suspension was then drawn through an 18 guage syringe approximately 20

times to hydrodynamically shear the DNA. The resultant foaming of the lysis buffer was cleared or reduced by a 15 min. centrifugation in a clinical centrifuge. The cell lysate was diluted up to 9 ml with the guanidinium lysis buffer and layered on top of a 3.0 ml cushion of CsCl buffer in a Beckman SW41 centrifuge tube. The suspension was then centrifuged at 20°C in a SW41 Ti Beckman rotor at 25,000 rpm (180,000 × g) for 18 hours. The top guanidinium isothiocynate layer and the CsCl cushion including the band of DNA were removed with a pasteur pipette and the tube was cut slighly above the pellet. The sides of the centrifuge tube and the pellet were washed once with guanidinium lysis buffer and then twice with 70% ethanol plus 0.1% SDS. The RNA pellet was resuspended in 500 μl sterile RNA water, transferred to a microfuge tube and precipitated overnight at -20°C by the addition of 1/10 volume 3 M sodium acetate (pH 5.5) and 2 volumes of absolute ethanol. The sample was then centrifuged 14,000 × g for 10-15 minutes in a benchtop microcentrifuge at 4°C. The pellet containing total cellular RNA resuspended in 100 to 150 μl of sterile RNA water and stored at -70°C.

The concentration of RNA was determined by usually diluting 5 μl of RNA in 995 μl deionized water and measuring the absorbance at both 260 and 280 nm. Only those preparations having $OD_{260\lambda}/OD_{280\lambda}$ ratios of 1.8 to 2.1 were used. Using the formula: 1 $OD_{260\lambda}$ = 40 μg/ml RNA, when the 260λ/280λ ratio is 2.0 (Maniatis *et al.*, 1982), allows for calculation of the RNA concentration.

6.3.2. Rapid Cytoplasmic RNA Isolation

Rapid RNA prep buffer A:
10mM Tris-HCl (pH 7.5)
0.15 M Na Cl
1.5 mM $MgCl_2$
0.65% Nonidet P-40 (NP-40)
(Sigma Chemical Co.)

Rapid RNA prep buffer B:
7 M urea
1% SDS
0.35 M NaCl
10 mM EDTA
10 mM Tris-HCl (pH 7.5)

Alternatively, RNA was also prepared using a rapid method for cytoplasmic RNA isolation (Gough, 1988). Briefly one to three 150 mm plates of subconfluent cells were harvested, washed in PBS, and pelleted in a 1.5 ml microfuge tube (5 min., 800 × g). The cell pellet was resuspened in 200 μl cold Rapid prep buffer A. After vigorous mixing, the nuclei were removed by centrifugation (5 min., 800 × g) and the cytoplasmic lysate carefully removed from the nuclear pellet and transferred to a fresh microfuge tube. To the cytoplasmic lysate, 200 μl of Rapid prep buffer B and 400 μl of phenol/chloroform (1:1) was added, mixed vigorously, and centrifuged 14,000 × g, 10 min. The aqueous layer was removed and extracted with 400 μl of chloroform. After mixing and centrifuging at 14,000 × g, 5 min., the aqueous layer was removed and ethanol precipitated in 800 μl 100% ethanol overnight at -20°C.

6.4. Northern Blot Analysis

5× Mops buffer:	0.1 M morpholinopropanesulfonic acid pH 7.0. 40 mM sodium acetate 5 mM EDTA
6× RNA Gel Loading buffer:	0.25% bromphenol blue 0.25% xylene cyanol 1 mM EDTA (pH 8.0) 50% glycerol

The RNA was prepared for Northern blot analysis as follows: to 4.5 μl RNA sample containing 20 μg of cellular RNA was added 2.0 μl MOPS buffer, 3.5 μl formaldehyde and 10.0 μl formamide. The sample was then incubated at 55°C for 15 minutes to denature the RNA. The sample was then chilled on ice and 2.0 μl of 6 × RNA gel loading buffer added. The sample was then loaded onto a 1% formaldehyde-agarose gel containing 18% formaldehyde and 1 × MOPS (Lehrach *et al.*, 1977; Miller, 1987) and electrophoresed overnight in 1 × MOPS buffer at 30 volts. An RNA sample containing ribosomal RNA was loaded into a separate lane as a relative mobility (Rf) reference marker. The marker lane was cut from the gel and stained with ethidium bromide (0.5 μg/ml in 0.1 M ammonium acetate) for 30-45 minutes and then destained with distilled deionized water for 30-45 minutes. The marker RNA bands (28s and 18s ribosomal RNA) were visualized and the Rf measured under ultraviolet illumination. The remainder of the gel was blotted onto a nitrocellulose or nylon membrane using 10 × SSC as previousy described for Southern blots but without any pretreatment of the gel. The membranes were again baked at 80°C for 2 hours and the blots prehybridized, hybridized, washed and developed as outlined for Southern

blots except that prehybridization and hybridization solutions contained 750 μl/ml denatured salmon sperm DNA to allow more efficient blocking.

6.5. RNA Dot/Slot Blot Analysis

Analysis of relative mRNA levels for M1 and M2 protein was carried out using the cytoplasmic dot hybridization technique (White and Bancroft, 1982). RNA was denatured by incubation at 60°C for 15 minutes in 6 × SSC plus 7.4% formaldehyde. Following dilution with 6 × SSC to the appropriate concentration, RNA was applied to a nitrocellulose membrane using the Bio-Rad Dot Blot or Bio-Rad Slot Blot apparatus. Blots were prehybridized and hybridized as described for Northerns above.

6.6. Isolation of Polysomal RNA

Polysome isolation buffer:
250 mM KCl
10 mM $MgCl_2$
20 mM Hepes, pH 7.5
0.25 M sucrose
2 mM DTT
150 μg/ml cycloheximide
100 units/ml RNasin (Promega, Inc.)

Sucrose Gradient buffer:
500 mM KCl
10 mM $MgCl_2$
20 mM Hepes, pH 7.5
0.25 M sucrose
2 mM DTT
150 μg/ml cycloheximide
0.5 mg/ml sodium heparin (Sigma Chemical Co.)

NETS buffer:

100 mM NaCl
2.5 mM EDTA
1% SDS
20 mM Tris-HCl, pH 7.4

Polysomal RNA was isolated by the method of Aziz and Munro (1986). Medium was aspirated from logarithmically growing H^R-1.5 and H^R-5.0 cells and the cells were washed with ice-cold PBS. Trypsin/EDTA was used to remove the cells and the trypsin was inactivated with α-MEM plus 10% FBS quickly as possible. A small aliquot was counted to determine the total number of cells from which polysomal mRNA was to be isolated. The cells were immediately placed on ice until they were centrifuged at 4°C and washed with ice-cold PBS. The following steps were performed with ice-cold solutions and samples kept on ice whenever possible. The cells were resuspended in 4 pellet volumes of polysome isolation lysis buffer. Non-idet P40 (NP40) was added to the resuspended cells to a final concentration of 0.5% to lyse the plasma membrane but still leave the nuclei intact. The sample was mixed by gentle mixing and incubated on ice for 5 minutes. The sample was then centrifuged at 500 × g for 10 minutes, 4°C. The cytosolic supernatant was carefully removed from the pelleted nuclei. The cytosolic extract was then layered onto a 30 ml 10% to 50% continuous sucrose gradient in a Beckman allopolymer SW27 centrifuge tube. The samples were spun at 24,000 rpm at 4°C for 4 hours. The bottom of the centrifuge tube was punctured and fractions were collected. To each fraction 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol was added, and the cytoplasmic RNA allowed to precipitate at -20°C. The cytoplasmic RNA was recovered by pelleting at

14,000 × g for 10 minutes. The RNA was resuspended in 400 μl of NETS buffer and extracted with equal volume of phenol/chloroform (1:1). Following ethanol precipitation with sodium acetate each RNA pellet was resuspended in 9.0 μl of RNase free water and the whole fraction probed on Northern blots for M2 mRNA.

7. Labeling of cDNA probes with ^{32}P-dCTP

cDNA probes between the size of 100 bp and 2000 bp were labeled using the hexanucleotide method of Feinberg and Volgelstein (1983) to specific activities between 0.5 to 1.0 x 10^9 cpm/ug using ^{32}P-dCTP. Approximately 500 ng of an appropriate cDNA probe in a volume of 25 μl was boiled for 5 min., and then immediately quenched on ice. Labeling of the single stranded cDNA probe was then performed using the hexanucleotide labeling kit (Amersham) or the random primer kit (Bethesda Reasearch Laboratories [BRL] Life Technologies, Inc.) using procedures described by each kit literature. In general, the probes were labeled adding 1 μl of DNA polymerase I ('Klenow' fragment: 1 unit/μl, stored in 50 mM potassium phosphate, pH 6.5, 10 mM β-mercaptoethanol, and 50% glycerol), 15 μl of multiprimer buffer solution (containing random hexanucleotides in a concentration buffer solution of Tris-HCl, pH 7.8, magnesium chloride, and β-mercaptoethanol), 2.0 μl each of dTTP, dATP and dGTP, and 5 μl ^{32}P-dCTP (specific activity 3000 Ci/mmol) to the probe. Following incubation at room temperature from a minimum of 3 hours to overnight, the reaction mixture was passed through a 1 ml spin column of sephadex G-50 (Pharmacia)

and the labeled probe was eluted with 100 μl TE. The incorporation of label into the probe was determined by counting 1 μl of the effluent using a ^{32}P Cerenekov scintillation counter program. An appropriate volume was then added to the hybridization mixture to achieve 1×10^6 cpm/ml. For probes larger than 2.0 kb, labeling was done by the Nick Translation System (Amersham; BRL). Again 500 ng of probe was diluted upto 28 μl with distilled water. To this 5 μl of the appropriate dNTPs in concentrated buffer was added plus 5 μl of DNA PolymeraseI/DNase I (100 units) and 7 μl ^{32}P-CTP (specific activity 3000 Ci/mmol). The mixture was then incubated at 12°C for 60 minutes and the probe purified from the free nucleotides by a spin column as described above.

8. Isolation and sources of cDNA probes and plasmids

8.1. Sources of cDNA probes and plasmids

Ribonucleotide reductase probes for the murine M1 cDNA (clone 65) and M2 cDNA (clone 10) were obtained from Dr. L. Thelander of the Karolinska Institute, Stockholm (Thelander and Berg, 1986). The β-actin cDNA was from the rat β-actin cDNA (McClarty *et al.*, 1987). Glyceraldehyde-3-phosphate-dehydrogenase cDNA was obtained from Dr. C.L.J. Parfett, Cancer Research Laboratory, University of Western Ontario, London, Ontario (Edwards *et al.*, 1985). The ornithine decarboxylase cDNA was obtained from Dr. A.E. Pegg, Department of Physiology, Milton S. Hershey Medical Center, Pennsylvania State University (Berger *et al.*,

1984). The transferin receptor probe (kindly provided by Dr. F. Ruddle, Department of Biology, Yale University) was expression vector pcD-TR1 containing full-length human transferin receptor cDNA (Kuhn *et al.*, 1984). The heavy (H) and light (L) chain ferritin probes were kindly provided by Dr. H. Munro (Department of Nutrition and Food Services, Massachusetts Institute of Technology). The H subunit cDNA was isolated from rat heart (Murray *et al.*, 1987) and the L subunit cDNA was isolated from rat liver (Leibold *et al.*, 1984). The probes required were obtained from plasmid preparations cut with the appropriate restriction endonucleases and purified as described below.

8.2. Large scale plasmid preparation

8.2.1. Transformation of *E. coli*

LB medium:	1% w/v bacto-tryptone (Difco) 0.5% w/v yeast extract (Difco) 1% w/v NaCl pH 7.5
STET:	0.1 M NaCl 10 mM Tris-HCl (pH8.0) 0.1 mM EDTA 0.5% v/v Triton X-100

Large scale preparations of plasmid DNA were obtained according to the procedures of Manniatis *et al.* (1982). A single colony of HB101 *E. coli* transformed with the appropriate plasmid was inoculated into 5 ml LB medium containing the appropriate plasmid selection antibiotic

(100 μg/ml tetracycline for GAPDH, and 50 μg/ml ampicillin for the others) and incubated at 37°C overnight. The entire suspension was then inoculated into 1 litre of LB medium and incubated until an $OD_{600\lambda}$ value of 0.6 to 0.8 was reached, at which time 5 ml of a 54 mg/ml solution of spectinomycin was added and the cells were incubated overnight in an orbital shaker at 250 rpm. The cell suspension was then centrifuged at 4,000 × g for 10 minutes and the supernatant was discarded while the pellet was resuspended in 25 ml ice-cold STET solution and recentrifuged. The pellet was then resuspended in 10 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 10% w/v sucrose. To the preparation was added 20 mg lysozyme in a volume of 2.0 ml followed by addition of 8.0 ml of 0.25 M EDTA. The sample was incubated on ice for 10 minutes and then 4 ml of 10% SDS was added and mixed quickly. Immediately afterwards, 6 ml of 5 M NaCl was added, gently mixed by inversion, and the sample set on ice for 1 hour. The sample was then centrifuged for 30 minutes at 30,000 rpm at 4°C in order to remove high molecular weight DNA and cellular debris. The supernatant was saved, extracted twice with phenol/chloroform and then twice with chloroform. To the aqueous phase was added 0.54 volumes of isopropanol and the sample mixed and precipitated at -20°C for a minimum of 1 hour. The sample was centrifuged at 15,000 × g for 30 min. The supernatant was discarded and the pellet washed once with 70% ethanol, dried under vacuum and resuspended in 4 ml TE. For each ml of the DNA solution 1 g of solid cesium chloride was added and dissolved. The sample was then transferred to a Beckman Quick seal centrifuge tube and 0.2 ml of a 10 mg/ml ethidium bromide solution was layered on top. The tube was filled to the rim with mineral oil, balanced and then sealed using a

Beckman heat sealer. Immediately before centrifugation the tubes were mixed by inverting and quickly placed in a Beckman Ti70.1 fixed angle rotor and centrifuged at 42,000 rpm overnight at 20°C. Two bands were visible under UV light, the lower band consisting of closed circular plasmid DNA was removed by puncturing the bottom side of the tube with a 21 guage syringe. The ethidium bromide was removed from the plasmid DNA by extracting the sample 4 to 5 times with an equal volume of TE/CsCl saturated butanol. The sample was then extensively dialized against 3 two litre changes of TE. The concentration of plasmid DNA in the preparation was determined by measuring the absorbance of the sample at 260nm as previously described. The plasmid preparation was stored at 4°C with a couple of drops of chloroform.

8.3. Purification of probes

To isolate the appropriate sequence of DNA required as a probe the plasmids were first cut with the appropriate restriction endonucleases at 37°C for 1 hour using 3 units/μg DNA. The C10 plasmid containing the cDNA sequence encoding the M2 subunit of ribonucleotide reductase was digested with Sal I and Pst I to yield the appropriate 1487 bp band corresponding to M2 cDNA. The D65 plasmid harboring the cDNA sequence encoding the M1 subunit of ribonucleotide reductase was digested with Nco I to yield the appropriate 2000bp band corresponding to M1 cDNA. The β-actin probe was a 1.2-kilobase BgII-BgII fragment. The ferritin plasmids and the transferin receptor plasmids were nick translated and the whole plasmid used for probing.

Following digestion the required bands were purified away from the rest of the plasmid on 1% agarose gels in ½ × TBE. After running the samples overnight at 30 volts in ½ × TBE running buffer the appropriate band was removed from the gel by visualizing the bands under UV light and excising the band. The gel slice containing the band was then placed in dialysis tubing containing the most minimal amount of ½ × TBE buffer, approximately 1 ml, and the insert was then electroeluted from the gel by passing a current of 100 volts for 2 to 3 hours across the sample. Once all of the DNA had migrated out of the gel the current was reversed and the dialysis bag containing the DNA was back electrophoresed for 60 seconds to remove any DNA fragments that may have stuck to the dialysis bag during elution. The probe fragment in ½ × TBE buffer was concentrated by passage through a mini-column-D (Sigma Chemical Co.) and eluted with 0.5 ml high salt buffer. The DNA was then precipitated by the addition of 2 volumes of ethanol and overnight at -20°C. The sample was centrifuged, washed once with 70% ethanol and resuspended in TE buffer in a concentration of approximately 500 ng/μl for subsequent labeling.

9. Western Blot Analysis

Tris-buffered saline (TBS):	50 mM Tris-HCl, pH 7.5 150 mM NaCl
TBS-Tween:	TBS 0.5% Tween 20 (Sigma Chemical Co.)
Carbonate-$MgCl_2$ buffer:	80 mM sodium bicarbonate 1 mM $MgCl_2$

Pelleted cells resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM DTT were sonicated and debris was centrifuged at 5000 × g for 5 min. Various amounts of protein were analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Proteins were then transferred to nitrocellulose membranes by the method of Towbin *et al.* (1979). The transfer was carred out at 100 volts for 1 hour. After transfer, the nitrocellulose membranes were blocked in TBS-Tween containing 1% bovine serum albumin. The membranes were then incubated with either AD203 anti-M1 mouse monoclonal antibody (Engstrom *et al.*, 1984) or JB4 anti-M2 rat monoclonal antibody (Engstrom and Rozell, 1988) for 3 hours, washed three times for 30 minutes each in TBS-Tween followed by incubation with the appropriate second antibody for 3 hours. Goat anti-mouse IgG conjugated with alkaline phosphatase (AP) (Sigma Chemical Co.) was used for detecting Protein M1, and rabbit anit-rat IgG conjugated with alkaline phosphatase (Sigma Chemical Co.) was used for detecting Protein M2. After one hour incubation, the membranes were again washed in TBS-Tween 3 times for 30 minutes each. The bound antibodies were visualized by the development of the AP reaction, as described by Blake *et al.* (1984) in carbonate-$MgCl_2$ buffer (pH 9.6).

^{125}I-labeled sheep anti-rat IgG was also used in detection of Protein M2 on immunoblots. Instead of using AP conjugated anti-rat IgG, the blots were incubated for one hour with 100 μCi of labeled anti-rat IgG and the membranes washed in TBS-Tween 4 times for 30 minutes each. The blots were then exposed on Kodak X-OMAT AR film at -70°C.

Western blot analysis for H and L ferritin subunits was carried out as previously described (McClarty *et al.*, 1987; Choy *et al.*, 1988) with the following modifications. Following cell extract preparations, total cell extract protein content was determined and then a given amount of protein was analyzed on a SDS 10% to 17.5% gradient pore polyacrylamide gel. After protein transfer and blocking, membranes were incubated with a rabbit polyclonal anti-human ferritin IgG (Boehringer Mannheim) which recognizes both mouse and human ferritins (Rouault *et al.*, 1987). Goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) was used for H and L subunit detection.

10. Immunochemical Analysis Methods

10.1. Immunoprecipitaion of Proteins

Solubilizing buffer 150 (SB150):	1% Triton X-100 0.5% sodium deoxycholate 150 mM NaCl 25 mM Tris-HCl, pH 7.5 5 mM EDTA
Solubilizing buffer 250 (SB250):	1% Triton X-100 0.5% sodium deoxycholate 250 mM NaCl 25 mM Tris-HCl, pH 7.5 5 mM EDTA
Tris-glycine transfer buffer:	25 mM Tris-HCl, pH 8.3 25 mM glycine

The cell lines were grown in culture for at least 24 hours before radionucleotide labeling. Equal numbers of cells were plated for protein half-life determinations. Labeling of logarithmically growing cells was carried out in plastic culture plates in methionine-free α-MEM (Flow Laboratories) containing 50 μCi of [^{35}S]methionine (3000 Ci/mmol/ml) and 5% (v/v) dialyzed fetal bovine serum (Gibco). The cells were incubated overnight to label the total protein pool or pulsed labeled for 30 minutes to 1 hour for biosynthetic rate determinations. The labeling medium was aspirated, and the cells were washed twice with phosphate-buffered saline. Trypsin/EDTA was used to harvest the cells, centrifuged, and washed again with phosphate-buffered saline. The resulting cell pellet was resuspended in SB250 solubilizing buffer to a final densisty of approximately 2×10^7 cells/ml. Solubilization was carried out at room temperature for 15 minutes. The cell extract was clarified by centrifugation in a microfuge at 12,000 $\times$ g for 10 minutes.

Immunoprecipitation was carried out by using saturating amounts of AD203 anti-M1 mouse monoclonal antibody or JB4 anti-M2 rat monoclonal antibody according to the method of Firestone *et al.*, (1982), except only one round of immunoprecipition was performed on the total cell extracts, and formalin-fixed *Staphylococcus aureus* cells (Pansorbin) was purchased from Calbiochem. The labeled lysate was precleared of non-specific immune complexes that could form with the fixed *S. aureus* cells. An aliquot of 20 μl of a 10% suspension was added to 200 μl of extract and incubated at room temperature with occasional mixing for 15 minutes. The *S. aureus* cells were removed by centrifugation (12,000 $\times$ g, 2 minutes) and the precleared extract was recovered. The fixed *S. aureus* cells were

complexed with the secondary antibody, either rabbit anti-mouse IgG (against AD203) or rabbit anti-rat IgG (against JB4) (Sigma Chemical Co.). The *S. aureus*-IgG immune complex was preloaded with cell free extract of non-labeled mouse L cells to reduce the amount of non-specific binding of the labeled extract with the immune complex. The mouse L cell free extract was prepared by resuspending 1×10^7 cells in 1 ml of SB150 solubilization buffer and incubated at room temperature for 15 minutes before removing cellular debris by centrifugation (12,000 × g, 20 minutes). An aliquot of a 10% suspension of *S. aureus* cells and the appropriate IgG was mixed with 2× volume of the non-labeled cell free extract at room temperature for 15 minutes. The preloaded *S.aureus*-IgG immune complexes were recovered by centrifugation and resuspended in SB250 buffer to make a 10% cell suspension. This cell suspension was used to precipitate the antigen-antibody complexes.

Prior to immunoprecipitation, a 5 μl sample from each labeled extract was treated with 10% trichloroacetic acid (TCA) and the TCA-precipitable radioactivity was determined. The same number of TCA-precipitable counts were used in the subsequent immunoprecipitation. A volume of 200 μl of labeled extract containing the desired amount of TCA-precipitable radioactivity was mixed with either AD203 (anti-M1) or JB4 (anti-M2) monoclonal antibodies and 100 μl of SB250 solubilizing buffer containing 50 mg/ml of BSA (Sigma Chemical Co.). After incubating at room temperature for 15 minutes, 15 μl of 10% preloaded *S. aureus*-IgG was added to the mixutre and incubated for another 5 minutes. The entire reaction mixture was layered on 600 μl of 1 M sucrose solution in a microfuge tube. The mixture was centrifuged for 3 minutes at 12,000 × g. The top layer

was aspirated and the side of the tube was washed with 2 M urea solution. The sucrose layer was aspirated and the immunecomplex pellet was first washed with SB250 buffer and then with TE buffer. The resulting precipitated products were analyzed on 10% SDS-PAGE gels. After equilibration in Tris-glycine transfer buffer, the gels were dried and exposed to Kodak X-OMAT AR film for 12 to 36 hours. Autoradiograms were scanned, and peak areas were quantitated (Beckman DU-8 spectrometer or Bio-Rad Densitometer model 620).

10.2. Determination of Protein Half-Life

Cells were metabolically labeled as described above, overnight. The cells were then chased with methionine free growth medium supplemented with unlabeled methionine and 10% fetal calf serum. Cells were harvested at various time points by removal from plates with trypsin/EDTA solution, and then centrifuged again. Immunoprecipitation was carried out as described above. The samples were run on a 10% SDS-PAGE, autoradiographed, and quantified by densitometry (Beckman Spectrophotometer DU-8). The protein half-life was determined from the reduction in [^{35}S]methionine labeled protein over a period of time.

10.3. Determinations of Protein Biosynthetic Rates

Protein M2 biosynthetic rates were determined by [^{35}S]methionine pulse labeling. Cells were labeled in the [^{35}S]methionine-labeling medium described above, except 500 μCi of [^{35}S]methionine/ml were used, and labeling time was 20 minutes at 37°C. Immediately following the pulse label, cells were harvested and lysed, and [^{35}S]methionine incorporation into protein was determined by trichloroacetic acid precipitation. Immunoprecipitation (3×10^6 cpm) using JB4 anit-M2 monoclonal antibody, 10% SDS-PAGE, autoradiography, and densitometry was performed as described above.

10.4. Analysis of Iron in Protein M2

10.4.1 ^{59}Fe Labeling of Cells

Monolayer cultures of logarithmically growing hydroxyurea resistant mouse L cells (H^R-5.0) were washed three times with warm phosphate buffered saline (PBS), and then refed with α-minimal essential medium containing 1% fetal calf serum and ^{59}Fe-diferric transferrin (22.5 μg/ml) prepared as described by Klausner *et al.* (1983). Following incubation at 37°C for 18 hours, the ^{59}Fe-labeled cells were washed, then harvested and used for immunoprecipitation experiments.

10.4.2. Incubation of Ribonucleotide Reductase Preparations with Hydroxyurea and Immunoprecipitation of ^{59}Fe labeled Protein M2

Hydroxyurea treated protein M2 was obtained by incubating ^{59}Fe-labeled enzyme preparations, prepared as previously described (McClarty *et al.*, 1986a) in the presence of various concentrations of hydroxyurea for 10 minutes on ice. ^{59}Fe-labeled or non-labeled protein M2 was then immunoprecipitated by using a saturating amount of JB4 anti-M2 rat monoclonal antibody (Engstrom and Rozell, 1988) according to the method of Firestone *et al.* (1982) except only one round of immunoprecipitation was performed. The resulting precipitated products were either analyzed on 10% SDS-polyacrylamide gels and autoradiography, or quantitated directly by using a gamma counter (LKB, Compugamma model).

10.4.3. In Vivo Labelling of Ferritin H and L Subunits

The apparent rate of ferritin H and L subunit biosynthesis in the presence or absence of hydroxyurea and/or iron was determined by measuring the incorporation of [^{35}S] methionine into the two subunits. Cells (9×10^5) were biosynthetically labeled at 37°C with a one hour pulse of 500 μCi [^{35}S] methionine per ml of methionine free medium plus 5% (V/V) dialyzed fetal calf serum. Immediately following the pulse label, cells were harvested and lysed as previously described above. For measurement of radioactivity incorporated into total soluble protein, 10 μl aliquots of the labeled cell extracts were precipitated with 10% trichloroacetic

acid followed by filtration and liquid scintillation counting. For chase experiments, cells were labeled for one hour, washed three times with α-minimal essential medium, and then incubated in the same medium plus 10% fetal calf serum for the indicated period of time.

Immunoprecipitation with a saturating amount of ferritin polyclonal antibody (Boehringer Mannheim), SDS 10 to 17.5% gradient pore polyacrylamide gel electrophoresis, autoradiography and densitometry (Bio-Rad Model 620) were carried out.

11. Nuclear DNA binding proteins

11.1. Nuclear protein isolation

Nuclear Extract buffer A:
- 0.3 M sucrose
- 10 mM HEPES-KOH, pH 7.9
- 10 mM KCl
- 1.5 mM $MgCl_2$
- 0.1 mM EGTA
- 0.5 mM DTT
- 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.)
- 2 μg/ml each of antipain, leupeptin, and pepstatin A (Sigma Chemical Co.)

Nuclear Extract buffer B:
- 400 mM NaCl
- 10 mM HEPES-KOH, pH 7.9
- 1.5 mM $MgCl_2$
- 0.1 mM EGTA
- 0.5 mM DTT
- 5% glycerol
- 0.5 mM PMSF

Nuclear Extract buffer C: 20 mM HEPES-KOH, pH 7.9
75 mM NaCl
0.1 mM EDTA
0.5 mM DTT
20% glycerol
0.5 mM PMSF

Nuclear protein extracts were prepared using a modified procedure by Dignam *et al.* (1983). Nuclei were harvested from 5×10^8 to 10^9 cells. HeLa cells were grown in suspension in 500 ml spinner bottles and harvested by centrifugation for 10 minutes at 3000 × g, 4°C. 3T3 BALB/c cells were grown in tissue culture plates, harvested using a rubber policeman and pelleted at 3000 × g, 4°C. Nuclei were prepared from non-treated 3T3 BALB/c cells and cells treated with 0.1 μM TPA for 30 minutes. All the cell pellets were resuspended in 1 ml of PBS, transferred to 1.5 ml eppendorf tubes and centrifuged at 3000 × g, 4°C. The cell pellet was then resuspended in 5 pellet volumes of Nuclear Extract buffer A. Cells were lysed by 8 to 12 strokes with a B pestle in a Dounce glass homogenizer (Kontes) and 1 to 2 strokes in the presence of 0.3%-0.4% Nonidet P-40 (NP-40). The homogenate was then centrifuged at 1200 × g for 10 minutes, 4°C, and the pelleted nuclei washed twice with Nuclear Extract buffer A without NP-40.

The nuclei were then resuspended in 2.5 pelleted nuclei volumes of Nuclear Extract buffer B. The resuspended nuclei were stirred slowly for 30 minutes at 4°C, followed by centrifugation for 60 minutes at 100,000 × g using a Type 70Ti rotor (Beckman). After dialyzing the supernatant for 2 to 4 hours against 50 volumes of Nuclear Extract buffer C., the extract was clarified by centrifugation at 25,000 × g for 15

minutes, which removes precipitated material completely and partially removes lipid (Dignam *et al.*, 1983). The protein concentration was measured by the Bradford method (Bio-Rad kit). The nuclei protein extracts were frozen in small 50 μl to 100 μl aliquots at -70°C. Repeated thawing and freezing was avoided whenever possible.

11.2. DNA Electromobility shift assays

2× Gel Shift buffer:
20 mM Tris-HCl, pH 7.5
100 mM KCl
10 mM $MgCl_2$
2 mM DTT
2 mM EDTA
12.5% glycerol

DNA electromobility shift assay (EMSA), or gel-shift assay, were performed based on methods by Fried and Crothers (1981) and Garner and Revzin (1981) with modifications by Strauss and Varshavsky (1984). Crude nuclear protein (approximately 1 μg) were titrated with increasing amounts of poly(dI·dC) in 15 μl of 2× gel shift buffer. The sample is made up to 30 μl with double distilled water and incubated for 15 minutes at room temperature. After further incubation for 20-40 minutes in the presence of about 1 ng of labeled probe, 2 μl of 0.1% bromphenol blue was added and the samples were separated in a 1 × TBE, 4% polyacrylamide gel. Prior to loading the samples, the gel was prerun for 1 hour at 20 mA. The gel was electophoresed until the bromphenol blue had run 3/4 length of the gel. After fixing the gel for 10 to 20 minutes in 10% acetic acid and 10% methanol, the gel was transferred to filter paper support. The gel was

dried under vacuum in a conventional gel dryer (Bio-Rad) at 80°C for 1.5 hours, and subjected to autoradiography.

Results

1. Molecular Mechanisms of Drug Resistance

1.1. Drug Sensitivity and Ribonucleotide Reductase Levels in a Series of Mutant Cell Lines

A series of mouse L-cells lines were sequentially selected in the presence of increasing concentrations of hydroxyurea for the ability to proliferate in normally cytotoxic drug concentrations, as described in Materials and Methods. The colony-forming abilities of the parental wild-type population and the various drug-selected cell lines in the presence of drug-supplemented medium are shown in Fig. 6. All drug-selected cell lines exhibited a reduced sensitivity to the the cytotoxic effects of hydroxyurea when compared to parental wild-type cells. For example, when the D_{10} values for hydroxyurea are considered (Table 1), cells selected in the presence of the lowest drug concentration (0.35 mM hydroxyurea) exhibited approximately an 8-fold elevation in drug resistance, whereas cells selected at the highest concentration (5 mM hydroxyurea) were about 35-fold more resistant to the drug. The drug resistance properties of the cell lines signifigcantly increased at each of the drug selection steps tested (0.35 mM, 1.5 mM, 2.0 mM, 3.0 mM, 4.0 mM, 5.0 mM hydroxyurea). In keeping with the resistance characteristics, each drug-selected line also exhibited an elevation in ribonucleotide reductase activity when compared to the wild-type cell line (Table 1). There was a sequential increase in enzyme activity with each selection step, with the least resistant line showing a modest increase in activity, and the highest resistant line exhibiting the greatest increase in enzyme activity of approximately 24-fold.

Figure 6. Relative colony-forming abilities of wild type L60 cells and hydroxyurea-resistant cells in the absence and presence of various concentrations of hydroxyurea of (♦) wild type, (▲) H^R-0.35, (Δ) H^R-1.5, (■) H^R-2.0, (□) H^R-3.0, (●) H^R-4.0 and (○) H^R-5.0 cell lines. Experiments were performed as described in Materials and Methods. The y-axis scale is logarithmic.

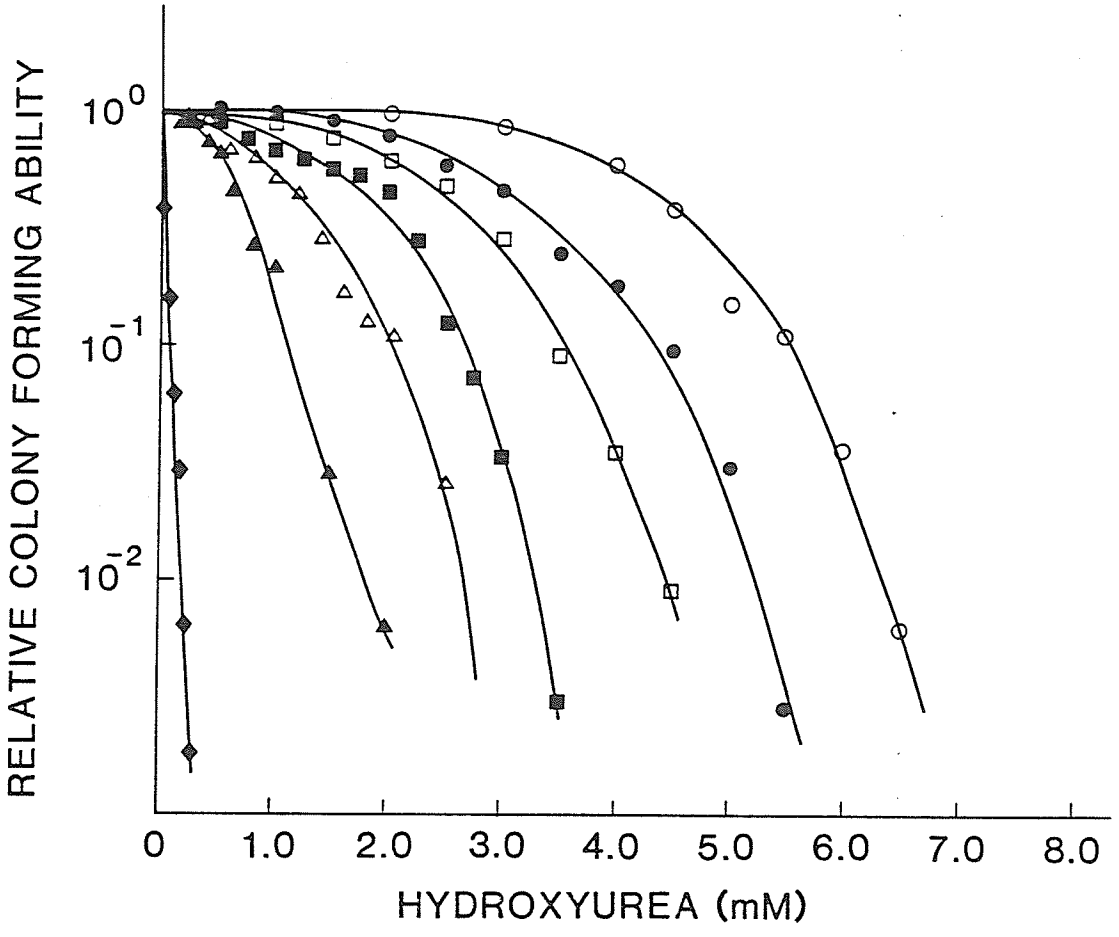

RELATIVE COLONY FORMING ABILITY
10^0
10^{-1}
10^{-2}
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
HYDROXYUREA (mM)

TABLE 1 - CHARACTERISTICS OF WILD TYPE AND DRUG RESISTANT CELL LINES

Cell Line	D_{10} Values[a]	CDP Reductase Activity[b]	Relative M1 DNA Hybridization[c]	Relative M2 DNA Hybridization[c]	Relative M1 RNA Hybridization[d]	Relative M2 RNA Hybridization[d]	Relative M2 Levels[e]
Wild Type	0.15	0.7	1.0	1.0	1.0	1.0	1.0
H^R-0.35	1.2	1.2	1.2	1.5	0.9	11	---
H^R-1.5	2.0	1.95	0.9	2.9	1.3	25	8.6
H^R-2.0	2.7	2.2	0.9	3.0	1.3	21	---
H^R-3.0	3.4	3.2	1.2	3.4	0.8	24	---
H^R-4.0	4.4	4.6	1.0	3.8	1.0	23	---
H^R-5.0	5.5	17	1.3	3.9	1.8	23	52

a) D_{10} value is the concentration of drug that reduces relative colony-forming efficiency to 10% and was determined as described in Materials and Methods.

b) Enzyme activity is expressed as nmoles CDP reduced/hour/mg protein.

c) Determined from densitometric measurements of the most prominent band hybridizing with M1 or M2 cDNA with DNA from drug resistant cells, relative to measurements of the most prominent band hybridizing with DNA from wild type cells.

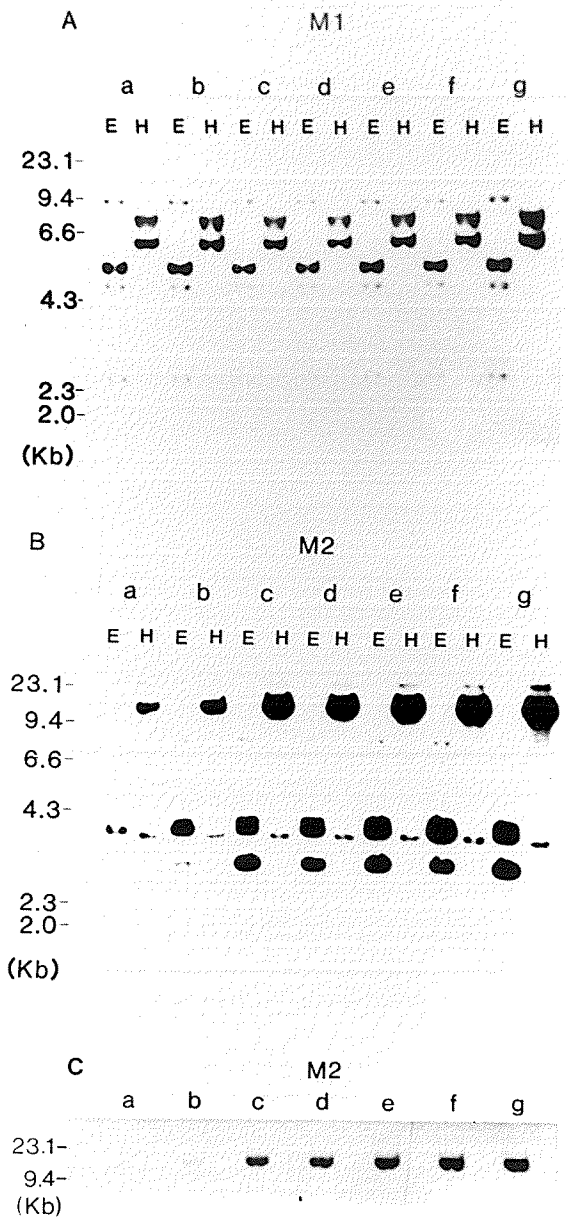
d) Determined from densitometric scanning of dot blot hybridizations carried out with M1 or M2 cDNA and RNA from drug resistant and wild type cells, and expressed relative to the wild type result.

e) Determined from tyrosyl free radical concentrations estimated by EPR signals with drug resistant and wild type cells, and expressed relative to the wild type level.

1.2. Analysis of M1 and M2 DNA from Wild-Type and Hydroxyurea Resistant Cell Lines

To compare the relative number of M1 and M2 gene copies in wild-type and drug-resistant cells, DNA isolated from the various lines was digested to completion with *Eco*RI or *Hind*III endonucleases, and Southern blots of the digested mixtures were hybridized with ^{32}P-labeled M1 or M2 cDNA probes (Fig. 7, A and B). There were no indications of M1 gene amplification in any of the drug-resistant cell lines, since the intensity of the restriction bands was about the same with DNA obtained from wild-type and durg-resistant populations (Fig. 7A). The similarity of the banding patterns also suggested that there were no obvious M1 gene rearrangements in any of the drug-resistant cell lines. In contrast to the above observations, the M2 gene was clearly amplified in all the drug-resistant populations (Fig. 7, B and C). None of the cell lines appeared to contain gross M2 gene rearrangements. As observed in previous studies of dug-resistant cell lines (Wright *et al.*, 1987), only selected bands were amplified (Fig. 7B)., and this may be due to the presence of M2 pseudogenes in mammalian cells (Yang-Feng *et al.*, 1987). Densitometry of the most intense bands of the M2 cDNA-probed autoradiograms gave estimates of 1.5, 2.9, 3.0, 3.4, 3.8, and 3.9 more hybridization with DNA from H^R-0.35, H^R-1.5, H^R-2.0, H^R-3.0, H^R-4.0, and H^R-5.0 cells, respectively, when compared to the wild-type condition (Table 1). It is interesting to note (Fig. 7, B and C) that there was relatively little change (approximately 35%) in hybridization intensity with DNA isolated from

Figure 7. Southern blot analysis of M1 and M2 genes in genomic DNA of the wild type and drug resistant cell lines. Twenty μg of high molecular weight DNA was digested to completion with either Eco RI (E) or Hind III (H) (A and B) or with Hind III only (C). Blots were hybridized with a ^{32}P-labeled Nco I fragment of the M1 cDNA (A) or a ^{32}P-labeled Pst I fragment of the M2 cDNA (B and C), as described in Materials and Methods. Autoradiograms were exposed for 20 hours (A and B) or 4 hours (C) at -70°C with intensifying screens. The lanes are (a) wild type, (b) H^R-0.35, (c) H^R-1.5, (d) H^R-2.0, (e) H^R-3.0, (f) H^R-4.0 and, (g) H^R-5.0.


A
M1
a b c d e f g
E H E H E H E H E H E H E H
23.1-
9.4-
6.6-
4.3-
2.3-
2.0-
(Kb)
B
M2
a b c d e f g
E H E H E H E H E H E H E H
23.1-
9.4-
6.6-
4.3-
2.3-
2.0-
(Kb)
C
M2
a b c d e f g
23.1-
9.4-
(Kb)

cells selected between 1.5 mM and 5.0 mM hydroxyurea (between H^R-1.5 and H^R-5.0 cells). This is in contrast to the significant increases in the levels of cellular resistance and ribonucleotide reductase activities that occur during selection of these drug-resistant lines (Table 1).

1.3. Analysis of M1 and M2 Message Levels in Wild-Type and Mutant Cell Lines

Northern blot analysis using M1 cDNA is presented in Fig. 8A. These results are consistent with other investigations of mammalian cell lines which showed the presence of a single M1 mRNA species of about 3.1 kilobases (Thelander and Berg, 1986; Wright *et al.*, 1987). Although no major changes in M1 mRNA concentrations were detected in most of the drug-resistant cell lines, an increase of about 1.8 fold, as determined by densitometry analysis, was routinely observed when RNA from wild-type and H^R-5.0 cells was compared. Interestingly, M2 cDNA-probed Northern blots, presented in Fig. 8B, showed significant elevations in the M2 mRNAs levels in all the resistant cell lines. A 2.1 kb M2 mRNA species was clearly observed in both wild-type and mutant cell lines; upon longer exposure a 1.6 kb M2 mRNA species also became evident in all the cell lines. Thelander and Berg (1987) and Wright *et al.* (1987) have previously described the presence of two M2 mRNA speceis of 2.1 and 1.6 kb in mouse cell lines, and they have suggested that these species may be generated through the use of different polyadenylation sites at the 3′ untranslated region of the M2 gene transcript.

Figure 8. Northern blots of M1 and M2 mRNA in the wild type and drug resistant cell lines. For Northern blots, 25 μg of total cellular RNA isolated from (a) wild type, (b) H^R-0.35, (c) H^R-1.5, (d) H^R-2.0, (e) H^R-3.0, (f) H^R-4.0 and (g) H^R-5.0, were denatured and run on 1% agarose-formaldehyde gels. After transfer to nitrocellulose, the filters were probed with a [^{32}P]-labelled NcoI fragment of the M1 cDNA (A) or a [^{32}P]-labelled Pst I fragment of the M2 cDNA (B). Equal loading of RNA was determined by reprobing with β-actin cDNA (C) as indicated in Materials and Methods. The positions of 28S and 18S rRNA are indicated. Autoradiograms were exposed for 4 hours (A) or 1 hour (B and C) at -70°C with intensifying screens.

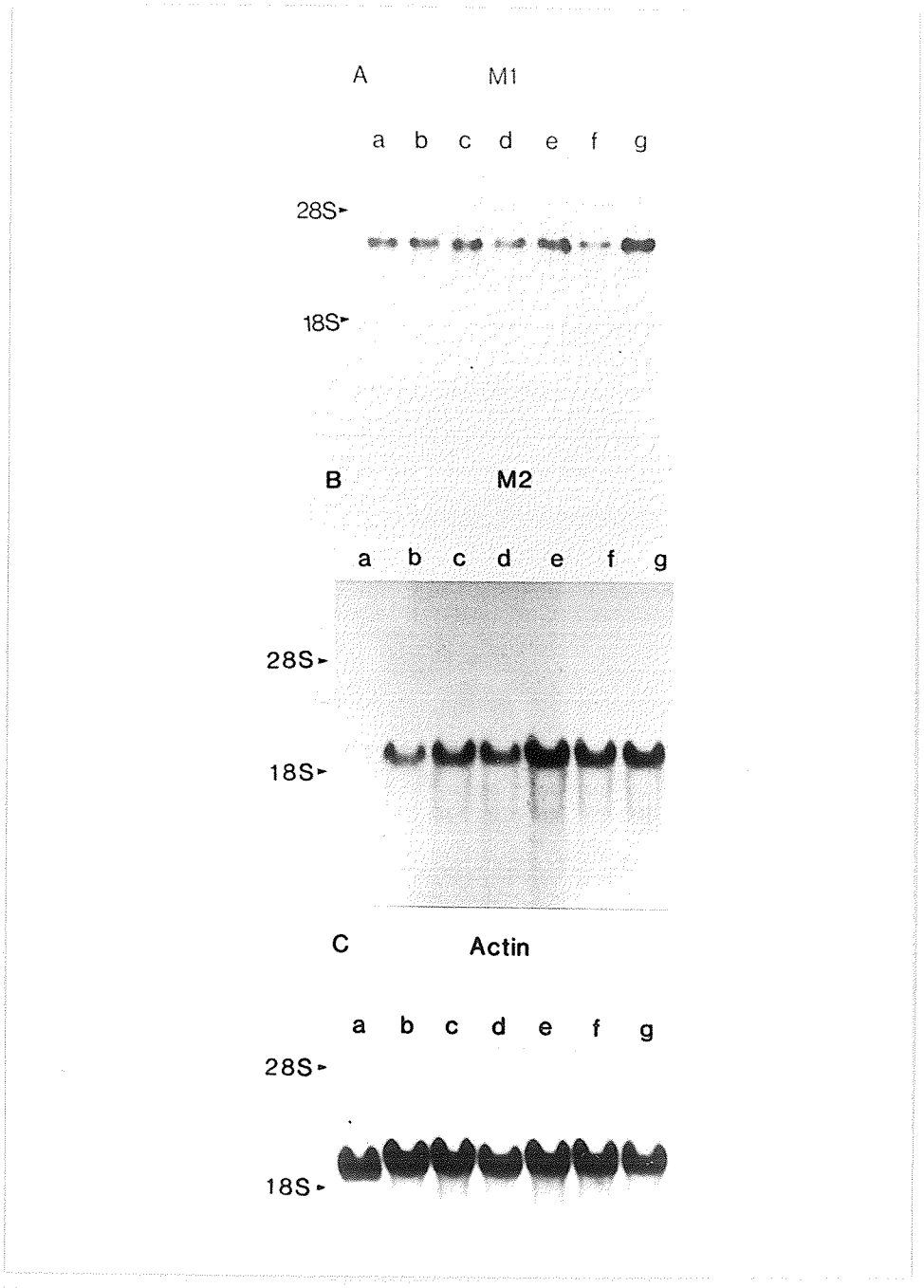
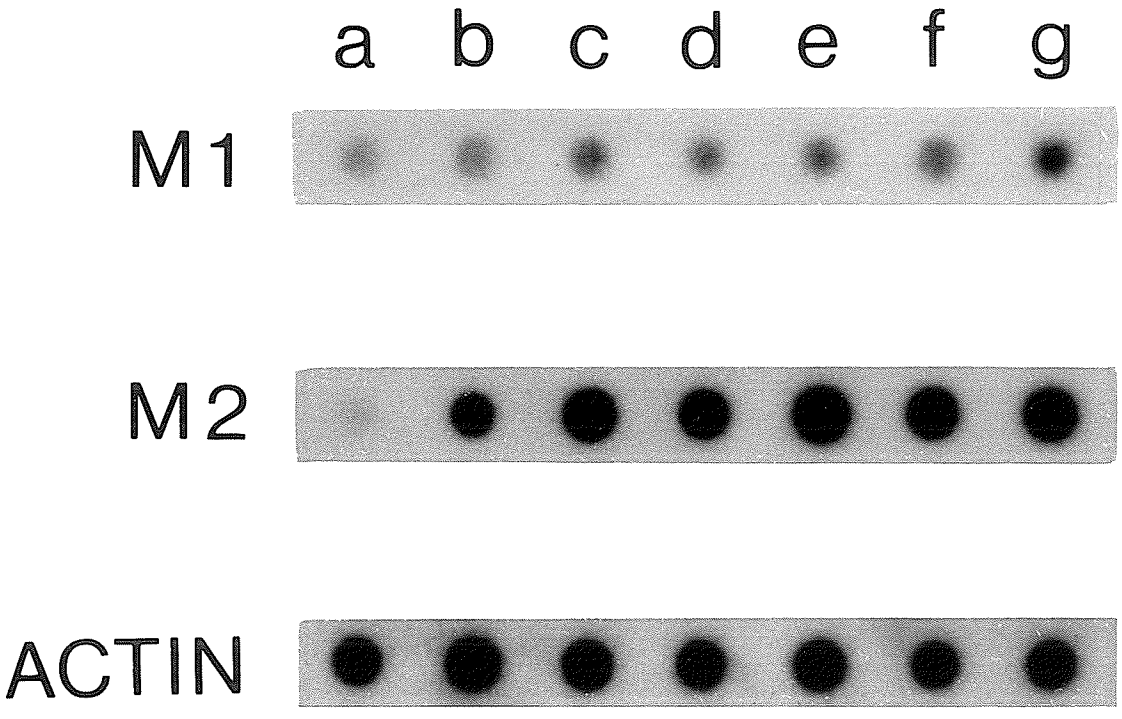

A
M1
a b c d e f g
28S
18S
B
M2
a b c d e f g
28S
18S
C
Actin
a b c d e f g
28S
18S

Figure 9. M1 and M2 mRNA Dot blots. (a) wild type, (b) H^R-0.35, (c) H^R-1.5, (d) H^R-2.0, (e) H^R-3.0, (f) H^R-4.0, (g) H^R-5.0. Two μg of total cellular RNA was loaded per lane and was probed with M1, M2, or β-actin cDNAs.


a b c d e f g
M1
M2
ACTIN

Quantitation of mRNA levels was carried out by densitometry of dot blots (Fig. 9) (McClarty *et al.*, 1987; White and Bancroft, 1982) of total cellular RNA probed with either M1 or M2 cDNA (Table 1). In agreement with Northern blot analysis (Fig. 8A) only the H^R-5.0 cell line showed a significant increase in M1 message of about 2-fold. Also, in agreement with Northern blot studies (Fig. 8B) large elevations in M2 mRNA levels were observed in all drug-resistant lines when compared to the wild-type population. The least resistant, H^R-0.35 line, showed an 11-fold increase in M2 mRNA concentration, and interestingly, the rest of the drug-resistant lines (H^R-1.5, H^R-2.0, H^R-3.0, H^R-4.0, and H^R-5.0) had approximately the same elevation in the M2 message of about 23-fold (Table 1).

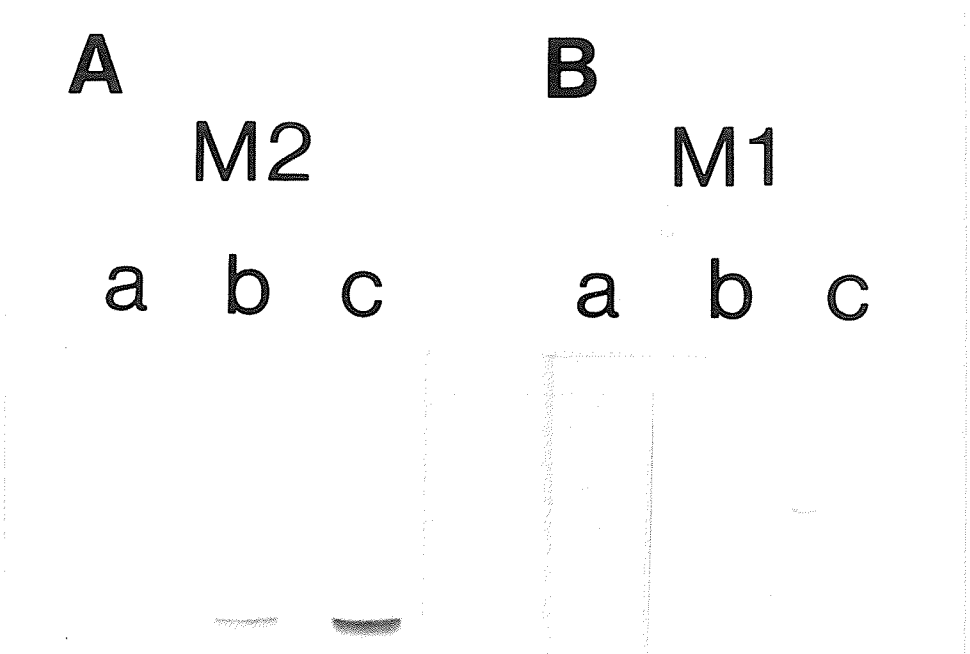
1.4. EPR Spectroscopy and Immunoblot Analysis

The increase in M2 mRNA levels in drug-resistant lines suggested that these cells contained elevations in the M2 component. Since this protein has a tyrosyl free radical necessary for enzyme activity, it is possible to determine expression of the M2 protein in whole cells by measuring the free radical signal characteristic of a functional M2 component (Thelander and Berg, 1986; Wright *et al.*, 1987; McClarty *et al.*, 1987). An EPR analysis was performed on three representative cell lines; the wild-type line; the moderately resistant H^R-1.5 line; and the most resistant line H^R-5.0. The concentration of free radical during exponential growth was 0.14 μM, 1.2 μM, and 7.0 μM for wild-type, H^R-1.5,

and H^R-5.0 cells respectively. Although H^R-1.5 and H^R-5.0 lines contained about the same level of M2 mRNA (Fig. 8B; Table 1), there was a significant difference between these two cell lines in tyrosyl free radical content. Purification of the M2 protein and preparation of specific M2 monoclonal antibodies (Thelander, M. *et al.*, 1985; Engstrom *et al.*, 1984) have provided the ability to directly measure the levels of M2 protein by Western blot analysis. Cell extracts prepared from wild-type , H^R-1.5, and H^R-5.0 cells were subjected to SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose, and M2 protein was detected using anti-M2 monoclonal antibody (Fig. 10A). The results indicated that a M_r 44,000 band corresponding to M2 protein was markedly overproduced in both drug-resistant cell lines. The increases observed in the Western blots correlated with the 8.6-fold and 50-fold elevations in tyrosyl free radical content determined by EPR spectroscopy (Table 1). These results are also consistent with previous observations that there is normally no pool of radical-free M2 protein present in mammalian cells (Eriksson *et al.*, 1984; McClarty *et al.*, 1987a).

The availability of monoclonal antibodies specific for protein M1 (Engstrom *et al.*, 1984) makes it possible to compare the levels of the M1 protein in wild-type, H^R-1.5, and H^R-5.0 cells by Western blot analysis. The results of these experiments are shown in Fig. 10B. The anti-M1 mouse monoclonal antibody detected a high molecular weight band of about 90,000, corresponding to protein M1. The level of M1 protein in the H^R-1.5 cells did not change from that of the wild-type cells, whereas the highly resistant H^R-5.0 showed an approximately 2-fold increase in M1 protein over the wild-type line. This latter observation is consistent with the

Figure 10. Western blot analysis of total protein in wild type, H^R-1.5 and H^R-5.0 cells. The cell-free preparation and the Western blot procedure were carried out as described in Materials and Methods. (A) The immunoblots of protein M2 show (a) 100 μg wild type, (b) 20 μg H^R-1.5, and (c) 10 μg H^R-5.0. (B) The immunoblots of protein M1 show (a) 40 μg wild type, (b) 40 μg H^R-1.5, and (c) 40 μg H^R-5.0.

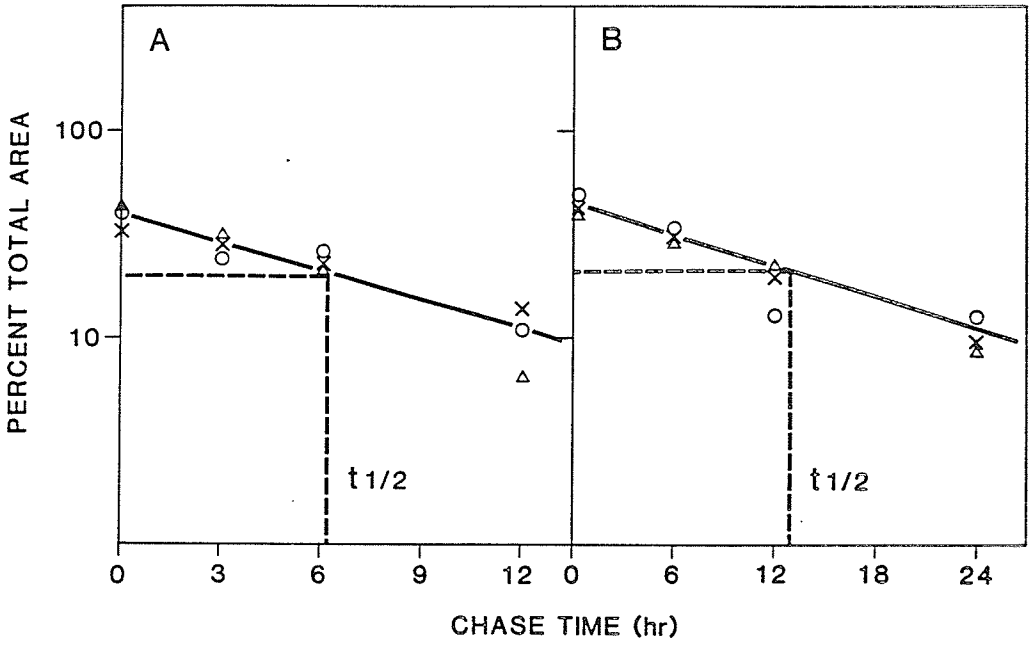

A
M2
a b c
B
M1
a b c

finding of approximately a 1.8-fold elevation of M1 mRNA over the wild type condition, with H^R-5.0 cells (Fig. 8A, Table 1).

1.5. Posttranscriptional Modulation of Protein M2

The difference in protein M2 pools between H^R-1.5 and H^R-5.0 cells, given the similar levels of M2 mRNA, suggests that changes in M2 protein occur by a posttranscriptional mechanism, such as an increase in protein half-life or through an increase in protein M2 biosynthetic rate in the highly resistant line. Protein turnover was measured in [^{35}S]methionine pulse-chase experiments as described in "Materials and Methods." These studies indicated that the M2 protein half-life was 6.3 hours in both H^R-1.5 and H^R-5.0 cells and was unchanged from the value determined for the wild-type M2 protein (Fig. 11A). Also, as expected, no differences in the M1 protein half-life of 12.5 hours were found (Fig. 11B). The rates of M2 biosynthesis in the H^R-1.5 and H^R-5.0 cells were estimated following a 20-minute pulse labeling with [^{35}S]methionine and immunoprecipitation with JB4 anti-M2 monoclonal antibody as described in "Materials and Methods." The results of such an immunoprecipitation are shown in Fig. 12. The M2 protein band with an apparent molecular weight of 44,000 is clearly visible. Densitometric quantitation of the bands indicated that there was approximately a 2-fold increase in the metabolically labeled M2 immunopreicipitate in the H^R-5.0 cell line compared to the H^R-1.5 line. This increase in protein M2 biosynthetic rate would contribute to the

Figure 11. Pulse-chase analysis of protein M1 (A) and protein M2 (B) half-lives in wild type (×), H^R-1.5 (Δ), and H^R-5.0 (○). The $t_{1/2}$ (half-life) is determined from the time of chase when half the initial labeled M1 protein or M2 protein is remaining. Following immunoprecipitation with M1 or M2 monoclonal antibodies and SDS-gel electrophoresis, the labeled proteins were detected by autoradiography and quantitated by densitometric analysis as described in Materials and Methods. The y-axis scale is logarithmic.


A
B
PERCENT TOTAL AREA
100
10
t1/2
t1/2
0
3
6
9
12
0
6
12
18
24
CHASE TIME (hr)

elevation in cellular M2 content observed in the H^R-5.0 cell line (Fig. 10).

1.6. Distribution of M2 mRNA in the Cytoplasm of H^R-1.5 and H^R-5.0 Cells

The analysis of total mRNA in H^R-1.5 and H^R-5.0 cell lines presented an interesting problem as both cell lines contained almost equal amounts of M2 mRNA yet had disproportionate amounts of M2 protein. Protein M2 half-life in H^R-1.5 and H^R-5.0 cells were not changed (Fig. 11), but the biosynthetic rate of protein M2 was elevated by 2 fold in the H^R-5.0 line compared to the H^R-1.5 line (Fig. 12). It is possible that the H^R-5.0 cells may be more efficient in translating the M2 mRNA than H^R-1.5 cells. The other possibility is that in both cell lines the mRNA are translated with equal efficiency but only certain pools of M2 mRNA is translated and a larger pool of translatable message is available in the H^R-5.0 line. To test this idea, the distribution of M2 mRNA was analysed with respect to actively translated message, *i.e.* mRNA-ribosome complex (polysomal RNA) and inactive mRNA, *i.e.* mRNA sequestered in a ribonucleoprotein complex (RNP).

Polysomal, monosomal and ribosomal subunit RNA were separated from free mRNP (non-polysomal RNA) on sucrose gradients as described in Materials and Methods. The actively translated mRNA is complexed with more ribosomes and thus has a higher density. Fractions collected from the gradient were run on a denaturing 1% formaldehyde gel and stained with

Figure 12. Immunoprecipitation of protein M2 in H^R-1.5 and H^R-5.0 cells. Immunoprecipitation of ^{35}S-methionine pulse labeled cellular proteins with M2 specific monoclonal antibody of (a) H^R-1.5 and (b) H^R-5.0. Immunoprecipitation (3×10^6cpm) was performed as described in Materials and Methods.

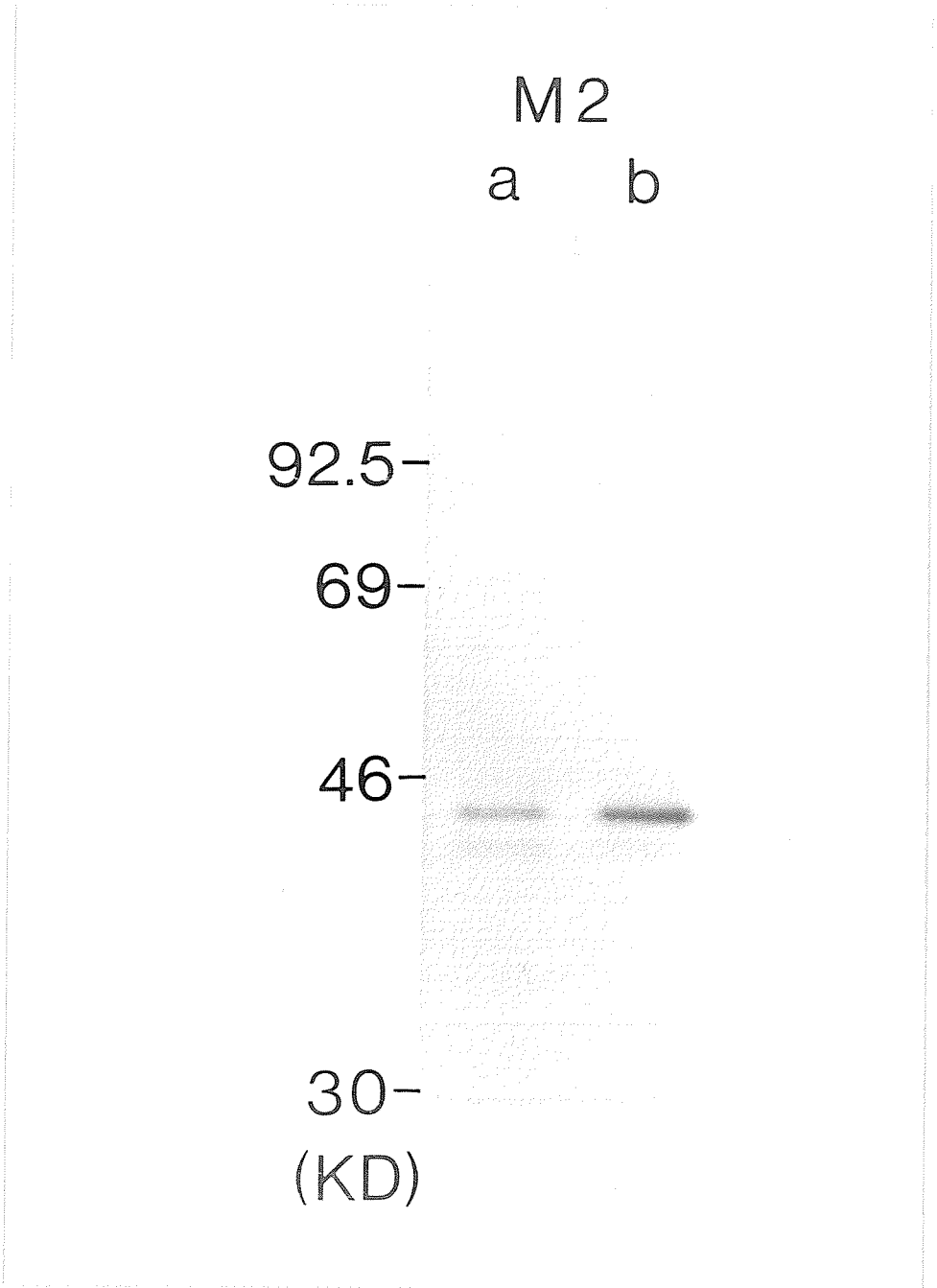
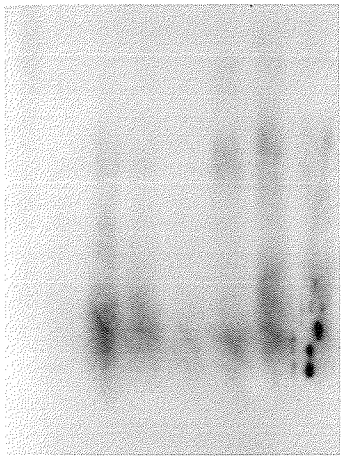

M2
a
b
92.5-
69-
46-
30-
(KD)

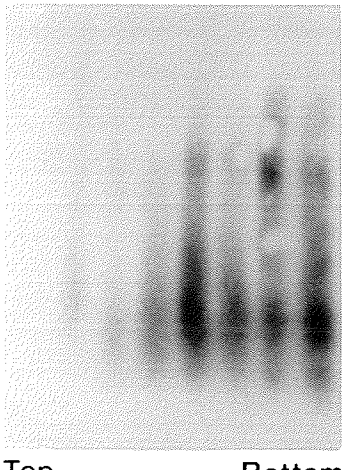
Figure 13. Cytoplasmic distribution of M2 mRNA in H^R-1.5 and H^R-5.0. Logarithmically growing cells were harvested and cytoplasmic polysomal mRNA were prepared, run on a 1% formaldehyde denaturing gel, blotted, and probed with M2 cDNA as described in Materials and Methods. Fractions from the top and bottom of the sucrose gradient is indicated in the figure. Actively translated mRNA pools distribute near the bottom of the density gradient. The panels show cytoplasmic mRNA from (A) H^R-1.5 and (B) H^R-5.0 cells.

A. $H^R1.5$



Top Bottom

B. $H^R5.0$



Top Bottom

ethidium bromide to identify the distribution of the ribosomes and their subunits.

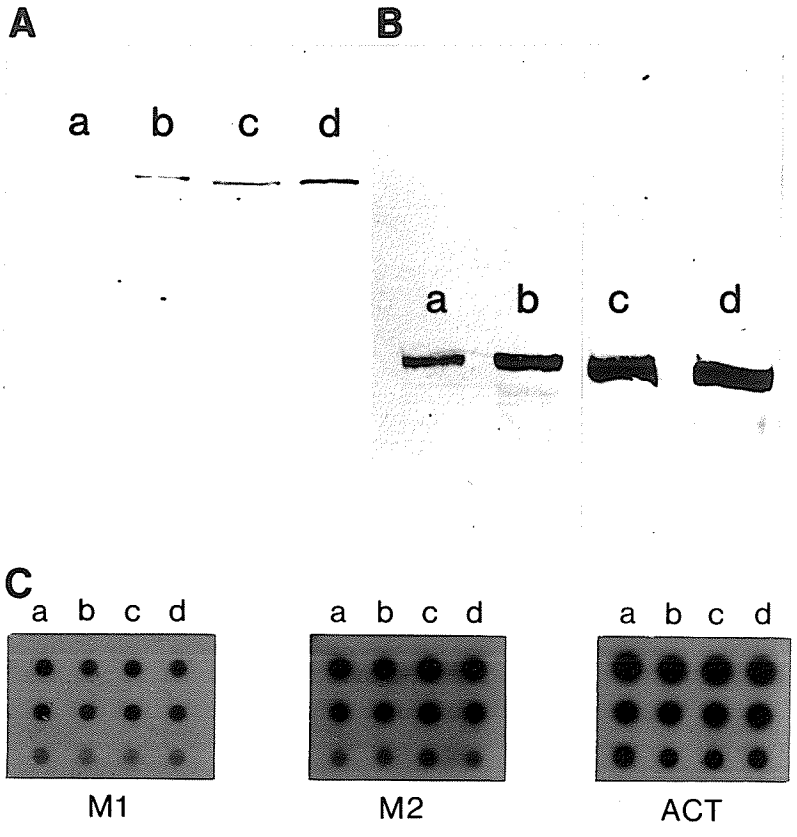
Northern blot analysis was performed to determine the distribution of M2 mRNA along the gradient (Fig. 13). The RNA samples shows some degradation due to the elongated polysomal RNA isolation procedure, nonetheless, a difference between the two cell lines was observed when probed for M2. For the H^R-5.0 cells , the M2 mRNA appeared to be more concentrated towards the bottom of the gradient signifying that the majority of the M2 message is in the denser fractions. The H^R-1.5 cells had a more even distribution of the M2 mRNA among the fractions.

2. Effects of Hydroxyurea on Resistant Cells

2.1. Effects of Hydroxyurea Concentration on M1 and M2 Protein and mRNA Levels

A previous study of the cell line SC2, a subclone of the H^R-5.0 population, reported that the ribonucleotide reductase activity increased in a drug concentration dependent manner when the cells were cultured in the presence of hydroxyurea (McClarty *et al.*, 1986a). The increase in enzyme activity could be the result of increased protein levels of the M1, M2, or both subunits. In order to examine this possibility, SC2 cells previously cultured in the absence of drug were grown in the presence of various concentrations of hydroxyurea for three days, harvested and the M1 and M2 protein levels were analyzed by Western blots (Fig. 14). In

Figure 14. Effect of hydroxyurea on cellular M1 and M2 protein and mRNA levels. Cell extract preparations, Western blot procedure, and cytoplasmic dot hybridization were carried out as described under Materials and Methods. (A) Western blot analysis for protein M1; 25 μg of cell extract protein was loaded in each lane. (B) Western blot analysis for protein M2; 10 μg of cell extract protein was loaded in each lane. Individual lanes represent cell extract protein prepared from SC2 cells grown in the (a) absence and presence of (b) 1, (c) 5, and (d) 10 mM hydroxyurea for 3 days. (C) Cytoplasmic dot hybridization for M1, M2, and β-actin mRNAs in SC2 cells grown in the (a) absence and presence of (b) 1, (c) 5, and (d) 10 mM hydroxyurea for 3 days. Cytoplasmic extract was prepared from SC2 cells and treated with 7.4% formaldehyde as previously described (White and Bancroft, 1982). Treated extract was then spotted in serial 2-fold dilutions onto nitrocellulose followed by hybridization with either an M1 cDNA fragment, and M2 cDNA fragment, or a β-actin cDNA probe to quantitate the relative amounts of M1 and M2 mRNAs in each preparation. The autoradiographs were exposed for 24 hours for the M1 blot and 12 hours for both M2 and β-actin at -70°C with intensifying screens. Several very faint low molecular weight bands in B are probably due to some M2 protein degradation.


A
B
a b c d
a b c d
C
a b c d
a b c d
a b c d
M1
M2
ACT

cell culture, typically hydroxyurea has been used as a S-phase blocking cell synchronizing agent (Young *et al.*, 1967; Ashihara and Baserga, 1979). The SC2 cells only have slight variations in cell cycle distribution as shown by flow cytometry when the cells are grown in the presence of 5 mM hydroxyurea (McClarty *et al.*, 1987), and even when cultured in the presence of 10 mM hydroxyurea, there is only a 6% increase in the number of cells in S phase (McClarty *et al.*, 1988). Therefore, the drug induced increase in ribonucleotide reductase activity is not due to a significant increase in the porportion of the cell population in S phase. The induction of protein levels of M1 and M2 are shown in Fig. 14A and 14B respectively for SC2 cells grown in 0 mM, 1 mM, 5 mM, and 10 mM hydroxyurea. The results clearly show increases to M1 and M2 protein levels in a drug concentration dependent manner.

The SC2 cells grown in the presence of drug as described above were analyzed quantitatively for mRNA levels by cytoplasmic RNA dot blots (Fig. 14C). The blots were probed with M1- and M2-specific cDNA probes. The results were in agreement with previous findings (McClarty *et al.*, 1987a) that the levels of M1 and M2 transcripts were essentially unchanged when the cell line was cultured in the presence hydroxyurea.

2.2. Kinetics of Protein M1 and M2 Induction

The kinetics of protein M1 and M2 induction by hydroxyurea was examined with respect to the mechanism responsible for the elevation in ribonucleotide reductase activity. SC2 cells cultured for several days in α-MEM supplemented with FBS, without hydroxyurea were returned to medium with 5 mM hydroxyurea, and a various time points, cells were harvested, crude protein extracts prepared and protein M1 and M2 levels analyzed by immunoblots. Induction of the M1 subunit occured very slowly with no significant increase in protein levels until about 6 hours (Fig. 15A, *cf.* lanes a and d). In contrast, the M2 subunit appears to respond much more rapidly to hydroxyurea treatment with noticable increases in protein as early as 1 to 3 hours after the addition of drug (Fig. 15B, *cf.* lanes a, b, and c). Even after 24 hours of culture with 5 mM hydroxyurea, the induction of M1 and M2 in the cells were still not complete. SC2 cells cultured continuously (more than two weeks) in the the presence of hydroxyurea have accumulated significantly higher levels of M1 and M2 protein (Fig. 15A, 15B, *cf.* lanes e and f).

2.3. Effect of Hydroxyurea on the Degradation Rates of Proteins M1 and M2

The increases in protein M1 and M2 levels in SC2 cells grown in the presence of hydroxyurea, in the absence of elevations in their corresponding mRNA levels, suggest that the protein increases occurred by

Figure 15. Kinetics of protein M1 and M2 induction. Cell extract preparation and Western blot procedure were carried out as described in Materials and Methods. Western blot analysis for (A) protein M1 and (B) protein M2 in SC2 cells cultured in the (a) absence and presence of 5 mM hydroxyurea for (b) 1 hour, (c) 3 hours, (d) 6 hours, (e) 24 hours, and (f) continuously, (> 2 weeks). For protein M1, 25 μg of cell extract protein was loaded in each lane, and 10 μg of cell extract protein was loaded in each lane for protein M2.

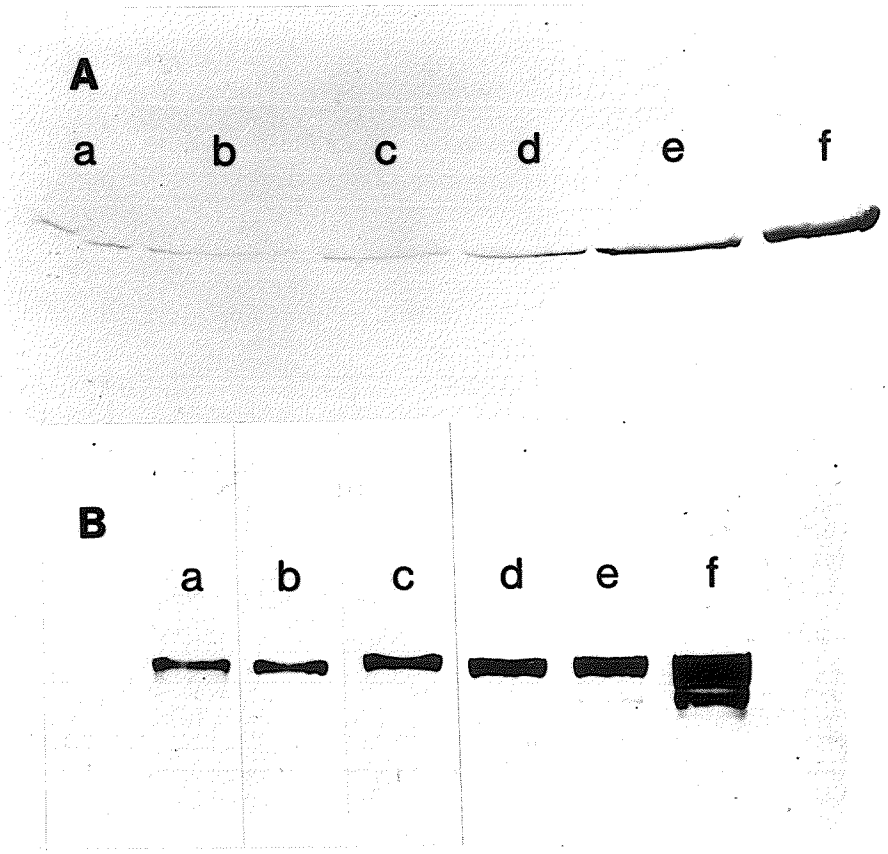
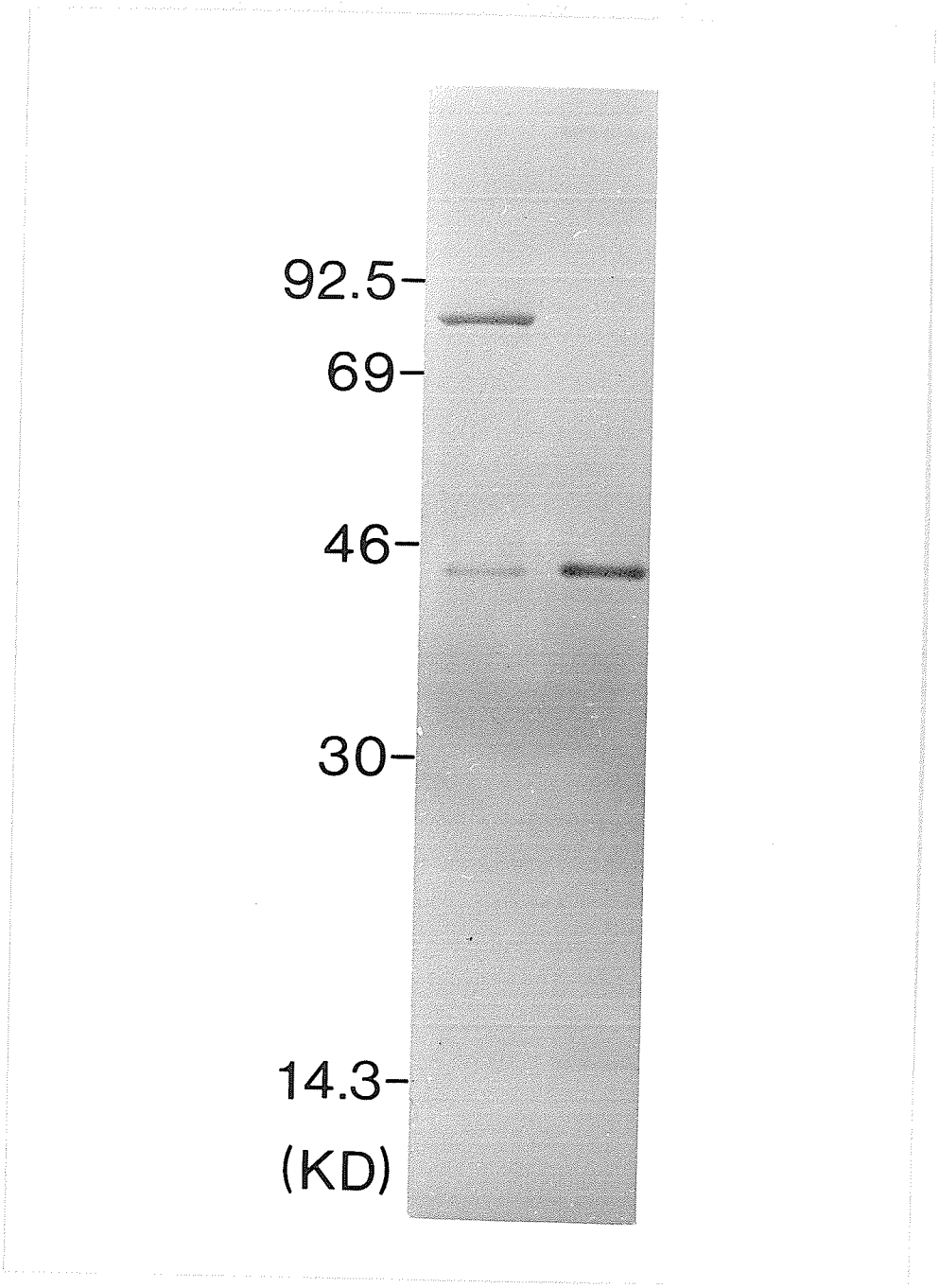

A
a
b
c
d
e
f
B
a
b
c
d
e
f

Figure 16. M1 and M2 immunoprecipitation in SC2 cells. Immunoprecipitation of protein M1 and M2 from metabolically labeled cell extracts was carried out as described under Materials and Methods, and in Firestone *et al.* (1982). The specificity of the M1 immunoprecipitation is shown on the left and the M2 immunoprecipitation is shown on the right. The band at 44,000 daltons in the M1 immunoprecipitation appears to be protein M2 that coprecipitates with protein M1 as a holoenzyme.


92.5
69
46
30
14.3
(KD)

a posttranscriptional mechanism. It seemed possible that an alteration in the stability of M1 and M2 protein could account for the hydroxyurea-induced increases observed for both subunits. In order to examine this possibility, the decay rate of [^{35}S]methionine labeled M1 and M2 protein was compared in SC2 cells grown in the presence ($SC2^+$) and absence ($SC2^-$) of hydroxyurea. $SC2^-$ and SC^+ cells were labeled for 16 h with [^{35}S]methionine, followed by culturing in chase medium free of label; then at various intervals, cells were harvested, and immunoprecipitation was carried out using monoclonal antibodies specific for protein M1 or M2. The specificity of the immunoprecipitation of metabolically labeled M1 (M_r 85,000) and M2 (M_r 44,000) is shown in Fig. 16. Following SDS gel electrophoresis and autoradiography, quantitative determinations were carried out by spectrometric densitometry. The results of this pulse-chase experiment indicated a half-life of 15.25 h for protein M1 and of 6.75 for protein M2 in SC2 cells grown in the absence of hydroxyurea. These protein half life values obtained are similar to values obtained in earlier experiments with LHF cells (Fig. 11; Choy *et al.*, 1988). When SC2 cells were cultured in the presence of hydroxyurea, the half-lives of both proteins M1 and M2 increased by approximately 2-fold to 25.75 and 12.25 h, respectively (Fig. 17A,B).

2.4. Rates of Protein M1 and M2 Biosynthesis

Even though M1 and M2 mRNA levels are unchanged in SC2 cells cultured in the presence of hydroxyurea, altered rates of M1 and M2 polypeptide biosynthesis may also contribute to the regulation of M1 and

Figure 17. Half-life determinations for proteins M1 and M2 in SC2 cells. Pulse-chase analysis of (A) protein M1 and (B) protein M2 half-life; (●) SC2 cells grown in the absence of hydroxyurea and (○) SC2 cells grown in the presence of 5 mM hydroxyurea > 2 weeks. The $t_{1/2}$ is determined from the time of chase when half the initial labeled M1 or M2 protein is remaining. Following immunoprecipitations with M1 or M2 monoclonal anitbodies and SDS gel electrophoresis, the labeled proteins were detected by autoradiography and quantitated by densitometric analysis as described in Materials and Methods. The *y*-axis scale is logarithmic.

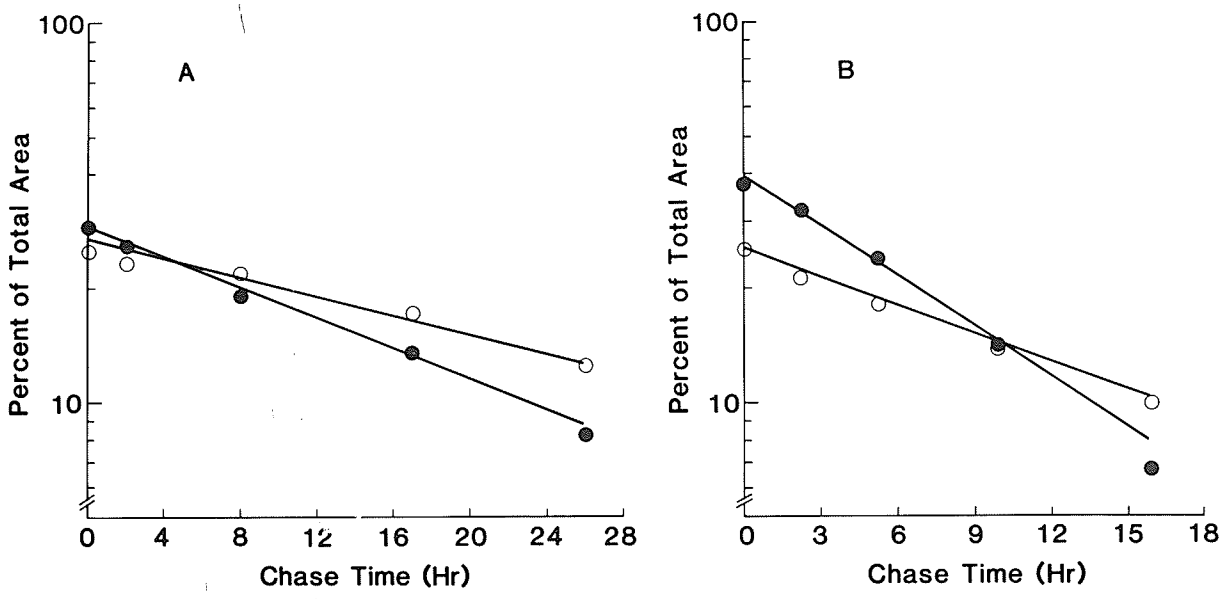

100
10
A
Percent of Total Area
0
4
8
12
16
20
24
28
Chase Time (Hr)
100
10
B
Percent of Total Area
0
3
6
9
12
15
18
Chase Time (Hr)

Figure 18. Effect of hydroxyurea and iron on the rate of M1 and M2 biosynthesis. SC2 cells were cultured in the absence or presence of 5 mM hydroxyurea and/or 150 μM ferric ammonium citrate prior to pulse-labeling with [^{35}S]methionine for 30 minutes. Following labeling, cells were washed and lysed, and then the supernatant was immunprecipitated with anti-M1 or anti-M2 monoclonal antibody and subjected to SDS-PAGE and autoradiography as described in Materials and Methods. (A) Immunoprecipitation for protein M1 and (B) immunoprecipitation for protein M2 from (a) SC2 cells + no treatment, (b) SC2 cells + 150 μM ferric ammonium citrate for 3 hours, (c) SC2 cells + 5 mM hydroxyurea for > 2 weeks, and (d) SC2 cells + 150 μM ferric ammonium citrate for 3 hours + 5 mM hydroxyurea for > 2 weeks.

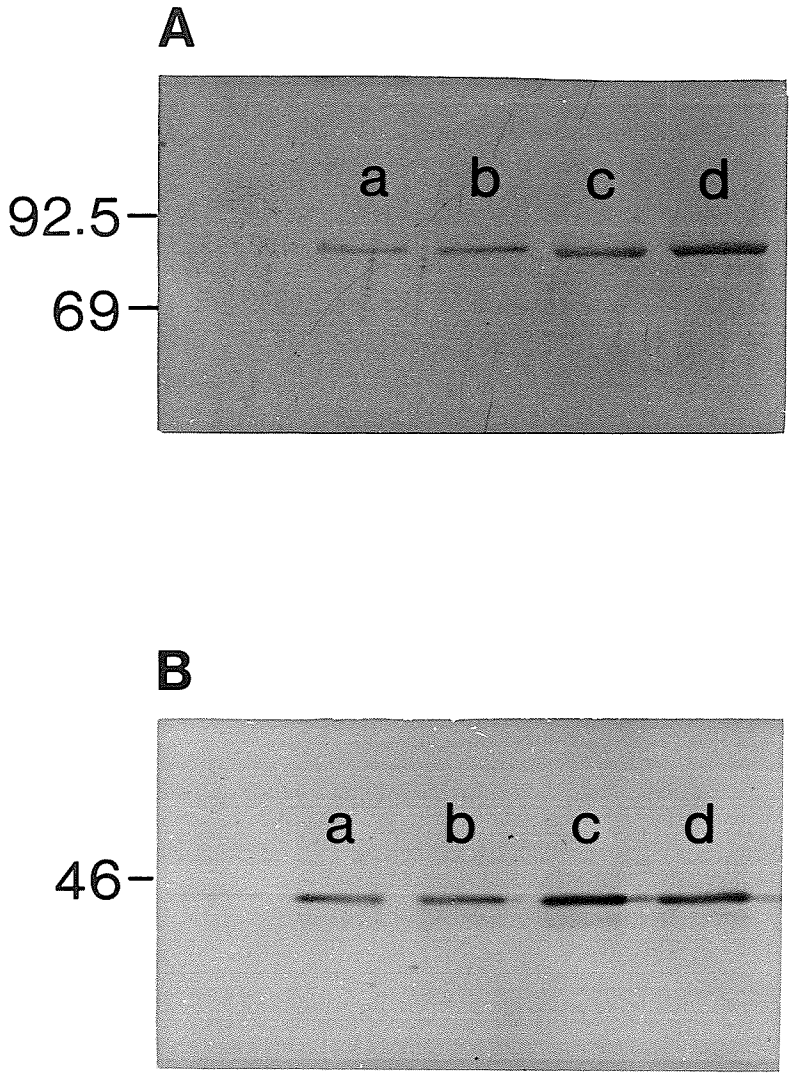

A
92.5
69
a
b
c
d
B
46
a
b
c
d

Table 2

Effect of Hydroxyurea and Iron on the Rates of Biosynthesis of Proteins M1 and M2[a]

Hydroxyurea Treatment[b]	Ferric Ammonium Citrate Treatment[c]			
	Protein M1		Protein M2	
	-	+	-	+
Not Treated	1.00	1.11 ± 0.14	1.00	0.97 ± 0.08
Treated	3.30 ± 0.39	4.26 ± 0.55	1.92 ± 0.15	1.26 ± 0.13

[a] The average (± S.E.) of four densitometric quanitations of labeled M1 and M2 immunoprecipitates under the different experimental conditions. For easy comparison, the values have been normalized such that the level of [^{35}S]methionine incorporated into protein M1 or M2 in SC2 cells grown in the absence of hydroxyurea and iron has been arbitrarily set at 1.

[b] 5 mM for > 2 weeks.

[c] 150 μM for 3 hours.

The incorporation of [^{35}S]methionine for SC^- and $SC2^+$ were 6.4×10^4 cpm/μg of protein and 6.1×10^4 cpm/μg of protein, respectively.

M2 protein levels by hydroxyurea. To compare the rates of biosynthesis of M1 and M2 in SC2 cells grown in presence and absence of hydroxyurea, cells were pulse-labeled with [^{35}S]methionine for 30 minutes, and then cell lysates were prepared and immunoprecipitated with M1 or M2 monoclonal antibodies followed by SDS-PAGE and autoradiography. The results are shown in Fig. 18. The rates of biosynthesis of both protein M1 (Fig. 18A, *cf* lanes a and c) and protein M2 (Fig. 18B, *cf* lanes a and c) have increased in the SC2 cell line grown in the presence of hydroxyurea when compared to the same cells grown in the absence of drug. The result shown is representative of four such experiments. The average of the four densitometric quantitations of the labeled M1 and M2 immunoprecipitates under the different experimental conditions is presented in Table 2. For easy comparison, the results have been normalized such that the level of [^{35}S]methionine incorporated into protein M1 or M2 in SC2 cells grown in the absence of hydroxyurea has been arbitrarily set at 1. It is important to note that little or no effect on total protein synthesis was observed after treatment with hydroxyurea. $SC2^-$ cells incorporated 6.4×10^4 cpm/μg of protein whereas $SC2^+$ cells incorporated 6.1×10^4 cpm/μg of protein during the 30 minute [^{35}S]methionine labeling period.

In order to determine how rapidly the increase in the rate of M1 and M2 biosynthesis occurs, in response to hydroxyurea, a kinetic study was conducted. $SC2^-$ cells were cultured in the presence of hydroxyurea for various lengths of time prior to pulse labeling with [^{35}S]methionine for 30 minutes. Immediately following the pulse-label, cells were harvested, cell lysates immunoprecipitated, run on SDS PAGE and autoradiographed. Densitometric quantitation of the various time points

Table 3

Kinetics of the Hydroxyurea Effect on the Rates of Biosyntheis of Proteins M1 and M2

Time After Hydroxyurea Addition (hours)	Protein M1	Protein M2
	1.00	1.00
1	0.99 ± 0.04	0.94 ± 0.02
3	1.02 ± 0.04	1.04 ± 0.01
6	1.06 ± 0.02	0.99 ± 0.06
24	1.90 ± 0.06	1.27 ± 0.05
48	2.43 ± 0.10	1.73 ± 0.03
> 2 weeks	3.02 ± 0.08	1.93 ± 0.04

The average (± S.E.) of two densitometric quanitations of labeled M1 and M2 immunoprecipitates at various time points following addition of hydroxyurea to the growth medium. For easy comparison, the values have been normalized such that the level of [^{35}S]methionine incorporated into protein M1 or M2 in SC2 cells grown in the absence of hydroxyurea has been arbitrarily set at 1.

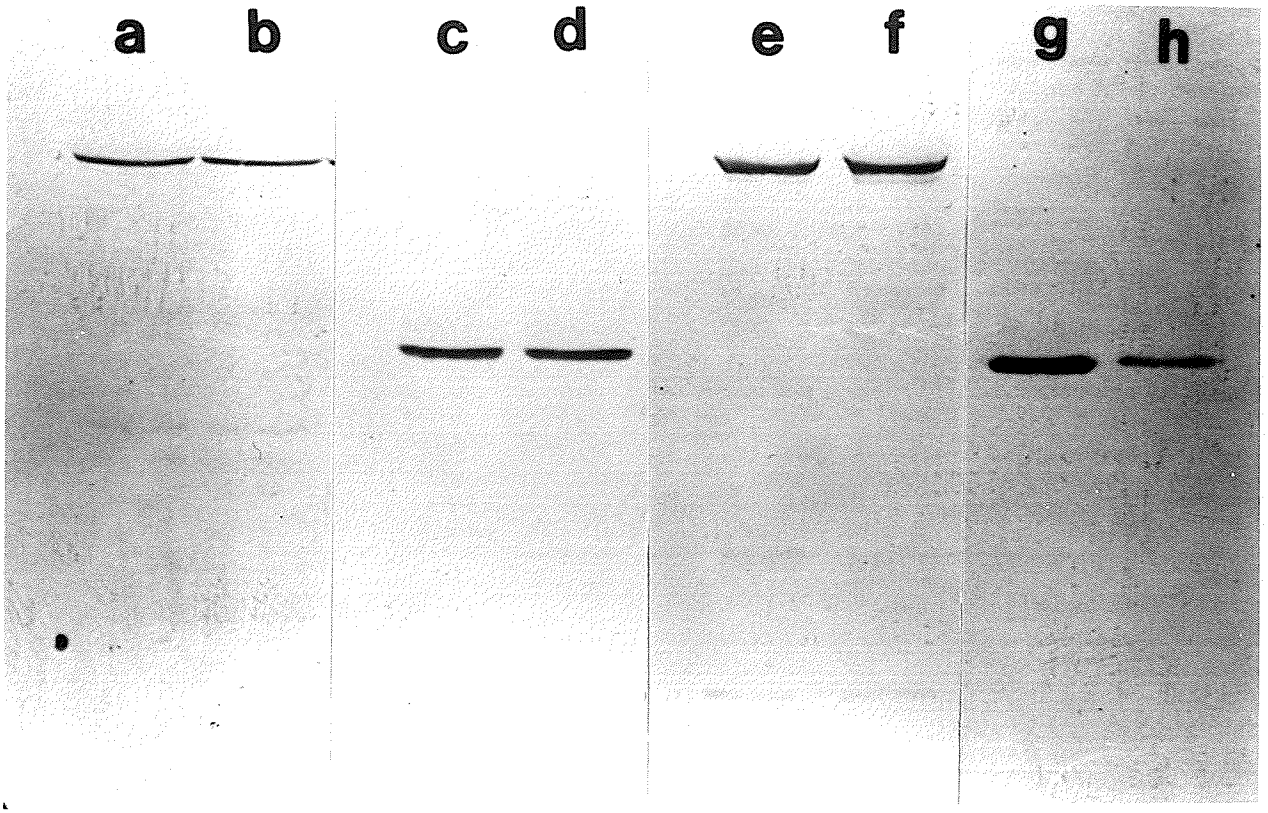
The incorporation of [^{35}S]methionine for SC^- and $SC2^+$ were 6.4×10^4 cpm/μg of protein and 6.1×10^4 cpm/μg of protein, respectively.

was then carried out. The average peak area of two such determinations for each time point is shown in Table 3. Once again, the values have been normalized such that the level of [^{35}S]methionine incorporated into protein M1 or M2 in SC2 cells grown in the presence of hydroxyurea has been set at 1. The rate of biosynthesis of both proteins M1 and M2 responds slowly to the presence of hydroxyurea with neither subunit showing any significant increase in synthesis rate until 24 hours after drug addition. Thereafter, the rates of M1 and M2 biosynthesis steadily increased until they reached the level occurring in SC2 cells growing continuously (> 2 weeks) in the presence of hydroxyurea.

2.5. Effect of Iron on Protein M1 and M2 Levels and Rates of Biosynthesis

Ferritin is the major iron storage protein in eukaryotic cells (Theil, 1987). Biosynthetic rates of ferritin and cellular ferritin levels are regulated by changes in iron availability, and this regulation occurs mainly through posttranscriptional mechanisms (Aziz and Munro, 1986; Mattia *et al.*, 1986; Theil, 1987). Protein M2 contains stoichiometric amounts of non-heme iron which is essential for enzyme activity (Thelander *et al.*, 1985). It has been shown that hydroxyurea inhibits ribonucleotide reductase by destroying the tyrosyl free radical of protein M2 (Graslund *et al.*, 1982); however, it is also known that hydroxyurea has iron chelating properties (Young *et al.*, 1967). It seemed possible that the effect hydroxyurea has on ribonucleotide reductase levels and biosynthetic rates could be a result of changes in iron

Figure 19. Effect of hydroxyurea and iron on total cellular M1 and M2 protein levels. Cell extract preparation and Western blot procedure were carried out as described in Materials and Methods. SC2 cells were grown in the absence or presence of 5 μM hydroxyurea and/or 30 μM ferric ammonium citrate for the indicated period of time prior to cell extract preparation. Western blot analysis for protein M1 (a,b,e, and f) and protein M2 (c,d,g, and h). (a) SC2 cell extract, no treatment, 40 μg of protein; (b) SC2 cell extract, 30 μM ferric ammonium citrate for 3 days, 40 μg of protein; (c) SC2 cell extract, no treatment, 15 μg protein; (d) SC2 cell extract, 30 μM ferric ammonium citrate for 3 days, 15 μg of protein; (e) SC2 cell extract, 5 mM hydroxyurea for > 2 weeks, 25 μg protein; (f) SC2 cell extract, 5 mM hydroxyurea for > 2 weeks + 30 μM ferric ammonium citrate for 3 days, 25 μg of protein; (g) SC2 cell extract, 5 mM hydroxyurea for > 2 weeks, 10 μg protein; (h) SC2 cell extract, 5 mM hydroxyurea for > 2 weeks + 30 μM ferric ammonium citrate for 3 days, 10 μg of protein;


a
b
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h

availability when cells are cultured in the presence of the drug. In order to test this hypothesis, the effect of iron, on the M1 and M2 protein levels and rates of biosynthesis in SC2 cells grown in the presence or absence of hydroxyurea, was determined.

$SC2^-$ and $SC2^+$ cells were cultured in the presence of various concentrations of ferric ammonium citrate for 3 days; then total cellular levels of proteins M1 and M2 were determined by Western blot analysis. In Fig. 19, at a concentration of 30 μM ferric ammonium citrate, iron had no obvious effect on M1 levels in SC2 cells whether they were cultured in the absence of hydroxyurea (Fig. 19, *cf* lanes c and d); however, the presence of iron caused a dramatic decrease in protein M2 levels in SC2 cells cutured in the presence of hydroxyurea (Fig. 19, *cf* lanes g and h).

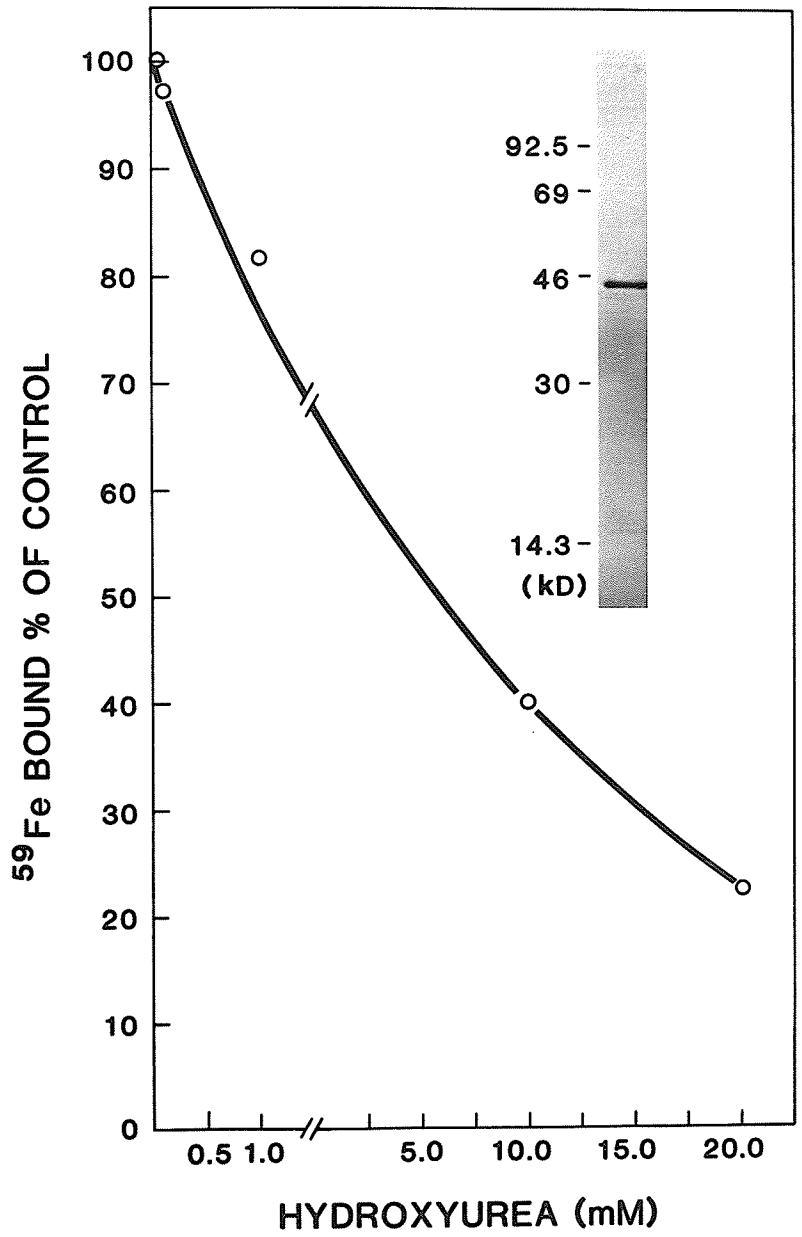
The effect of iron on the rate of protein M1 and M2 biosynthesis in $SC2^-$ and $SC2^+$ cells is shown in Fig. 18. For these experiments, $SC2^-$ and $SC2^+$ cells were cultured in the presence of 150 μM ferric ammonium citrate for 3 hours, and then the cells were pulse labeled for 30 minutes with [^{35}S]methionine. Immediately following labeling, the cells were harvested and cellular extracts were immunoprecipitated with M1 or M2 monoclonal antibody followed by SDS PAGE and autoradiography. Iron had no significant affect on the rate of synthesis of either protein M1 or M2 in SC2 cells grown in the absence of hydroxyurea (Fig. 18A, *cf* lanes a and b; and Fig. 18B, *cf* lanes a and b). In contrast, when iron was added to SC2 cells growing in the presence of hydroxyurea, the rate of biosynthesis of both M1 and M2 was affected. Interestingly, the rate of M2 synthesis appeared to increase slightly whereas the rate of M2 biosynthesis showed a small but consistent decrease (Fig. 18A, *cf* lanes c and d; and Fig. 18B,

cf lanes c and d). The average of densitometric quantitations from four such experiments is presented in Table 2.

2.6. Inactivation of Protein M2 with Hydroxyurea Leads to ApoM2 Formation

Treatment of the B2 subunit from *E. coli* or the M2 subunit from mammalian sources with hydroxyurea or certain other free radical scavengers either *in vivo* or *in vitro* leads to the reduction of the tyrosyl free radical and subsequent loss of enzymatic activity (McClarty *et al.*, 1987; Reichard, 1988). In the case of B2, treatment with hydroxyurea gives rise to a stable structure in which the tyrosyl radical is destroyed but the iron center is retained (Barlow *et al.*, 1983). To determine if the iron center of inactive protein M2 is stable, like its *E. coli* counterpart, mouse L cells were prelabeled with ^{59}Fe to tag the iron center. The mouse cells (H^R-5.0) used in these experiments exhibited a high resistance to hydroxyurea as a result of a >50-fold overproduction of protein M2 (Table 1, Fig. 10), and were therefore useful as a source of M2 for *in vivo* labeling experiments. These cells were cultured in medium containing ^{59}Fe labeled transferrin for 18 hours prior to harvesting the cell extract preparation. This ^{59}Fe labeled cell extract was divided into five equal portions, and then hydroxyurea was added to achieve a variety of final concentrations ranging from 0 to 20 mM. Following incubation on ice for 10 minutes, the cell extracts were subjected to immunoprecipitation using a monoclonal antibody specific for protein M2. The specificity of the immunoprecipitation reaction for metabolically

Figure 20. Effect of Hydroxyurea on the Iron Centre of Protein M2. Whole cell extracts were prepared from ^{59}Fe-labeled hydroxyurea resistant mouse L cells, H^R-5.0, as previously described in Materials and Methods. The cell extract was then incubated at 0°C for 10 minutes in the presence of various concentrations of hydroxyurea. Protein M2 was immunoprecipitated from the treated extracts using a M2 specific monoclonal antibody (Engstrom and Rozell, 1988) as described under "Experimental Procedures". Following extensive washing, the ^{59}Fe-labelled immunoprecipitates were quantitated by gamma counting. Inset: An entire gel lane of a similar immunoprecipitation of [^{35}S] methionine labelled cell extract is shown to indicate the specificity of the monoclonal antibody for protein M2 (Mr 44,000). 100% control is equal to the quantity of ^{59}Fe-labelled protein M2 immunoprecipitated from a quantity of cell extract which had not been treated with hydroxyurea (100% = 1004 cpm).


100
90
80
70
60
50
40
30
20
10
0
^{59}Fe BOUND % OF CONTROL
92.5
69
46
30
14.3
(kD)
0.5 1.0
5.0
10.0
15.0
20.0
HYDROXYUREA (mM)

labeled M2 (Mr 44,000) is shown in Figure 20 (inset). Following such an immunoprecipitation, the amount of ^{59}Fe bound to protein M2 can be directly determined by gamma counting. The results shown in Figure 20 clearly indicate that as the concentration of hydroxyurea was increased in the incubation mixture, progressively less ^{59}Fe remained bound to protein M2. Approximately 50% of the M2 bound ^{59}Fe was released following treatment of the cell extract for 10 minutes at 0°C with 5 mM hydroxyurea.

3. Ferritin Expression in Hydroxyurea Resistant Cells

3.1. Altered Expression of Ferritin Heavy and Light Chain Genes in Hydroxyurea Resistant Cell Lines

Previous studies indicated that when hydroxyurea resistant mouse cells were cultured in the presence of hydroxyurea, they contained high levels of protein M2 as estimated by Western blot analysis (McClarty *et al.*, 1987). However, by using electron paramagnetic resonance (EPR) spectroscopy, it was shown that the vast majority of this protein M2 is lacking its tyrosyl free radical and is, therefore, inactive (McClarty *et al.*, 1987). This finding when coupled with the above result, indicating that hydroxyurea inactivated M2 possesses an unstable iron center which readily releases iron, suggested that there may be a disruption in iron homeostasis in hydroxyurea resistant cell lines. Therefore, alterations in ferritin expression in a variety of hydroxyurea resistant cell lines were examined.

The cell lines used were: a) a human (HeLa S3 H^R-1) mutant cell line selected for resistance in the presence of 2 mM hydroxyurea and its parental HeLa S3 wild type; b) a gat$^-$ Chinese hamster ovary (gat$^-$ CHO H^R-12SC8) mutant cell line selected for drug resistance in the presence of 0.33 mM hydroxyurea and its gat$^-$ CHO parental wild type; c) a rat L6 myoblast (L6 H^R-1) mutant cell line selected for drug resistance in the presence of 1 mM hydroxyurea; and d) a mouse L (H^R-5.0) mutant cell line selected for resistance in the presence of 5 mM hydroxyurea and its mouse L parental wild type. All of these drug resistant cell lines have previously been shown to overproduce ribonucleotide reductase activity as a result of elevated levels of M2 protein, which occur as a direct consequence of increased M2 mRNA levels and amplification of the M2 gene (Wright *et al.*, 1987). cDNA clones encoding the heavy (H)-subunit and light (L)-subunit of rat ferritin were used as specific hybridization probes to determine the relative amounts of H and L-mRNA in the hydroxyurea resistant and sensitive cell lines. The Northern blots shown in Figure 21 indicate that there are wide variations in the concentrations of H and L-mRNAs when the individual drug resistant cell lines are compared to their respective parental wild types. Interestingly, despite multiple attempts to probe Northern blots, even under low stringency conditions, no detectable amounts of L-ferritin mRNA in either the wild type or drug resistant gat$^-$ CHO cell lines. Densitometric measurements of H and L-mRNA autoradiograms yielded the values summarized in Table 4. There was no obvious pattern of H and/or L mRNA overproduction throughout the various hydroxyurea resistant cell lines examined. It is important to note, however, that there was an increase in ferritin mRNA expression

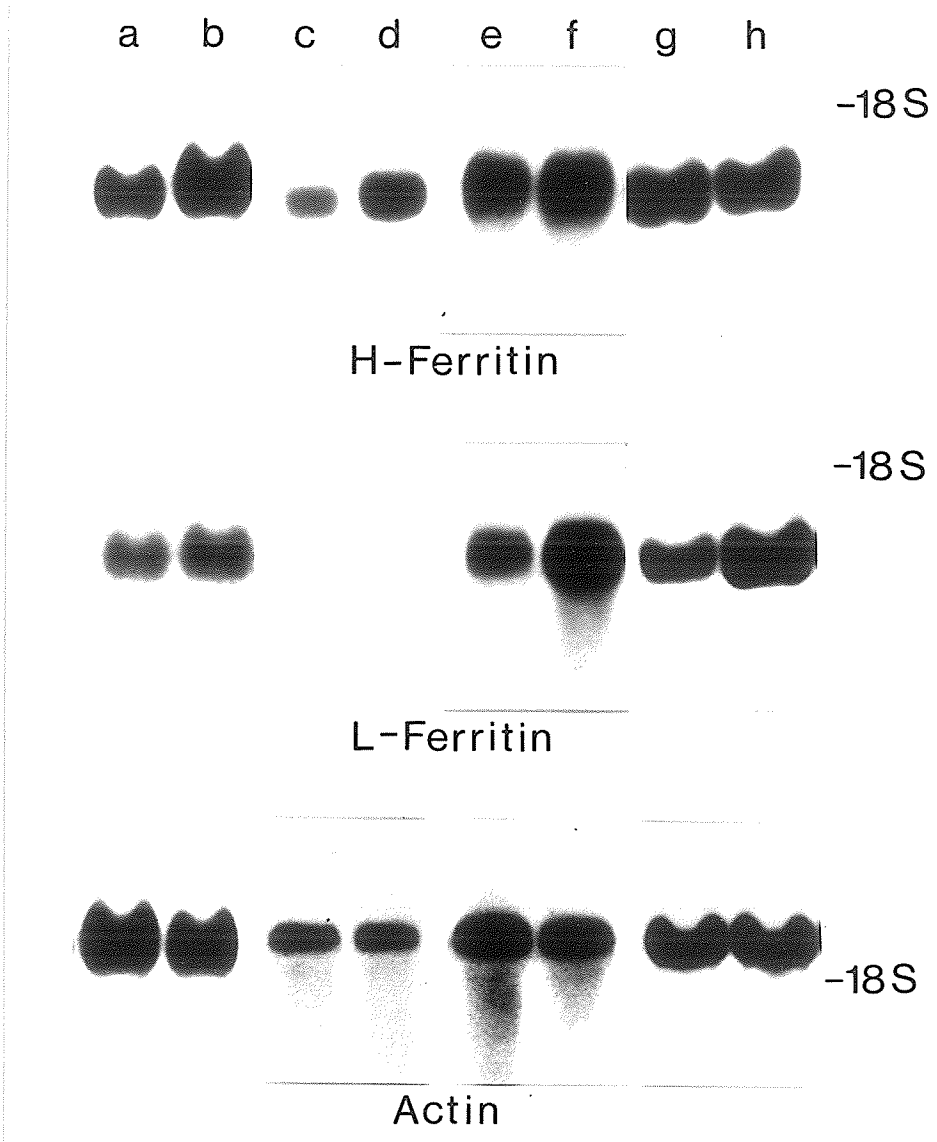
Table 4

Ferritin mRNA Content of Mutant Cell Lines Relative to Parental Wild Type Cell Lines

Cell Lines	Relative Ferritin L-Chain mRNA Hybridization	Relative Ferritin H-Chain mRNA Hybridization
W.T. Hela	1.0	1.0
H^R-1 Hela	1.45 ± 0.31	2.21 ± 0.43
W.T. gat^- CHO	-	1.0
H^R-12SC8 gat^- CHO	-	3.85 ± 0.92
W.T. Rat L6	1.0	1.0
H^R-1 Rat L6	3.77 ± 1.01	1.29 ± 0.18
W.T. Mouse L	1.0	1.0
H^R-5.0SC2	2.58 ± 0.78	1.10 ± 0.13

Data was determined from densitometric scanning of Northern hybridizations (Figure 21) carried out with H or L cDNA and RNA obtained from wild type (W.T.) and hydroxyurea resistant cell lines, and expressed relative to the wild type result. The values presented are the average of 3 determinations ± S.E.

Figure 21. Northern Blot Analysis of Ferritin mRNA Levels in Wild Type and Hydroxyurea Resistant Cell Lines. 20 μg of total RNA from HeLa WT (a), HeLa H^R-1 (b), CHO gat^- WT (c), CHO gat^- H^R-12SC8 (d), rat L6 WT (e), rat L6 H^R-1 (f), mouse L WT (g), and mouse L H^R-5.0 (h), cells was denatured then run on a 1% agarose/formaldehyde gel followed by transfer to a nylon membrane. The filter was then probed with the appropriate (H ferritin, L ferritin or β-actin) ^{32}P-labelled cDNA. The autoradiograms were exposed for 24-72 hours at -70°C with intensifying screens. The position of the 18S rRNA is indicated.

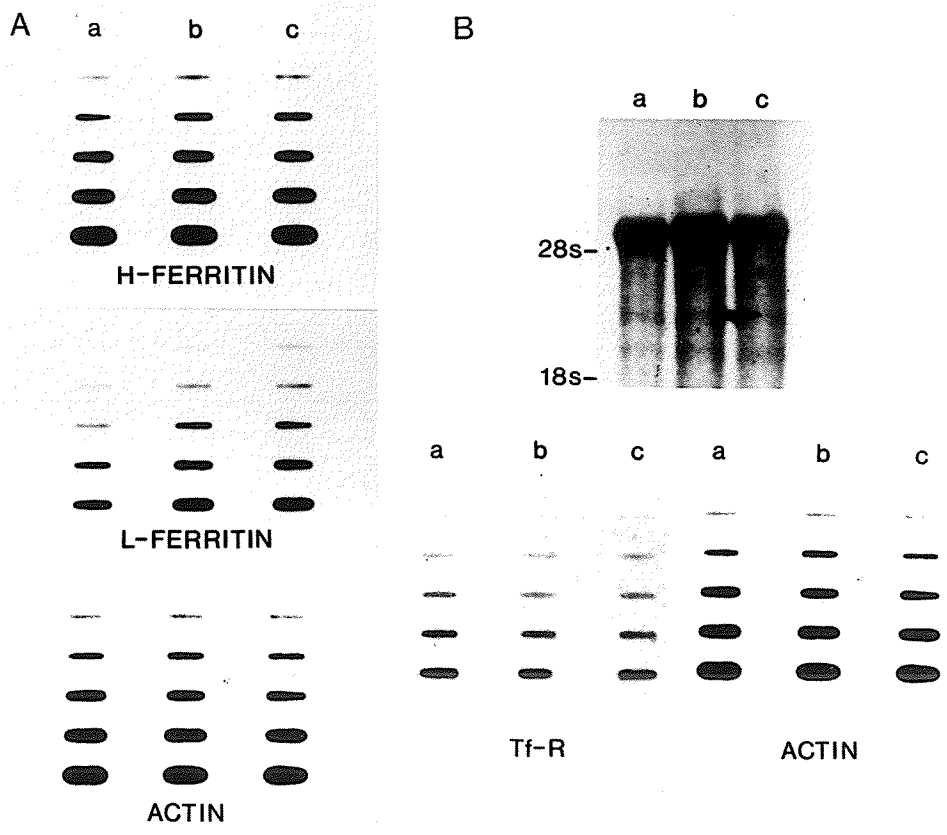

a b c d e f g h
-18S
H-Ferritin
-18S
L-Ferritin
-18S
Actin

(H and/or L) in all of the hydroxyurea resistant cell lines. In addition, elevations have been observed for H and/or L-ferritin mRNA in all the other hydroxyurea resistant cell lines that were screened including rat L8 myoblasts, CHO gat$^-$SC17, CHO gat$^-$ SC13, HeLa H^R-2 and a series of mouse L cell lines H^R-1.5, H^R-2.0, H^R-3.0 and H^R-4.0, whose drug resistance properties have been detailed (this thesis; McClarty *et al.*, 1986a; Wright *et al.*, 1987; Choy *et al.*, 1988; Creasey and Wright, 1984; Lewis and Wright, 1979).

To determine whether H and L mRNA levels were changed when the hydroxyurea resistant cells were grown in the presence of hydroxyurea, a more quantitative slot blot analysis was carried out. For this study, the cell line used was the hydroxyurea resistant mouse L cell line, H^R-5.0, since it is the best characterized of the mutant cell lines. It has been previously shown that hydroxyurea can regulate ribonucleotide reductase expression in this cell line (McClarty *et al.*, 1986a, 1987, 1988). The results of the slot blot analysis clearly indicated that there was essentially no change in H-mRNA levels but approximately a 2- to 3-fold elevation in L-mRNA levels when the parental wild type cells were compared to the hydroxyurea resistant mutant cells (Figure 22A, Table 4). There was no significant change in concentration of either H or L-mRNA when resistant cells grown in the absence of hydroxyurea were compared to the same cells grown in the presence of 5 mM hydroxyurea (Figure 22A).

Another key protein involved in the regulation of iron uptake and availability is the transferrin receptor (TfR)(Bomford and Munro, 1985). Like ferritin, the expression of the TfR in proliferating cells is regulated by iron availability. Increased intracellular iron levels lead

Figure 22. Effect of Hydroxyurea on Ferritin and Transferrin Receptor mRNA Levels. (A) Slot blot analysis of H ferritin, L ferritin and β-actin mRNA levels and (B) Northern and slot blot analysis of transferrin receptor (TFR) mRNA levels in wild-type mouse L cells (a) and hydroxyurea resistant mouse L cells (H^R-5.0) grown either in the absence (b) or presence (c) of 5 mM hydroxyurea. TFR Northern blot analysis was carried out as described in the legend to Figure 2. The position of the 28S and 18S rRNA is indicated. For TFR, H and L ferritin, and β-actin slot blots, total RNA was loaded in serial 2 fold dilutions and hybridized with the appropriate ^{32}P-labeled cDNA to quantitate the relative amounts of the specific mRNAs in each preparation. The amount of RNA present in the least dilute spot is 4.0 μg each for wild-type and hydroxyurea resistant H^R-5.0 cells grown in the absence and presence of 5 mM hydroxyurea. The autoradiograms were exposed for 24 hours for H and L ferritin, 96 hours for TFR and 2 hours for β-actin at -70°C with intensifying screens.


A
a
b
c
H-FERRITIN
L-FERRITIN
ACTIN
B
a
b
c
28s-
18s-
a
b
c
a
b
c
Tf-R
ACTIN

to a decrease in the number of TfR as a result of a decline in the level of the mRNA for the receptor (Rao *et al.*, 1986; Casey *et al.*, 1988). To determine if selection for resistance to hydroxyurea leads to changes in TfR mRNA levels, a human TfR cDNA was used as a specific molecular probe for Northern and slot blot analyses. Once again, the highly resistant mouse L cells was used as an example of a hydroxyurea resistant cell line. The results of this analysis showed that there was no significant change in the level of TfR mRNA when wild type cells were compared to resistant cells grown either in the absence or presence of 5 mM hydroxyurea (Figure 22B).

3.2. Heavy and Light Chain Ferritin Genes in Wild Type and Hydroxyurea Resistant Cell Lines

Stable overexpression of mRNA in drug resistant cell lines is frequently a result of gene amplification (Stark *et al.*, 1989). Southern blot analyses were performed to determine if the elevated levels of ferritin H and/or L-mRNAs in the hydroxyurea resistant cell lines were a result of gene amplification. At the wash stringency used (0.2 × SSC and 0.1% SDS at 60°C), it has been shown that the H and L cDNAs do not cross-hybridize (Aziz and Munro, 1986). As expected, banding patterns detected with the H-cDNA (Figure 23) and L-cDNA (Figure 24) probes varied with the mammalian species of DNA examined (human, hamster, rat, mouse). There was no indication of ferritin H or L-gene amplification in any of the drug resistant cell lines, since the intensity of the bands was approximately

Figure 23. Southern Blot Analysis of H Ferritin Genes in Wild Type and Hydroxyurea Resistant Cell Lines. Genomic DNA (20 μg) from HeLa WT (a), HeLa H^R-1 (b), CHO gat^- WT (c), CHO gat^- H^R-12SC8 (d), rat L6 WT (e), rat L6 H^R-1.0 (f), mouse L WT (g) and mouse L H^R-5.0 (h) cells was digested to completion with (A) Hind III or (B) EcoRI restriction endonucleases, electrophoresed through a 0.75% agarose gel and then transferred to a nylon membrane. Blots were then hybridized with a ^{32}P-labeled H ferritin cDNA. Autoradiograms were exposed for 24-72 hours at -70°C with intensifying screens.

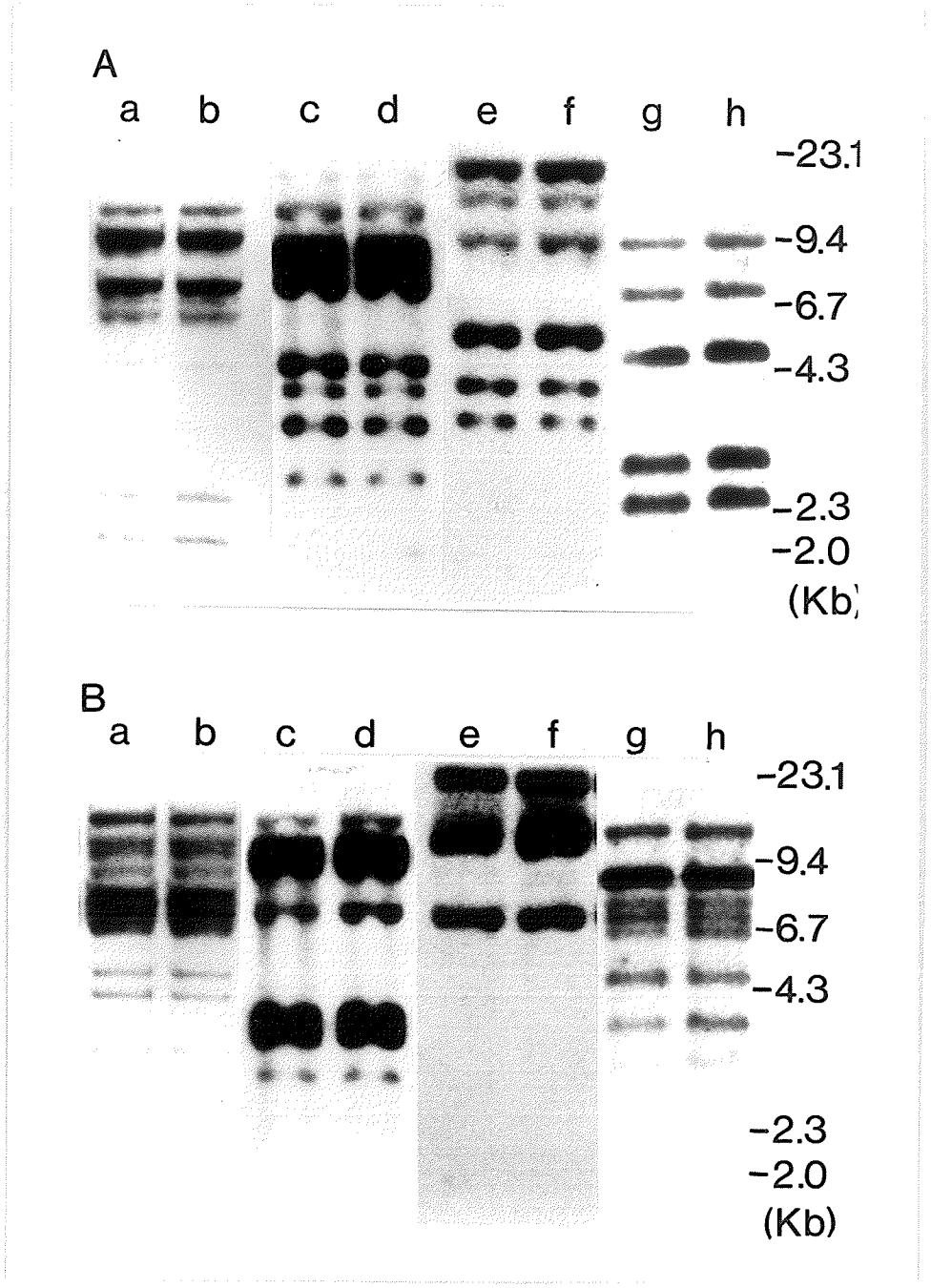
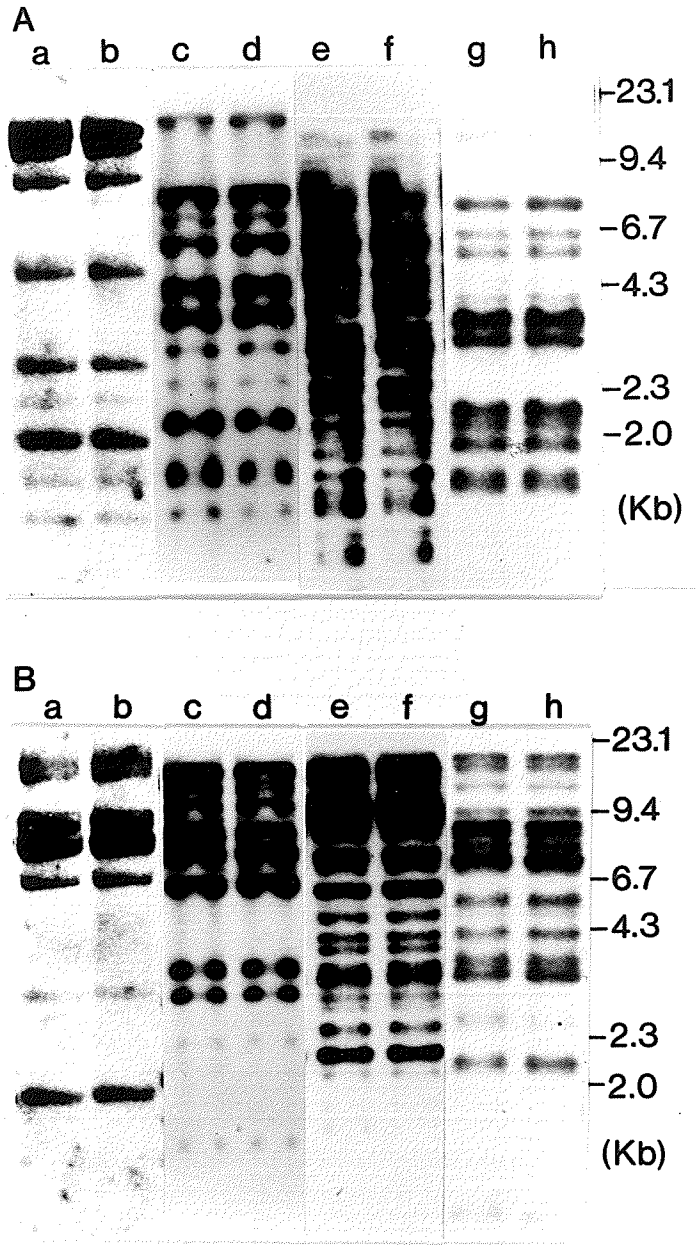

A
a b c d e f g h
-23.1
-9.4
-6.7
-4.3
-2.3
-2.0
(Kb)
B
a b c d e f g h
-23.1
-9.4
-6.7
-4.3
-2.3
-2.0
(Kb)

Figure 24. Southern Blot Analysis of L Ferritin Genes in Wild Type and Hydroxyurea Resistant Cell Lines. Southern blot analysis was carried out as described in the legend to Figure 4 except that the L ferritin cDNA was used as a hybridization probe. Genomic DNA from HeLa WT (a), HeLa H^R-1 (b), CHO gat^- WT (c), CHO gat^- H^R-12SC8 (d), rat L6 WT (e), rat L6 H^R-1.0 (f), mouse L WT (g) and mouse L H^R-5.0 (h) cells. (A) Hind III or (B) EcoRI endonuclease restriction digests. Auto-radiograms were exposed for 24-72 hours at -70°C with intensifying screens.

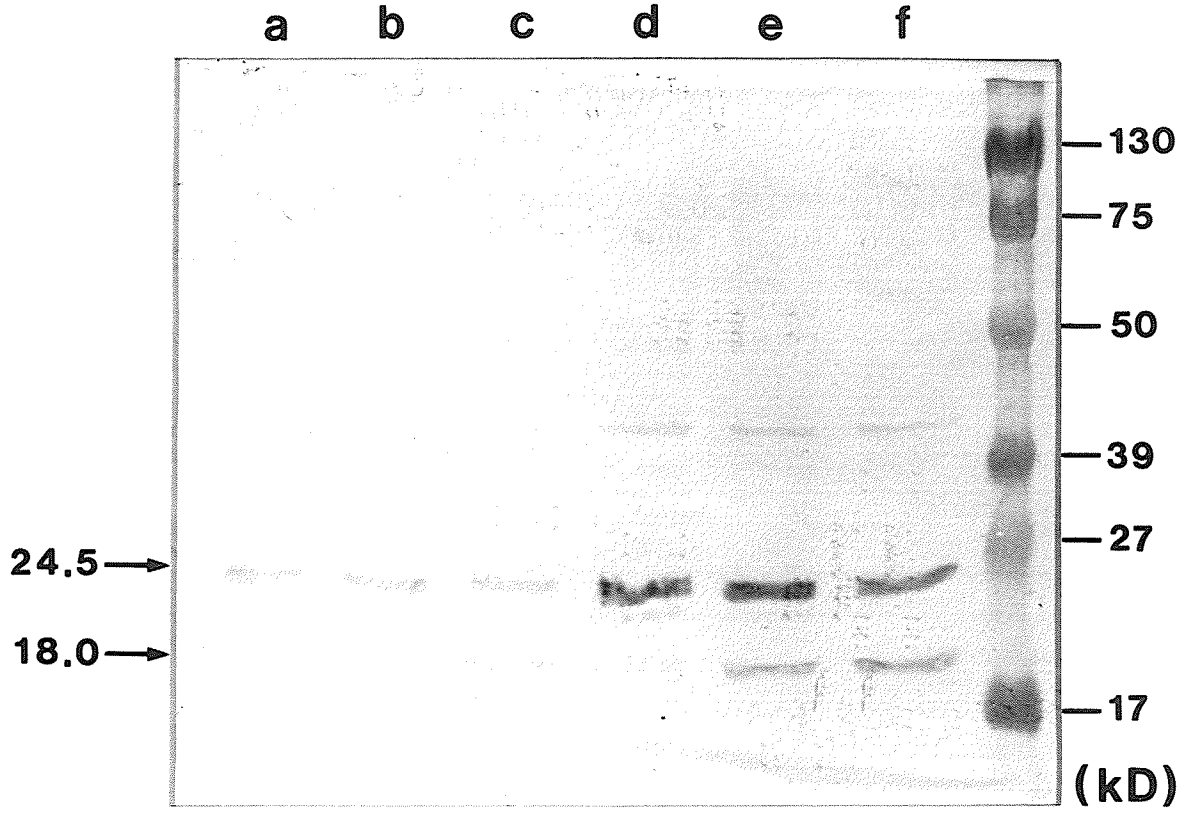

A
a b c d e f g h
23.1
9.4
6.7
4.3
2.3
2.0
(Kb)
B
a b c d e f g h
23.1
9.4
6.7
4.3
2.3
2.0
(Kb)

the same with DNA isolated from resistant and parental wild type cells. In addition, there was no evidence of H or L gene rearrangement in the resistant cell lines. Recent work with mouse cell lines selected for resistance to very high concentrations of hydroxyurea (15 mM and 30 mM drug) has shown that amplification of H- and L- ferritin genes can be obtained in culture if the drug selection pressure is exceedingly high (Hurta and Wright, 1990a). Interestingly, the rat L-chain cDNA hybridized extensively to DNA isolated from CHO gat$^-$ wild type and resistant cell lines (Figure 24; lanes c and d). This finding suggests that our inability to detect L-mRNA in these cell lines was unlikely to be a result of lack of cross-hybridization between the rat L-cDNA probe and gat$^-$ CHO mRNA.

3.3. Heavy and Light Chain Ferritin Subunits in Wild Type and Hydroxyurea Resistant Cells

To directly determine if the 2- to 3- fold elevation in ferritin L-mRNA in the hydroxyurea resistant mouse SC2 cell line results in a corresponding increase in L subunit protein, a commercially available polyclonal anti-ferritin IgG (Boehringer-Mannheim) was used in Western blot analysis. This anti-human ferritin IgG has been shown to recognize both mouse and human H and L ferritin subunits (Rouault *et al.*, 1987). Two predominant bands were detected using this antibody with apparent molecular weights of 24,500 and 18,000 (Figure 25). This result is in agreement with other analyses of mouse liver ferritin and mouse Friend

Figure 25. Western Blot Analysis for Ferritin Subunits in Wild Type and Hydroxyurea Resistant Mouse L Cells. Western blot analysis for ferritin subunits in wild-type mouse L cells (a and d) and hydroxyurea resistant mouse L cells (H^R-5.0) grown either in the absence (b and e) or presence (c and f) of 5 mM hydroxyurea. Electrophoresis, transfer to nitrocellulose and immunostaining were performed as described under "Experimental Procedures" and in McClarty *et al.* (1987). Lanes a-c, 25 μg cell extract protein and lanes d-f, 50 μg cell extract protein. The apparent molecular mass (kD) of the two ferritin subunits is indicated on the left. Previous studies by Beaumont *et al.* (1987, 1989) have shown that the 24.5 KD band represents the ferritin L chain subunit, whereas the 18 KD band corresponds to the ferritin H chain subunit.


a
b
c
d
e
f
130
75
50
39
27
17
(kD)
24.5
18.0

erythroleukemic cell ferritin, which also had apparent molecular weights of 24,500 and 18,000 (Beaumont *et al.*, 1987, 1989). From the relative distributions of the two ferritin subunits in liver and Friend erythroleukemic cells, by using antibodies with specificity for human L determinants, and by electrophoretic analysis of the subunits encoded by mouse H and L subunit cDNAs, Beaumont *et al.* (1987,1989) have conclusively shown that the 24,500 subunit corresponds functionally to the L subunit, whereas the 18,000 subunit corresponds to the H subunit. The result of our Western blot analysis is shown in Figure 25 and summarized in Table 5. No significant change in concentration of the 24,500 (L) subunit was detected, however, there was an increase in the level of the 18,000 (H) subunit in the hydroxyurea resistant cell line when compared to wild type cells.

3.4. Regulation of Ferritin Biosynthesis in Wild Type and Hydroxyurea Resistant Cells

A central feature of the biology of ferritin is the high degree of regulation of ferritin biosynthesis that takes place in response to changes in iron availability (Drysdale, 1988; Theil, 1987). It seemed quite possible that the apparently anomolous result of a decreased H/L ferritin mRNA ratio coupled with an increased H/L ferritin subunit ratio in the hydroxyurea resistant cell line compared to its parental wild type could be explained by specific changes in the rate of ferritin biosynthesis. In order to determine if this was the case, the rate of

Table 5

Effect of Iron and/or Hydroxyurea on Ferritin Synthesis and Accumulation

Cell Line + Culture Conditions	Ferritin L Chain	Ferritin H Chain
A. Wild Type Mouse L	1.0	1.0
W.T. Mouse L +Iron	1.85 ± 0.3	1.99 ± 0.34
H^R-5.0SC2 Mouse L	0.79	0.69
B. H^R-5.0SC2 Mouse L	1.0	1.0
H^R-5.0SC2 Mouse L + Iron	5.0 ± 1.13	3.3 ± 0.81
H^R-5.0SC2 Mouse L + Hydroxyurea	3.3 ± 0.85	4.9 ± 1.06
H^R-5.0SC2 Mouse L + Iron + Hydroxyurea	16.7 ± 4.11	18.0 ± 3.69
C. Wild Type Mouse L	1.0	1.0
H^R-5.0SC2 Mouse L	1.06 ± 0.2	2.62 ± 0.4
H^R-5.0SC2 Mouse L + Iron	0.86 ± 0.1	3.69 ± 0.4
D. H^R-5.0SC2 Mouse L	1.0	1.0
H^R-5.0SC2 Mouse L + Iron	0.81 ± 0.1	1.41 ± 0.2

A and B: Data was determined from densitometric scanning of immunoprecipitations (Figures 24 and 25) carried out with rabbit polyclonal anti-human ferritin IgG and ^{35}S-methionine labelled protein extracts prepared from wild type (W.T.) and hydroxyurea resistant H^R-5.0SC2 mouse L cells, and expressed relative to control cultures and/or control culture conditions. The values are the average of 3 determinations ± S.E.

C and D: Data was determined from densitometric scanning of Western blot experiments (Figure 23) carried out with rabbit polyclonal anti-human IgG and protein extracts prepared from wild type (W.T.) and hydroxyurea resistant H^R-5.0SC2 cells, and expressed relative to control cultures and/or control culture conditions. The values are the average of 3 determinations ± S.E.

biosynthesis of mouse ferritin subunits in wild type and hydroxyurea resistant cells was examined. Also determined was the effect of iron treatment on the ferritin biosynthetic rate. [^{35}S] methionine pulse-labeled subunits were immunoprecipitated from wild type and resistant cell lines with the same antibodies as were used for the Western blot analysis. The immunoprecipitates were resolved into subunits by sodium dodecyl sulfate gradient pore polyacrylamide gel electrophoresis, and estimates of synthesis were made from autoradiograms by scanning densitometry. To demonstrate the specificity of the immunoprecipitation reaction, an entire gel lane is shown on the left hand side of Figure 26. Two predominant bands were observed with apparent molecular weights of 24,500 and 22,500. The 24,500 subunit corresponds to the larger (L) subunit detected on the Western blot, however, the 22,500 subunit had no counterpart in mature ferritin shells (compare Figures 25 and 26). Also shown in Figure 26 are the ferritin bands immunoprecipitated from untreated wild type mouse L cells and hydroxyurea resistant mouse cells, and the immunoprecipitates from the same two cell lines treated with 150 μM ferric ammonium citrate for three hours prior to [^{35}S] methionine labeling. Densitometry of these bands yielded the quantitation shown in the accompanying bar graph and summarized in Table 5. The result shown is representative of three such experiments. Both the wild type and the hydroxyurea resistant cells responded to the presence of iron by rapidly increasing the intensity of the metabolically labeled immunoprecipitates, a result consistent with other studies (Beaumont *et al.*, 1987; Rogers and Munro, 1987; Mattia *et al.*, 1986).

Figure 26. Ferritin Immunoprecipitation and the Effect of Iron on Ferritin Biosynthesis in Wild Type and Hydroxyurea Resistant Mouse L Cells. The lane on the left-hand side shows the specificity of the immunoprecipitation of metabolically labelled ferritin. On the right-hand side are shown the ferritin bands immunoprecipitated from untreated wild-type mouse L cells (a), wild-type mouse L cells treated with 150 μM ferric ammonium citrate for 3 hours (b), untreated hydroxyurea resistant mouse L cells (H^R-5.0) (c), and H^R-5.0 mouse L cells treated with 150 μM ferric ammonium citrate for 3 hours (d). Following iron treatment, cells were pulse-labelled with [^{35}S] methionine (500 μCi/ml of methionine-free medium) for 1 hour. The labeled cells were washed, then lysed, and the supernatant was immunoprecipitated with anti-ferritin polyclonal antibody (Boehringer Mannheim), and subjected to SDS gradient pore polyacrylamide gel electrophoresis and autoradiography as described under Materials and Methods. The resulting autoradiogram was scanned and the quantitation of the ferritin bands is illustrated in the bar graph. Stripped bars refer to the 24.5 KD band (L-ferritin subunit) and open bars refer to a 22.5 KD band, which Beaumont *et al.* (1987, 1989) have shown is post-translationally processed to form the 18 KD (H-ferritin subunit) detected in mature mouse ferritin shells.

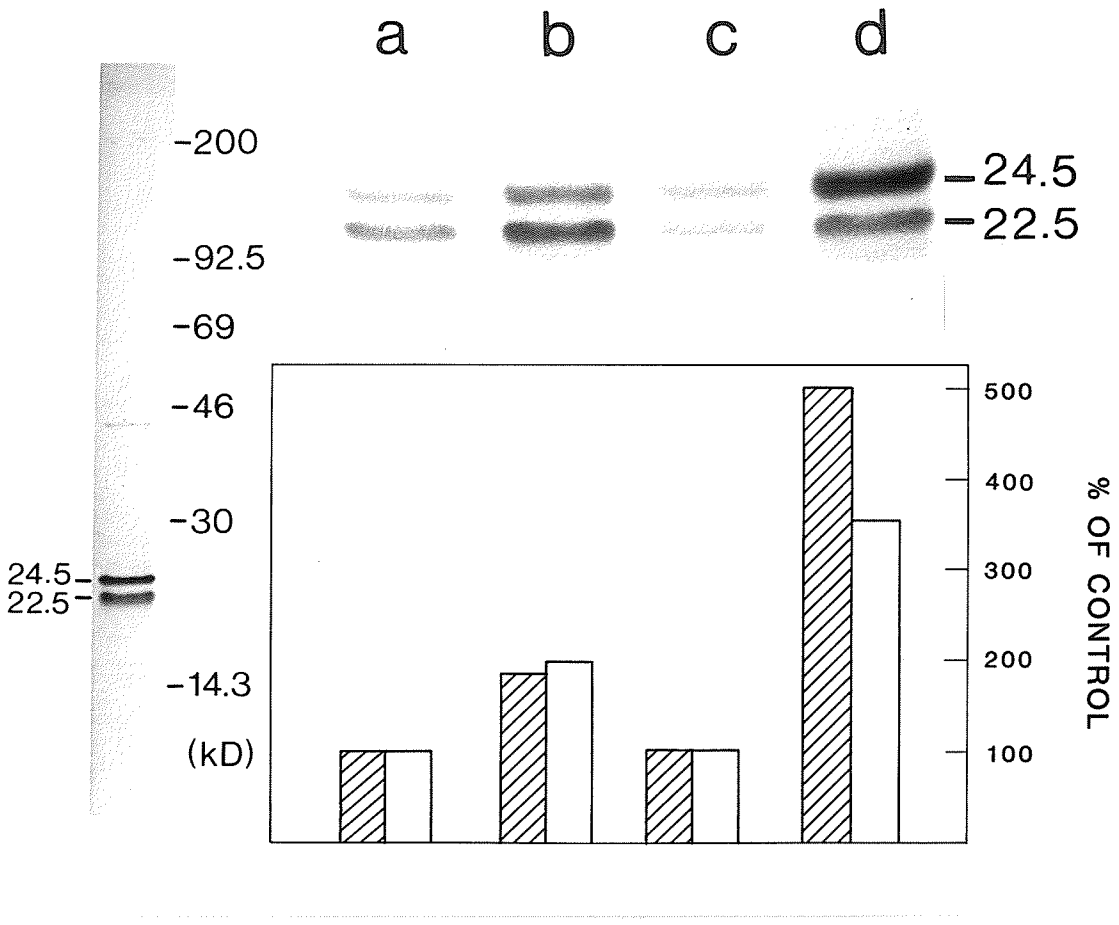
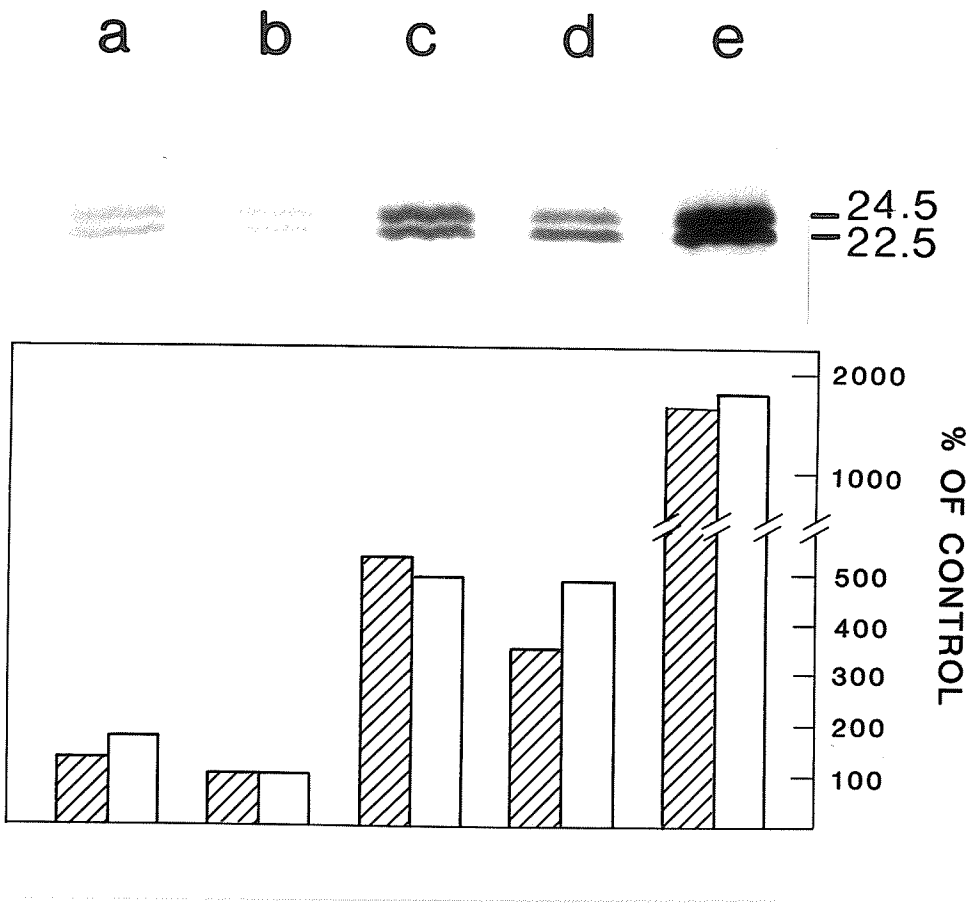
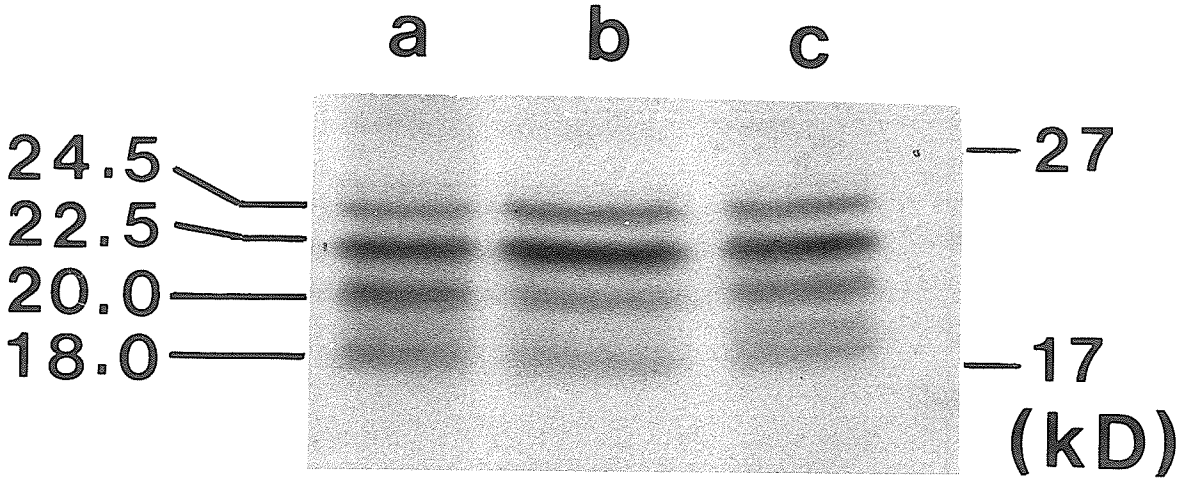

a
b
c
d
-200
-92.5
-69
-46
-30
-14.3
(kD)
24.5
22.5
24.5
22.5
500
400
300
200
100
% OF CONTROL

Figure 27. Effect of Hydroxyurea and Iron on Ferritin Biosynthesis in Hydroxyurea Resistant Mouse L Cells. Shown are the ferritin bands immunoprecipitated from hydroxyurea resistant mouse L cells (H^R-5.0) treated with 5 mM hydroxyurea for 3 hours (a), untreated mouse L H^R-5.0 (b), mouse L H^R-5.0 cells treated with 150 μM ferric ammonium citrate for 3 hours (c), mouse L H^R-5.0 cells treated with 5.0 mM hydroxyurea for > 2 weeks (d), and mouse L H^R-5.0 cells treated with 5.0 mM hydroxyurea for > 2 weeks and 150 μM ferric ammonium citrate for 3 hours (e). Following iron and/or hydroxyurea treatment, cells were pulse-labelled with [^{35}S] methionine (500 μCi/ml of methionine free medium) for 1 hour. Immunoprecipitation was carried out as described in the legend to Figure 26. The resulting autoradiogram was scanned and the quantitation of the ferritin bands is illustrated in the bar graph. Stripped bars refer to the 24.5 KD band (L-ferritin subunit) and open bars to the 22.5 KD band (H-ferritin subunit)


a
b
c
d
e
24.5
22.5
2000
1000
500
400
300
200
100
% OF CONTROL

In an earlier investigation using the same resistant mouse cell line, both hydroxyurea and iron affected the rate of biosynthesis of the M2 subunit of ribonucleotide reductase (Fig. 18, Table 2). Therefore, the question as to whether hydroxyurea could also affect the rate of ferritin biosynthesis was examined. Biosynthetic rates in the resistant cell lines were evaluated following a three hour exposure to 5 mM hydroxyurea or 150 μM ferric ammonium citrate (Figure 27). As before, the results shown are representative of three such experiments and densitometric quantitation of the immunoprecipitated ferritin bands is presented in the accompanying bar graph and summarized in Table 5. It is clearly evident that ferritin biosynthetic rates were increased by iron and hydroxyurea (Figure 27; compare lane b with a). Resistant cells that have been grown in the presence of hydroxyurea for an extended period of time (>2 weeks) synthesized ferritin at a rate approximately 5-fold greater than that observed in the same cells grown in the absence of hydroxyurea. The effect of iron treatment on resistant cells that had been growing in the presence of hydroxyurea for > 2 weeks was also determined. This combination of long term hydroxyurea treatment and short term iron treatment resulted in a very large increase in the rate of ferritin biosynthesis.

Figure 28. Post-Translational Processing of the 22,500 Ferritin Subunit in Wild Type and Hydroxyurea Resistant Mouse L Cells. Shown are the ferritin bands immunoprecipitated from wild-type mouse L cells (lane a) and hydroxyurea resistant mouse L cells (H^R-5.0) grown either in the absence (lane b) or presence of 5 mM hydroxyurea (lane c). Cells were labeled for 6 hours with [^{35}S] methionine (500 μCi/ml of methionine free medium) then immunoprecipitation with antiferritin antibody was carried out as described in the legend to Figure 26. To allow for the direct comparison of cell lines and culture conditions, equal quantities (dpms) of immunprecipitated ferritin protein were loaded in each lane (a, b and c) of the gel. The 24.5 KD band represents the ferritin L-chain subunit. The 22.5 and 20 KD bands are presumptive precursors of the 18 KD ferritin H-chain subunit.


a
b
c
24.5
22.5
20.0
18.0
27
17
(kD)

3.5. Post-Transcriptional Processing of the 22,500 Ferritin Subunit in Wild Type and Hydroxyurea Resistant Cells

Beaumont *et al.* (1987, 1989) have shown that the 22,500 subunit observed in labeling experiments is post-translationally processed to form the 18,000 (H) subunit detected in mature mouse ferritin shells. To determine if there was a difference in post-translational processing between wild type and resistant cell lines, a longer term [^{35}S] methionine labeling experiment was performed. Similar to the previous result (Figures 26,27), after one hour of labeling, only the 24,500 and 22,500 subunits were observed in wild type and resistant cells grown either in the absence or presence of hydroxyurea. However, after six hours in the presence of [^{35}S] methionine, the label also appeared in a 20,000 and an 18,000 molecular weight band in both the wild type and resistant cell lines (Figure 28). These results are in agreement with those of Beaumont *et al.* (1987, 1989), and suggest that both the wild type and the hydroxyurea resistant cell lines are capable of processing the 22,500 molecular weight precursor.

4. TPA Induction of Ribonucleotide Reductase

4.1. Ribonucleotide Reductase Activity After Treatment With TPA

Because of the role ribonucleotide reductase seems to play in proliferation of neoplastic cells, the effect of tumor promoters on the

TABLE 6

Elevation of Ribonucleotide Reductase Activity by TPA

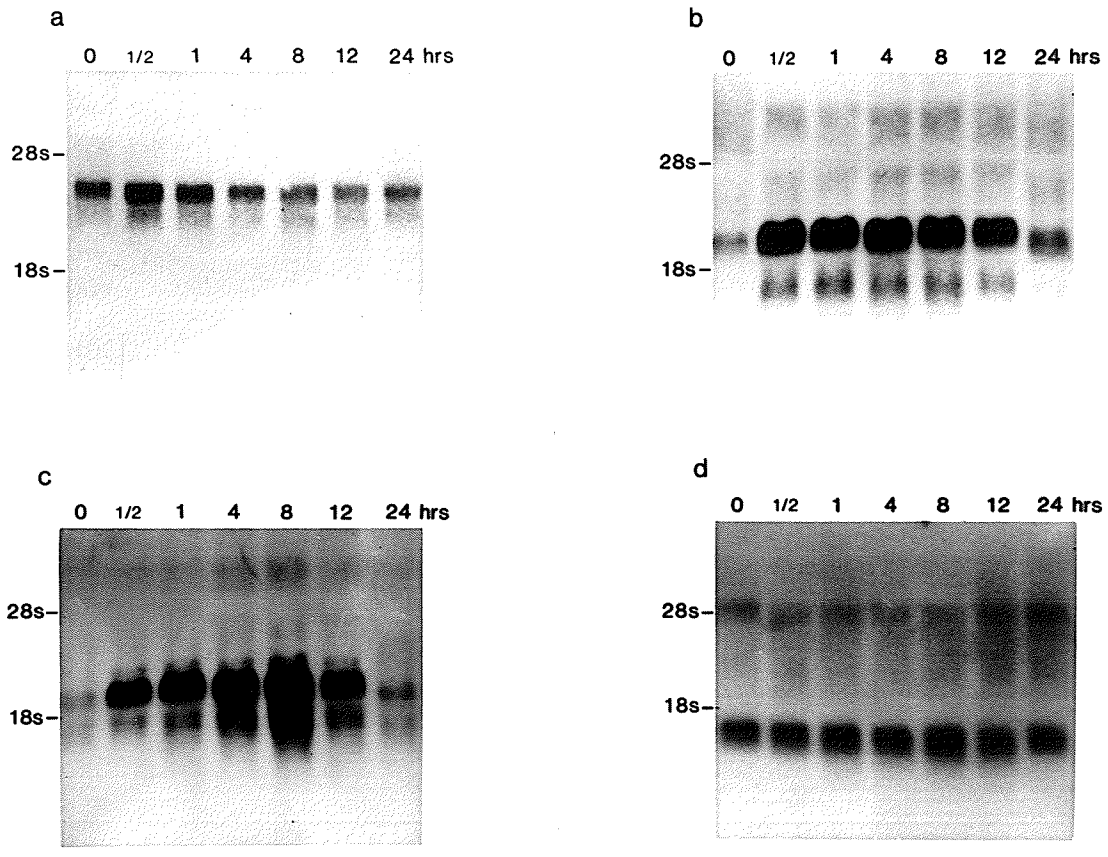
Hours Of 0.1μM TPA Treatment	Reductase Activity (nM CDP reduced/hr/mg)	Fold Increase
0	0.67	-
½	3.07	4.59
1	3.22	4.81
4	2.65	3.96
8	2.03	3.03
24	0.84	1.25
48	0.61	0.91

enzyme activity was examined (Choy *et al.*, 1989a). For this experiment, a very potent phorbol ester tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), was selected. A stock concentration of TPA was originally dissolved in dimethyl sulfoxide (DMSO) and then diluted in α-MEM medium. Proliferating BALB/c 3T3 cells were grown in the presence of 0.1 μM TPA for various lengths of time. A rapid elevation of ribonucleotide reductase activity by nearly five fold was observed within ½ to 1 hour of treatment of cells with the tumor promotor (Table 6). This increase in enzyme activity was transient and returned to near normal levels within 24 to 48 hours of treatment. The reduced levels of elevated activity with prolonged exposure is in agreement with other observations that continuous treatment leads to a feedback desensitization to TPA (Collins and Rozengurt, 1982; Pittelkow *et al.*, 1989).

4.2. Analysis of M1 and M2 mRNA Levels After TPA Treatment

Since TPA frequently affects the expression of numerous genes, the elevation in ribonucleotide reductase activity could result from *de novo* synthesis of the enzyme. The mRNA expression of M1 and M2 was examined in TPA treated 3T3 BALB/c cells. Results of Northern blot analysis using M1 specific cDNA as hybridization probe (Choy *et al.*, 1988; Thelander and Berg, 1986), revealed that the M1 message was elevated 1.4 to 1.8 fold after ½ hour treatment with TPA, and returned to control levels obtained with cells treated with 0.01% DMSO (the final concentration of DMSO when the stock TPA solution was diluted), within 24 hours (Figure 29a). M2

Figure 29. Northern blot analysis of TPA treated BALB/c 3T3 fibroblasts. The proliferating cells were cultured in α-MEM supplemented with 10% FBS. The cells were treated with 0.1 μM of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The total cellular RNA blots were probed with (a) M1 cDNA, (b) M2 cDNA, (c) ornithine decarboxylase (ODC) cDNA as a positive control, and (d) glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA as a control for loading.

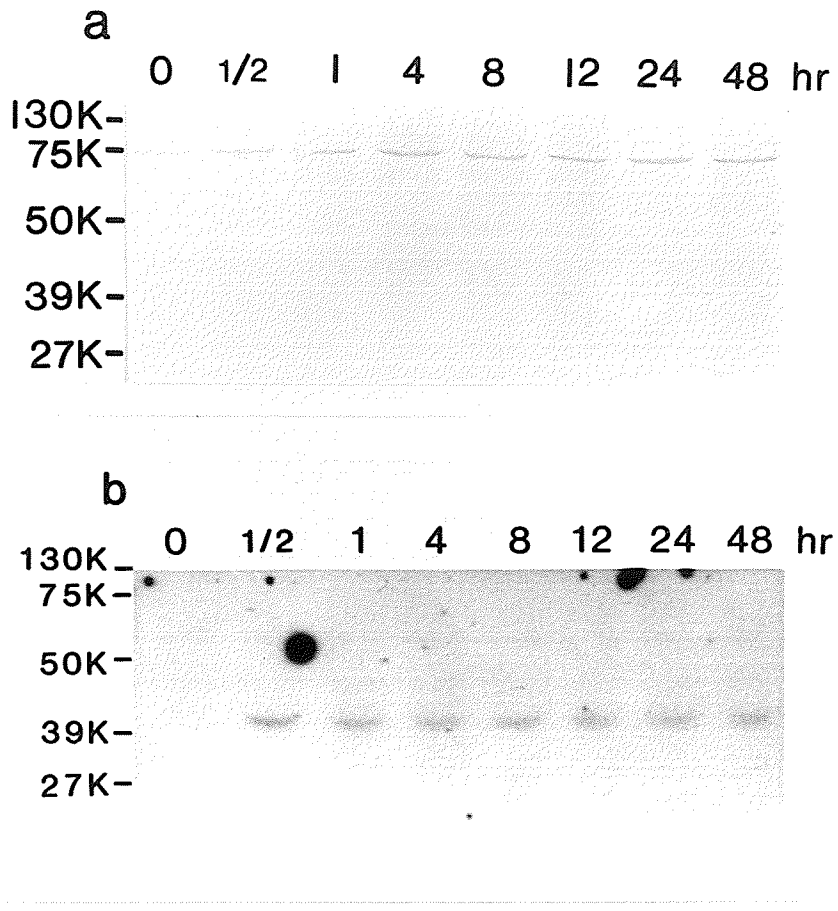

a
0 1/2 1 4 8 12 24 hrs
28s
18s
b
0 1/2 1 4 8 12 24 hrs
28s
18s
c
0 1/2 1 4 8 12 24 hrs
28s
18s
d
0 1/2 1 4 8 12 24 hrs
28s
18s

specific cDNA (Choy *et al.*, 1988; Thelander and Berg, 1986) probed blots showed a dramatic elevation of both M2 transcripts (2.1 and 1.6 Kb) usually observed in mouse cells (McClarty *et al.*, 1987; Thelander and Berg, 1986; Wright *et al.*, 1987). Within ½ hour of incubation with TPA, M2 message rose to about 18 fold above the values obtained with cells treated with 0.01% DMSO alone (Figure 29b), and returned to control levels within 24 hours. For comparison, the levels of ODC message was examined in 3T3 cells treated in an identical manner with TPA (Figure 29c). As observed by others (Gilmour *et al.*, 1985), ODC specific cDNA (Berger *et al.*,1984) detected several mRNA species (2.4 and 2.1 Kb) and they were transiently elevated approximately 16 fold by 8 hours (Figure 29c). As a loading control for each lane the Northerns were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase specific cDNA (Edwards *et al.*, 1985) (Figure 29d).

4.3. M1 and M2 Protein Levels After TPA Treatment

Although the M1 probed Northern shown in Figure 29a indicated a slight increase in M1 mRNA with TPA treated cells, a Western blot probed with monoclonal antibodies specific for protein M1 showed no obvious change in the levels of the protein in TPA treated cells (Figure 30a). This is in contrast to Western blots probed with M2 specific monoclonal antibodies, in which a clear increase in M2 protein was detected within ½ hour treatment with TPA, followed by a decline to lower levels upon further incubation in the presence of the tumor promotor (Figure 30b).

Figure 30. Western blot analysis of TPA treated BALB/c 3T3 fibroblasts. (a) For the Western blot of M1, 30 μg of protein for each lane was run, probed with a monoclonal antibody specific for the M1 subunit, and developed by an alkaline phosphatase conjugated second antibody. No fluctuations in the M1 protein level were detected. (b) The M2 western blot has 40 μg of protein loaded for each lane, probed with a monoclonal antibody to the M2 subunit, and developed by autoradiography of ^{125}I-labeled second antibody. The blot shows a large increase in the M2 protein within ½ hour. Although an increase in M2 protein at ½ hour is consistently observed, the amount of M2 detected at 0 time varies and in some experiments approaches the level observed after 48 hours of TPA treatment.


a
0 1/2 1 4 8 12 24 48 hr
130K-
75K-
50K-
39K-
27K-
b
0 1/2 1 4 8 12 24 48 hr
130K-
75K-
50K-
39K-
27K-

These changes in M2 protein resembled the observations of M2 mRNA levels in TPA treated cells (Figure 29b). Thus the increase observed in the ribonucleotide reductase activity seems to result primarily from the increase of M2 protein.

4.4. Transcriptional Control of the M2 Gene

4.4.1. M2 Promoter

The promoter regions of several phorbol ester inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor (Elholtz *et al.*, 1986; Angel *et al.*, 1987a; Fisch *et al.*, 1989). These genes carried a common 8 bp concensus sequence: 5'-TGAGTCAG-3'. This sequence was able to confer TPA inducibility on heterologous promoters and the transcription factor AP-1 was found to mediate this response (Angel *et al.*, 1987a; Fisch *et al.*, 1989). This AP-1 binding site is highly conserved in a number of TPA-inducible promoters (Angel *et al.*, 1987a).

The immediate 5' upstream 501 bp sequence of the murine M2 gene has been published (Thelander and Thelander, 1989). A search of this sequence revealed no sequence that was remotely similar to the concensus AP-1 site. It is possible that the AP-1 site is further upstream but the known AP-1 sites in TPA inducible genes are clustered between -72 bp and -185 bp (Karin and Richards, 1982; Fujita *et al.*, 1983; Matrisian *et al.*, 1986;

Angel *et al.*, 1987a) and in the SV40 promoter at +30 bp and +120 bp (Buchman *et al.*, 1980).

However, an upstream sequence resembling an AP-2 site was found at -184 bp of the M2 gene (Fig. 31). Transcription factor AP-2 is a 52,000 dalton protein that binds to enhancer regions of SV40 and human metallothionein IIA (hMT IIA). Purified AP-2 protein is able to stimulate *in vitro* transcription of the SV40 early and hMT IIA promoters (Mitchell *et al.*, 1987), and that this stimulation was abolished by deletion of the AP-2 DNA binding site. Imagawa *et al.* (1987) reported that transcription factor AP-2 is able to mediate induction by TPA. However, Kanno *et al.* (1989) reported that the AP-2 binding sites in the SV40 and metallothionein hMT IIA enhancers do not exhibit an ability to stimulate transcription by itself or through TPA stimulation, in contrast to the reports by Mitchell *et al.* (1987) and Imagawa *et al.* (1987).

Since there is lack of a proximal AP-1 binding sites and appearance of a very close concensus to the hMT IIA AP-2 site, it is possible that the TPA induction of ribonucleotide reductase is through this presumptive transcription factor AP-2.

4.4.2. Electromobility Shift Assay of the Presumptive AP-2 sequence

A 50 base oligonucleotide (MTAP-50) containing the M2 presumptive AP-2 binding site and approximately 20 flanking nucleotides, both 3′ and 5′ was synthesized by the Regional DNA Synthesizing Laboratory at the University of Calgary. A 15 base complementary sequence (MTAP-PR) was

Figure 31. Putative AP-2 binding site. The human metallothionein II_A ($hMT\text{-}II_A$) and murine M2 upstream promoter sequences are shown. The shaded nucleotides represent similiar nucleotides between the two sequences. The $hMT\text{-}II_A$ AP-2 binding site is situated from -170 to -179. The M2 upstream DNA sequence contains an homologous site from -176 to -183. The concensus AP-2 binding sequence is from Wingender, 1989.

MTAP-50 is a 50 base oligonucleotide containing the putative AP-2 site in the mouse M2 upstream promoter sequence. The 50 base is comprised of the region -154 to -203. MTAP-PR is a 15 base oligonucleotide of the complementary strand from -154 to -168. The 15 base is annealed to the MTAP-50 and serves to prime a DNA polymerase large fragment (Klenow) extension to yield MTAP-2, a double stranded 50bp DNA probe.

The sequence of the specific AP-2 competitor DNA is shown, and the AP-2 binding site is underlined.

AP-2 concensus 5′-CGCCCGCG-3′

-180v -170v

hMT-II_A 5′-AGGGAACTGACCGCCGCGCGGCCCGTGTG-3′

murine M2 5′-GCCCCCGCGAGCGCCCGGGCCACCCCGCCC-3′

-180^ -170^

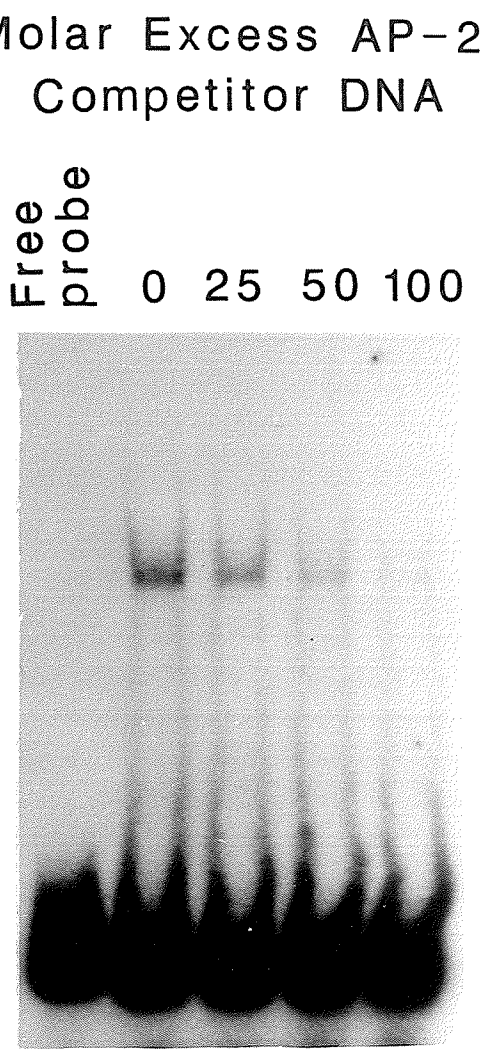
MTAP-50 5′-CCGGTTCCGGCCCCGCGAGCGCCCGCGCACCCGGCCGGCGCAGGCTCCTT-3′

MTAP-PR 3′-GCCGCGTCCGAGGAA-5′

AP-2 competitor 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′

3′-CTAGCTTGACTGGCGGGCGCCGGGCA-5′

Figure 32. Gel-shift of MTAP-2 with HeLa nuclear protein extract. MTAP-2 was end labeled with ^{32}P. One μg of HeLa nuclear protein extract was incubated with the labeled DNA probe in presence or absence of unlabeled AP-2 concensus sequence competitor DNA.

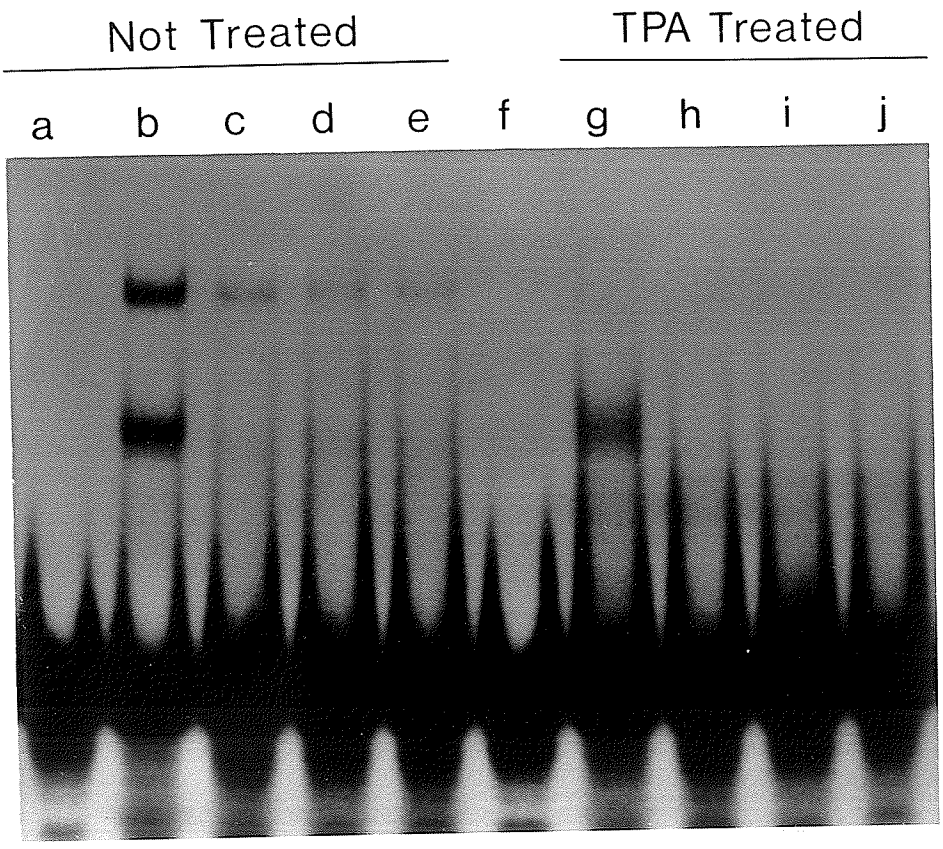

Molar Excess AP-2
Competitor DNA
Free probe
0
25
50
100

also synthesized as a primer to generate the complementary strand by Klenow extension to make the ^{32}P-labeled double stranded DNA probe (MTAP-2). These oligonucleotides sequences are shown in Fig. 31. Using 20 μg of HeLa nuclear extracts [from where AP-2 was originally purified (Mitchell *et al.*, 1987)], a single complex was found to form and retard in the electromobility shift assay (Fig. 32). Excess cold double stranded 26 bp competitor containing a concensus AP-2 binding site was able to compete with the MTAP-2 probe for the binding proteins. The competition experiment suggests that the complex is formed with AP-2, and is able to bind MTAP-2 tightly as 100 molar excess of competitor still did not abolish all complex formation.

Similar experiments with nuclear extract from BALB/c 3T3 cells formed the same complex and tight binding to the MTAP-2 probe (Fig. 33). However, two protein-DNA complexes were evident when the MTAP-2 probe was incubated with the BALB/c 3T3 nuclear extract (Fig. 33, lane b). The formation of two complexes with an AP-2 binding site is in agreement with results of Bishop *et al.* (1990) when a putative AP-2 binding site in the 5′ flanking region of the mouse proopiomelanocortin (POMC) gene was incubated with nuclear extracts from mouse corticotrope tumor cells. Again, both complexes could be competed off with molar excess of cold AP-2 competitor DNA. The lower complex was easily abolished with 25 molar excess competitor, while the higher complex was still visible even at 100 molar excess competitor. Surprisingly, nuclear extracts prepared from BALB/c 3T3 cells treated with TPA for ½ hour actually reduced complex formation. In each case, TPA treated and untreated, 35 μg of nuclear extract was used in the gel-shift assay. With the TPA treated nuclear

extract, only the lower complex was formed, and was very loosely bound as 25 molar excess AP-2 competitor was able to completely abolish the complex. It also appears that less of the lower complex was formed with the nuclear extract from TPA treated cells than the lower complex formed with the untreated cells.

Figure 33. Gel-shift of MTAP-2 with TPA treated and untreated nuclear protein extract. 35 μg of nuclear extract from BALB/c 3T3 cells, untreated and TPA treated for ½ hour, were incubated with end-labeled MTAP-2 probe and separated on a 4% polyacrylamide gel as described in Materials and Methods. (a,f) free probe, not incubated with nuclear extract. (b,g) no AP-2 competitor, (c,h) 25 molar excess AP-2 competitor to probe, (d,i) 50 molar excess AP-2 competitor, (e,j) 100 molar excess AP-2 competitor.


Not Treated
TPA Treated
a b c d e f g h i j

Discussion

DISCUSSION

1. Molecular Mechanisms of Drug Resistance

Ribonucleotide reductase is responsible for providing a continuous and balanced supply of deoxyribonucleotide precursors essential for DNA synthesis. Enzyme inhibitors such as the chemotherapeutic agent, hydroxyurea, inhibit DNA synthesis through ribonucleotide reductase, by altering deoxyribonucleotide concentrations, the substrates for DNA polymerase (Nicander and Reichard, 1985). A mechanism for achieving resistance to hydroxyurea and related drugs is through elevation of ribonucleotide reductase activity. This ensures that cells with high levels of enzyme activity will still have enough active enzyme, when grown in the presence of normally cytotoxic concentrations of drug, to provide the required deoxyribonucleotides for the synthesis of DNA.

Although ribonucleotide reductase contains the two nonidentical subunits M1 and M2, the precise site of action of hydroxyurea is at the M2 subunit, where the drug specifically destroys a tyrosyl free radical needed for enzyme activity. The M1 subunit appears to be nearly constant throughout the cell cycle of proliferating cells, whereas *de novo* synthesis of M2 correlates with S phase, suggesting that the reduction of ribonucleotides is directly dependent upon the concentration of M2 protein (Eriksson *et al.*,1984; Engstrom *et al.*, 1985). These observations are in keeping with the findings that hydroxyurea resistant cell lines with elevated levels of enzyme activity frequently contain increased levels of M2 (McClarty *et al.*,1986a; Thelander and Berg, 1986; Wright *et al.*,

1987). This point is strongly supported in the present study. Each successive drug selection step leading to the isolation of highly resistant cells was accompanied by stable increases in cellular resistance and ribonucleotide reductase activities. Furthermore, enzyme alterations at each step involved the M2 protein. Observations that changes in M2 occur independent of M1 in drug resistant cells is consistent with studies showing that M1 and M2 levels are controlled by different mechanisms in mammalian cells (Wright *et al.*, 1987; Engstrom *et al.*, 1985), and are in marked contrast to the situation in *E. coli*, where the two equivalent genes are located in one operon and their expression is coordinately regulated (Carlson *et al.*, 1984).

Several different molecular mechanisms were involved in the development of the hydroxyurea resistant phenotype. A very early event, occurring at the first step in the selection process, was the amplification of the M2 gene, which was accompanied by an elevation in M2 mRNA. There appeared to be a further increase in gene copy number after the first selection step (0.35 mM hydroxyurea), but only a modest change in M2 gene copies or mRNA levels were detected during the remaining selection steps (1.5 mM, 2.0 mM, 3.0 mM, 4.0 mM, and 5.0 mM drug). These observations contrast with the finding that cellular resistance increased significantly, especially during the latter stages of drug selection. Also, the highly drug resistant mouse cells used in this investigation do not appear to possess an enzyme activity less sensitive to hydroxyurea (McClarty *et al.*,1986b). EPR studies and Western blot analysis revealed that M2 pool sizes were markedly different among wild type, moderately resistant, and highly resistant cells, indicating that in addition to gene

amplification, post-transcriptional modification occurred during selection of hydroxyurea resistant cells. This additional mechanism was not due to changes in protein M2 half-life, but instead involved an increase in M2 protein biosynthetic rate. Total cellular protein synthesis, measured by incorporation of ^{35}S-methionine into proteins was essentially unchanged in the H^R-1.5 and H^R-5.0 cell lines (6.0×10^3 cpm/ug for H^R-1.5 and 6.1×10^3 cpm/μg for H^R-5.0 cells), which attests to the selectivity of increased M2 biosynthesis in H^R-5.0 cells. Therefore, by increasing the rate of protein M2 biosynthesis, without changing the rate of degradation, cells can accumulate very high levels of this key protein. Interestingly, this mechanism of increasing protein biosynthetic rates in the absence of mRNA elevations, has also been observed in other cell lines that overproduce highly regulated proteins such as ornithine decarboxylase (McConglogue *et al.*, 1986) or dihydrofolate reductase (Cowan *et al.*, 1986).

Mouse cells selected for the ability to proliferate in the presence of hydroxyurea concentrations as high as 4 mM exhibit changes that only involved the M2 subunit without detectable alterations to M1. However, both M1 and M2 are required for enzyme activity. It appears that the M2 subunit is limiting for activity in wild type mouse L cells, but as the level of M2 increases during selection of drug resistant variants, the M1 protein eventually becomes the limiting component for activity. This view is consistent with the observation that cells require an increase in the M1 protein, as well as M2, in order to survive concentrations of drug as high as 5.0 mM. Thus, an approximately 2-fold increase in M1 mRNA and protein was observed in the H^R-5.0 cell line, and this was probably important in achieving the very large elevation in ribonucleotide

reductase activity observed with these cells. The recent finding that selection for drug resistant mouse cell lines in the presence of very high concentrations of hydroxyurea (15 mM and 30 mM drug) can lead to the isolation of cell lines with M1 gene amplifications (Hurta and Wright, 1990b), is in agreement with the concept described above.

In summary, an early event that was observed in the establishment of a hydroxyurea resistant phenotype involves amplification of the M2 gene of ribonucleotide reductase. As cells become increasingly more resistant to this drug, other processes controlling the expression of the two subunits of ribonucleotide reductase are also altered (see Table 1). Furthermore, this study suggests that multiple mechanisms involving the regulation of the two proteins of ribonucleotide reductase are important determinants of chemotherapeutic sensitivity.

2. Effects of Hydroxyurea on Resistant Cells

In the past, it has been shown that the SC2 hydroxyurea resistant mouse cell line used in this study induces ribonucleotide reductase activity when grown in the presence of the chemotherapeutic agent hydroxyurea (McClarty *et al.*, 1986a, 1987). To better understand the mechanisms that underlie the altered activity in the mutant cell line and, perhaps, to learn something about the control of ribonucleotide reductase activity in normal cells, several factors were investigated. Any change in one of those factors could plausibly explain the drug induced increase in ribonucleotide reductase activity. The increase in reductase activity

in the SC2 cell line, upon exposure to hydroxyurea, has been shown to be both time and drug concentration dependent (McClarty *et al.*, 1986a). In order to determine whether these time and dose dependent increases were a result of elevations in protein M1, M2 or both M1 and M2, the cellular levels of both subunits were examined by Western blot analysis. The results presented in this thesis clearly demonstrated that both the M1 and M2 subunits of ribonucleotide reductase respond to hydroxyurea in a drug concentration dependent manner (Figure 13A and 13B). Both enzyme components were substantially elevated even in drug concentrations as low as 1 mM, and both subunits continued to increase even in concentrations of hydroxyurea as high as 10 mM. This result is in complete agreement with the earlier observations that enzyme activity continued to rise in response to increasing drug concentrations (McClarty *et al.*, 1986a). It is also evident from the results that the elevations in M1 and M2 protein occur in the absence of a corresponding change in mRNA levels for either subunit (Figure 13C).

The results of kinetic studies indicated that protein M2 levels rise much more rapidly, within 1 to 3 hrs, than protein M1 which showed no substantial increase until sometime between 6 and 24 hrs, after hydroxyurea addition (Figure 14A and 14B). It is interesting to note that no significant elevation in ribonucleotide reductase activity takes place until sometime between 6 and 18 hrs after drug addition (McClarty *et al.*, 1986a). Taken together, the data strongly suggests that, in contrast to wild type cells where protein M2 is normally limiting (Eriksson *et al.*, 1984; Choy *et al.*, 1988; Wright *et al.*, 1987; McClarty *et al.*, 1987), the M1 subunit is limiting in this mutant cell line and therefore regulates

the level of ribonucleotide reductase activity. Although one can not exclude the possibility that post-translational modification of either the M1 or M2 subunit may also play a role in the regulation of ribonucleotide reductase activity by hydroxyurea, the data supports the hypothesis that drug induced changes in enzyme activity occur mainly as a result of alterations in the amount of enzyme protein.

The results presented in this thesis clearly indicate that hydroxyurea can modulate ribonucleotide reductase expression post-transcriptionally. In this study, the rates of both synthesis and degradation of proteins M1 and M2 were directly determined by measuring the radioactivity of [^{35}S] methionine labeled M1 and M2 isolated from crude extracts by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The isolation procedure was satisfactory (Figure 15) and therefore this method gave accurate results. The half-lives of protein M1 and M2 in the SC2 mutant cell line grown out of drug are unchanged from that determined for both subunits in the parental wild type cell line. Measurements of the rate of turnover of proteins M1 and M2 in the mutant cell line grown in the presence of hydroxyurea showed that at least part of the drug induced elevation in both components could be accounted for by an approximate 2-fold increase in half-life (Figure 16A and 16B). The fact that there was no significant increase in cellular protein M1 levels until 6 to 24 hrs after hydroxyurea treatment as compared to 1 to 3 hours for protein M2 (Figure 14A and 14B) is likely a reflection of the longer half-life of the former. Numerous studies have reported on the effects of various metabolic inhibitors on the rate of degradation of dihydrofolate reductase (Alt *et al.*, 1976; Domin *et al.*, 1982; Cowan

et al., 1986), ornithine decarboxylase (Persson *et al.*, 1985; Pegg, 1986), and S-adenosylmethionine decarboxylase (Pegg, 1984; 1986; Shirahata and Pegg, 1985; Persson *et al.*, 1985). The mechanisms responsible for these stabilizations are not yet fully understood, however, it has been suggested that the presence of an inhibitor in the active site renders the enzyme less susceptible to proteolytic degradation (Schimke, 1973).

Changes in protein M1 and M2 stability account only in part for the regulation of ribonucleotide reductase levels by hydroxyurea. By measuring the rate of M1 and M2 protein biosynthesis directly in pulse labeling experiments, it was shown that an effect at the translational level, possibly increased translational efficiency, assumes a significant role in the hydroxyurea induced elevations of proteins M1 and M2 (Figure 17A and 17B, Table 2). In this connection, it is important to note that total cellular protein synthesis rates, measured by [^{35}S] methionine incorporation into proteins, was essentially unaffected by hydroxyurea treatment which attests to the selectivity of increased M1 and M2 biosynthesis rates in the presence of hydroxyurea. Inhibitors of ornithine decarboxylase activity alter polyamine pools and accumulating evidence from several laboratories indicates that it is the polyamines that regulate ornithine decarboxylase biosynthesis (Kahana and Nathans, 1985; Kanamoto *et al.*, 1986; Dircks *et al.*, 1986; Holtta and Pohjankelto, 1986; McConlogue *et al.*, 1986). Recently, it has been shown that polyamines have a direct inhibitory effect on the *in vitro* translation of mRNA for ornithine decarboxylase (Kameji and Pegg, 1987). Hydroxyurea is known to cause alterations in deoxyribonucleotide pools (Skoog and Nordenskjold, 1971; Nicander and Reichard, 1985), and hydroxyurea

resistant cell lines have been isolated, which exhibit changes in deoxyribonucleotide pools (Dick and Wright, 1984; Tagger and Wright, 1988). Whether or not any of the four deoxyribonucleotides can directly, or indirectly, through an unknown intermediate, affect M1 or M2 protein biosynthesis has not yet been elucidated.

Results of kinetic studies indicate that the increase in the rate of M1 and M2 biosynthesis in response to hydroxyurea occurs slowly with no significant effect until 24 hrs after the initial drug treatment (Table 3). The substantial increase in cellular protein M1 and M2 levels seen in SC2 cells continually (> 2 weeks) grown in the presence of hydroxyurea compared to cells exposed to drug for just 24 hrs (Figure 14A and 14B compare lanes e and f) is probably a result of this slow responding increase in biosynthesis rate of both proteins.

The effect that exogenously added iron has on M1 and M2 biosynthesis was experimentally explored in an attempt to gain some insight into the mechanism behind the translational regulation of ribonucleotide reductase. It has been known for quite a while that iron regulates the synthesis of ferritin, in many cells, at the level of translation (Zahringer *et al.*, 1976; Aziz and Munro, 1986; Mattia *et al.*, 1986; Theil, 1987). This regulation is believed to be mediated by a protein, which presumably binds iron and then interacts with a specific 5′ leader sequence of ferritin mRNA (Aziz and Munro, 1987; Hentze *et al.*, 1987). The results presented in this study indicate that exogenously added iron has no significant effect on protein M1 levels, however, iron treatment does lead to a decrease in cellular protein M2 levels but only when SC2 cells are grown in the presence of hydroxyurea (Figure 18). It is

interesting to note that iron treatment leads to an uncoupling of the M1 and M2 response to hydroxyurea (*i.e.*, M1 protein levels as estimated by Western blot were unaffected by iron whereas M2 levels decreased). This is the first preliminary indication that M1 and M2 may respond to independent regulatory signals. In addition it should be mentioned that iron treatment of cells, growing either in the absence or presence of hydroxyurea had no significant effect on M1 or M2 mRNA levels. However, iron did have a small but reproducible effect on the biosynthesis rate of both proteins M1 and M2, but once again this effect was only detected when SC2 cells were grown in the presence of hydroxyurea (Figure 17 and Table 2). The rate of M2 biosynthesis was decreased by iron treatment, not an unexpected result given the lower cellular protein M2 levels seen under similar conditions. Surprisingly, protein M1 biosynthesis rates were slightly elevated in the presence of iron, a result which is not reflected in cellular protein M1 levels which were unaffected by iron treatment. It was not determined whether the half-lives of proteins M1 and M2 were altered by iron treatment. Given that the effects of iron are only observed when cells are cultured in the presence of hydroxyurea, suggests that the metal may exert its action through hydroxyurea rather than directly itself. Clearly more work is required before it will be possible to determine the exact mechanism responsible for these effects. Interestingly, Fontecave *et al.* (1987) have recently described a ferric iron reductase in *Escherichia coli* that participates in the generation of the free radical of ribonucleotide reductase. They suggested that it may form part of a biological mechanism that regulates the activity of bacterial ribonucleotide reductase, by establishing the content of tyrosyl radical in the enzyme.

In summary, hydroxyurea increases the levels of ribonucleotide reductase without increasing the mRNA levels of either the M1 or M2 subunit. The results indicate that protein M1 and M2 levels rise proportionately with increasing hydroxyurea concentration. These elevations are brought about not only by increasing the stabilization of both proteins against degradation, but also by increasing their rates of biosynthesis. Furthermore, these changes are consistent with elevations in enzyme activity observed with cells grown in the presence of hydroxyurea. This is also the first observation that iron treatment affects the levels of M1 and M2 protein differently in the presence of hydroxyurea, indicating that the two enzyme components may respond to different regulatory signals.

3. Ferritin Expression in Hydroxyurea Resistant Cells

Upon exposure to hydroxyurea, whether *in vivo* or *in vitro*, the tyrosyl free radical of the non-heme iron containing M2 (B2) subunit of ribonucleotide reductase is reduced leading to a loss of enzyme activity (Reichard, 1988; McClarty, *et al.*, 1987). This thesis presents evidence indicating that hydroxyurea inactivated protein M2 possesses a destabilized iron center which can readily lose its iron.

Selection for resistance to hydroxyurea yields cell lines which overproduce the iron containing M2 subunit of ribonucleotide reductase, generally as a result of amplification of the M2 gene (Wright, 1989; Wright *et al.*, 1989). During such a selection procedure, cells are continually exposed to hydroxyurea which results in the reduction of the

tyrosyl free radical and destabilization of the iron center of protein M2. During selection for hydroxyurea resistance, there may be a need for ferritin to sequester excess iron released from inactive protein M2 and, at the same time, there could be a requirement for ferritin associated iron for regeneration of active M2. The fact that increased expression of H and/or L-ferritin mRNA was observed in all 13 hydroxyurea resistant cell lines surveyed (four of which are reported here) indicates that ferritin may well fulfill one, if not both, of these functions. These results imply that even though protein M2 is the primary target of hydroxyurea, changes in ferritin expression may have to occur simultaneously with changes in M2 expression in order to develop the drug resistance phenotype.

The stable elevations in H and/or L-mRNA seen in the variety of resistant cell lines surveyed could result from increased transcriptional activity or increased mRNA stability. If one assumes these changes are a result of increased transcriptional activity, then it suggests that the expression of H and L-genes can be, but need not be coordinated, and that different factors may regulate H and L transcriptional activity. A similar conclusion has been reached by others studying ferritin gene expression in response to iron (Cairo *et al.*, 1985; Dickey *et al.*, 1987; White and Munro, 1988), agents such as dimethyl sulfoxide and 12-*O*-tetradecanoylphorbol-13-acetate that induce differentiation (Beaumont *et al.*, 1987; Chou *et al.*, 1986), the hormone thyrotropin (Ursini and de Franciscis, 1988; Cox *et al.*, 1988), and the cytokine tumor necrosis factor (Torti *et al.*, 1988).

Results of the more comprehensive study on the hydroxyurea resistant mouse L cell line indicates that the regulation of ferritin expression in

the mutant cells can be exceedingly complex involving changes at the transcriptional, translational and post-translational level. Surprisingly, the increase in L-mRNA in the resistant mouse cells does not result in a corresponding elevation of L subunit protein as assessed by Western blot analysis. This discrepancy was clarified when it was discovered that there was also no increase in the rate of L subunit biosynthesis in the resistant cell line. Presumably, the additional L mRNA is incorporated into the non-translatable pool of ferritin mRNA in messenger ribonucleoprotein rather than into functional polysomes (Aziz and Munro, 1986; Zahringer, *et al.*, 1976). Results of the Western blot analysis indicated that there was an increase in the level of H subunit protein in the resistant cell line grown in the absence of hydroxyurea even though there was no corresponding elevation in H-mRNA levels or H subunit biosynthesis. In addition, results from the longer term (6 hr) [^{35}S] methionine labeling experiments suggest that both the wild type and the hydroxyurea resistant cell lines post-translationally process the 22,500 molecular weight precursor. The first processing event gives rise to the 20,000 molecular weight intermediate, which is subsequently processed to the 18,000 molecular weight band, presumably representing the mature H ferritin subunit observed on the Western blot (Figure 18).

It has previously been shown that H-rich ferritins accept and release iron more readily than L-rich ferritins (Wagstaff *et al.*, 1978; Jones *et al.*, 1978). Furthermore, H-rich ferritin shells turnover more rapidly than L-rich ferritin shells which, consequently perhaps, accumulate more iron (Kohgo *et al.*, 1980). These metabolic differences have led to suggestions that the H subunit plays a key role in intracellular traffic of iron, whereas the L subunit is better suited for

long term iron storage (Boyd *et al.*, 1985). The data in this thesis supports this hypothesis. Clearly, one can postulate that in the resistant cell line, ferritin is required for detoxification of protein M2 released iron, and as a source of readily available intracellular iron for M2 activation, it is advantageous to have H-rich isoferritins which readily take up and release iron. This is in contrast to situations of high iron "stress" where it appears, at least in the rat liver, to be advantageous to synthesize L-rich isoferritins which retain iron more firmly (White and Munro, 1988).

The observation that iron stimulates H and L subunit biosynthesis in both the wild type and resistant cell lines suggests that iron mobilizes ferritin mRNA from messenger ribonucleoprotein particles to functional polysomes as it does in other systems (Aziz and Munro, 1985; Zahringer *et al.*, 1976). An intriguing observation which resulted from our studies is that growth of the resistant cells in the presence of hydroxyurea leads to an increase in the rate of ferritin biosynthesis in the absence of changes in H or L-mRNA levels. Together, these findings suggest that hydroxyurea, either directly or indirectly, leads to a mobilization of ferritin mRNA to active polysomes (Aziz and Munro, 1986; Zahringer *et al.*, 1976). Similar to iron, hydroxyurea causes a coordinate elevation in both H and L subunit biosynthesis, a result consistent with activation of the translational regulatory mechanism. Surprisingly, the increase in H and L ferritin biosynthesis observed in the presence of hydroxyurea did not lead to an appreciable increase in cellular L ferritin subunit levels, although a small increase in H-ferritin subunit was observed. One way to rectify this anomolous result would be to increase the rate at which ferritin shells turnover in the resistant cells growing

in the presence of hydroxyurea. Results show that hydroxyurea can affect the half-life of both the M1 and M2 subunits of ribonucleotide reductase in this resistant mouse cell line.

Given the apparent instability of the inactive M2 iron centre, it is tempting to speculate that iron is released from the inactive M2 subunits and subsequently stimulates the rate of ferritin biosynthesis. However, the fact that there is no substantial change in TfR mRNA levels or receptor numbers, two components which have been shown to rapidly respond to alterations in intracellular iron levels (Rao *et al.*, 1985, 1986), suggests that there is no major change in intracellular iron homeostasis in resistant cells when they are grown in the presence of hydroxyurea. In addition, when resistant cells are treated with hydroxyurea and iron, there is a synergistic rather than additive effect on the rate of ferritin biosynthesis. This result also suggests that the two agents may affect biosynthesis through different mechanisms.

In summary, inactivation of the M2 subunit of ribonucleotide reductase with hydroxyurea leads to a destabilization of the protein iron centre allowing it to readily release iron. In addition, an early and apparently important event in the establishment of a hydroxyurea-resistant phenotype, involves alterations in the expression of the iron storage protein, ferritin. A detailed study using a hydroxyurea resistant mouse L cell line indicates that regulation of ferritin expression in the mutant cells can be complex. The hydroxyurea resistant cell lines described in this report will be useful for future studies on the importance of ferritin in the intracellular traffic of iron.

4. TPA Induction of Ribonucleotide Reductase

Phorbol esters such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) are potent tumor promoters, capable of potentiating the effect of a subcarcinogenic dose of an initiating carcinogen (Diamond, 1984). The prevailing hypothesis is that tumor promoters exert their biological effect by inducing an altered program of gene expression, a process that involves the activation of protein kinase C. It appears that this enzyme is the major target of TPA and other types of tumor promoters (Nishizuka, 1984). Among the genes whose transcription is induced by TPA are cellular proto-oncogenes like *c-fos* (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984), *c-myc* (Greenberf and Ziff, 1984; Kelly *et al.*, 1983), and *c-sis* (Colamonici *et al.*, 1986), which could be responsible for the loss of growth control. Other targets of TPA induction are the collagenase (Whitham *et al.*, 1986) and stromelysin (Matrisian *et al.*, 1986; Whitham *et al.*, 1986) genes, which may have a role in tumor invasiveness, metastasis (Mignatti *et al.*, 1986) and angiogenesis (Montesano and Orci, 1985).

The results of this study indicate for the first time that treatment of mammalian cells with a tumor promotor can cause a significant increase in ribonucleotide reductase activity, M2 message, and M2 protein. Interestingly, a highly regulated enzyme (Wright, 1989) which is normally coupled to the S-phase of the cell cycle (Thelander *et al.*, 1980; Lewis *et al.*, 1978) and is rate-limiting for DNA synthesis (Wright, 1989), can be markedly modified in a transient manner by the presence of TPA. The changes occurred very rapidly with proliferating BALB/c 3T3 cells (within ½ hour), which would appear to eliminate the possibility that a block at

S-phase or the movement of cells into S-phase could account for the obvious TPA-induced modifications of ribonucleotide reductase. It will be interesting to determine the effects of TPA treatment on cells synchronized at different phases of the cell cycle.

As a positive control, the time kinetics of ODC message induction in the presence of TPA was measured, and found to approximately follow previously reported observations (Gilmour *et al.*, 1985). Elevation of M2 message occurred more rapidly than ODC message, and appears to resemble more closely the TPA induction of *c-myc* (maximal at 1 hour) and *c-fos* (maximal at 15 to 30 minutes) transcripts (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984). The relationship between the TPA mediated effects upon ribonucleotide reductase and upon other early mitogenic responsive genes is not known, but may reflect a role for ribonucleotide reductase, particularly protein M2, in both the competence and progression stages of the cell cycle.

Although TPA activates protein kinase C (Nishizuka, 1986), the pathway responsible for transducing the signal generated by protein kinase C activation is not known. However, the promotor regions of several TPA inducible genes share a conserved region which confers TPA inducibility (Angel *et al.*, 1987). These TPA-responsive elements (TREs) are recognized by the transcription factor AP-1, suggesting that AP-1 is involved in a complex pathway responsible for transmitting the effects of phorbol ester tumor promoters to the transcriptional machinery of the cell (Lee *et al.*, 1987; Angel *et al.*, 1987). Purified AP-1 is able to activate transcription *in vitro* of the wild type human metallothionein II_A (hMT II_A) but not mutant hMT II_A promoters lacking AP-1 recognition sites. Multiple synthetic copies of the concensus AP-1 binding site can act as TPA

inducible enhancers in various plasmid constructs after transfection into HeLa and monkey Cos cells (Lee *et al.*, 1987; Angel *et al.*, 1987).

Whether the M2 promotor also possesses a TRE like element remains to be determined. Initial screenings for a TRE concensus in a published M2 DNA sequence (Thelander and Thelander, 1989) for first 501 base pairs immediately upstream of the M2 transcription initiation site yielded no such homologous sites. It is possible for a TRE like element further upstream may contribute to the TPA inducibility of the M2 gene. Only further cloning and sequencing of the 5′ upstream DNA sequences of the M2 gene would be able to answer this question.

Others have reported that transcription factor AP-2, a 52,000 dalton nuclear protein, can mediate, *in vitro* and *in vivo*, inducibility of heterologous promoters by TPA (Mitchell *et al.*, 1987; Imagawa *et al.*, 1987). A presumptive AP-2 binding site at -184 bp was located in the M2 promoter. This M2 presumptive AP-2 binding site contains a core concensus sequence and several flanking sequence that have a very high homology to the TPA inducible AP-2 site at -180 of the hMT II_A (Imagawa *et al.*, 1987). Out of 15 nucleotides in the homologous region, 13 nucleotides are conserved, and the core concensus 8 bp 5′-CGCCCGCG-3′ is conserved completely. Thus, it is conceivable that this site may mediate TPA inducibility in the M2 gene, or the M2 presumptive AP-2 binding site can mediate TPA inducibility in concert with an hitherto undiscovered AP-1 binding site in the M2 promoter.

DNA Electromobility Shift Assays showed that MTAP-2, a 50 bp DNA probe containing the presumptive AP-2 binding site, was able to form a tightly bound DNA-protein complex when incubated with HeLa and 3T3 nuclear protein extracts (Fig. 32, Fig. 33). Experiments with a DNA probe

containing an AP-2 binding site showed that the protein bound could be competed off the MTAP-2 probe by the competitor AP-2 binding site. This suggests that the protein bound to the MTAP-2 DNA probe may be AP-2.

The MTAP-2 probe appears to form only one complex with nuclear extracts prepared from HeLa cells, and forms two complexes with nuclear extracts prepared from BALB/c 3T3 cells. The formation of two complexes with a putative AP-2 binding site has been reported previously (Bishop *et al.*, 1990). The lower complex (Fig.33, lane b) binds to the MTAP-2 probe less tightly than the higher complex. Endogenous AP-2 protein purified by affinity chromatography has been shown to co-migrate in glycerol gradients with a 116 kD marker protein, in contrast to denaturing SDS-PAGE gels in which only a single 52 kD protein species is detected (Mitchell *et al.*, 1987). It has therefore been suspected that the native AP-2 DNA binding complex exists as a homodimer in solution. Since both complexes formed with the MTAP-2 probe can be competed with an AP-2 competitor probe, it is possible that AP-2 forms both complexes. The lower complex may result from a monomer AP-2 protein binding the putative AP-2 binding site, and the slower migrating higher complex may result from a homodimer AP-2 binding to the site. A similiar situation was shown to be true for the binding of the cAMP responsive element binding protein (CREB) to the cAMP responsive element (CRE) (Yamamoto *et al.*, 1988; Ganzalez *et al.*, 1989). A single monomer of CREB was able to weakly bind to the CRE, however when the protein is phosphorylated, CREB was able to bind the CRE tightly as a homodimer.

Reports of AP-2 transcriptional activation of genes showed that the addition of AP-2 protein contributed to enhanced *in vitro* transcription of a reporter gene (Mitchell *et al.*, 1987), and that TPA induction of a

heterologous reporter gene required the DNA sequences containing the AP-2 binding site from the hMT II_A gene. The gel shifts of MTAP-2 with nuclear extracts from TPA treated BALB/c cells displayed results that appears to rule out the involvement of this putative AP-2 site in TPA induction of the M2 gene. No increase in AP-2 binding or increase in the formation of the higher complex was observed with TPA treated nuclear extracts; on the contrary, the protein-DNA complex formation was signifigantly reduced when the M2 gene is induced *in vivo* by TPA. The reduction or loss of what is presumably an AP-2 protein when the cell is stimulated with TPA, resulting in increased M2 message levels, seems contrary to reports that AP-2 is a transcriptional activator (Mitchell *et al.*, 1987).

Whether this M2 putative AP-2 binding site is able to mediate TPA induction can only be answered by inserting this sequence upstream of a transcription reporter system. Current efforts are underway to construct plasmids that would contain one and multiple copies of the M2 putative AP-2 binding site upstream of the thymidine kinase (TK) TATA-box promoter and the chloramphenicol transferase (CAT) gene. This would determine if the putative AP-2 binding is able to and is sufficient for mediating TPA induction. Efforts are also underway to clone and insert 5′ genomic mouse and human M2 promoter DNA sequences into the TK-CAT plasmid. Through a series of deletion analyses of the 5′ promoter DNA sequences, it is hoped that the TPA inducible element may be found.

The fact that M2 is so quickly and signifigantly elevated by TPA, ribonucleotide reductase may play an important role in carcinogenesis. These future studies of the AP-2 site and a possible TPA inducible element

will attempt to understand the significance of ribonucleotide reductase in those events important to the process of tumor promotion.

5. Future Directions

The differences of the steady state protein M2 expressed between the moderately resistant H^R-1.5 cell line and the highly resistant H^R-5.0 cell line was striking. The differences appears to be a result of increased biosynthetic rates of protein M2 due to increased translational efficiency in H^R-5.0. Future studies would focus on the M2 mRNAs transcribed in the two cell lines. There may be differences in mRNA species; *i.e.*, differences in the 5′ and 3′ untranslated regions due to alternative splicing or RNA processing between the two cell lines. RNA polymerase chain reaction sequencing and S1 nuclease protection assays with the cloned cDNA may provide answers to alternate splice sites. If there are RNA sequence differences among wild type, H^R-1.5, and H^R-5.0, then these alternate RNA sequences may be reverse transcribed into double stranded DNA, and fused to heterologous reporter cDNAs to assay for any ability to modulate translational activity. These RNA fusion sequences would also be helpful in dissecting the regions, if there are any, that were responsible for the elevated M1 and M2 biosynthetic rates induced when the cells were cultured in the presence of hydroxyurea.

The data presented in this thesis strongly suggests a role for ferritin in developing the hydroxyurea resistant phenotype. Since cDNAs are available for both H and L ferritins, eukaryotic expression plasmids could be constructed. Co-transfection of ferritin expression plasmids

and M2 expression plasmids into wild type cells should augment or increase the degree of hydroxyurea resistance as compared to cells transfected with M2 expression plasmids.

The TPA inducibility of M2 data shows a possible involvement of an AP-2 binding site upstream of the promoter. In order to directly test if AP-2 can mediate the TPA inducible response, the 50 bp MTAP-2 sequence would be inserted upstream of an heterologous herpes simplex thimidine kinase (TK) promoter in the plasmid vector pBL2CAT. When the plasmid is transiently transfected in mammalian cells, the TK promoter is capable of only basal transcription and therefore a low level expression of the downstream reporter gene, chloramphenicol acetyl transferase (CAT). Insertion of MTAP-2 upstream of the TK promoter would show whether this sequence is able to confer the TPA response to a heterologous promoter. It is conceivable that the MTAP-2 sequence alone is not sufficient to elicit such a response. The other approach would involve inserting cloned 5′ upstream DNA sequences from the M2 gene into the pBL2CAT vector. Deletion and mutational analysis may define the regions involved.

Alternatively, Imagawa *et al.* (1987) have reported the AP-2 can also mediate a cyclic AMP (cAMP) inducible response of the hMT II_A gene. If AP-2 is the pathway for M2 induction by TPA, then M2 may also be induced by cAMP. Forskolin was used to stimulate adenylate cyclase in cells to show the hMT II_A gene induction by cAMP (Imagawa *et al.*, 1987). M2 mRNA and protein would be examined in forskolin treated cells. Cells transiently transfected with the various pBL2CAT - M2 promoter derivatives could also be treated with forskolin and the effects on the CAT reporter gene.

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