HYDROXYUREA RESISTANCE AND PROTEIN PHOSPHORYLATION OF RIBONUCLEOTIDE REDUCTASE IN G_0 / EARLY G_1 ARRESTED MOUSE CELLS

BY

HUNG YI LAU, B.Sc.(Hons.)

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

ABSTRACT

Mammalian ribonucleotide reductase occupies a key rate-limiting position in DNA synthesis and in cell proliferation. It is composed of two non-identical subunits, R1 and R2, and the R2 subunit is the target for the antitumor agent hydroxyurea. A hydroxyurea resistant mouse cell line, H^R3T3, was isolated and characterized in this study. The decrease in sensitivity to hydroxyurea observed in H^R3T3 cells was accompanied by amplification of the R2 gene, elevated levels of R2 mRNA, and elevated R1 and R2 protein levels when compared to parental wild type BALB/c 3T3 cells. H^R3T3 cells cultured in the presence of hydroxyurea showed a further increase in both R1 and R2 protein levels, and only the R2 mRNA level was elevated. The above observations may be due to regulatory changes at the level of transcription, post-transcription, and/or translation. It is known that the R2 protein can be phosphorylated at a serine residue near the N-terminus. Two-dimensional phosphopeptide maps of *in vivo* labeled R2 proteins isolated from wild type and mutant 3T3 cells showed no change in the phosphorylation pattern of the R2 protein when the two cell lines were compared, indicating that drug resistance does not involve an alteration of R2 phosphorylation. Results obtained from *in vitro* phosphorylation of recombinant R2 protein by various purified protein kinases suggested the possible involvement of protein tyrosine kinases in R2 phosphorylation. To examine ribonucleotide reductase regulation in quiescent cells, wild type and hydroxyurea-resistant cells were arrested by serum-starvation. Ninety percent of the cell population was arrested at G_0/G_1, as shown by flow cytometric studies. Low levels of R1 and R2 mRNAs were detected, which could be due to increased message half-lives. Decreases in protein levels were also detected in quiescent cells, and the extent of these decreases roughly paralleled the decreases detected in mRNA levels. R2 protein degradation was particularly noticeable in quiescent H^R3T3 cells. Two-dimensional phosphopeptide mapping suggested that R2 protein degradation is not mediated by changes in phosphorylation pattern.

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Special thanks and appreciation from the bottom of my heart to my parents, Shok-Chun and Hoi-Kai Lau, for their support, encouragement and endless love. Thank you for giving me this chance of education, Mom and Dad.

Also to my beloved brothers and sisters, thank you for your support, love and prayers.

Last but not least, I wish to give thanks to my Savior and my Lord, Jesus Christ, for granting me a renewed life filled with hope, joy, and love.

This Thesis Is Dedicated to My Parents

Shok-Chun and Hoi-Kai Lau

With Love

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ABBREVIATIONS

α-MEM	Alpha modified minimal essential medium
ADP	Adenosine 5'-diphosphate
AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 5'-monophosphate
cDNA	Complementary DNA
CDK	Cyclin dependent kinase
CDP	Cytidine 5'-diphosphate
CO_2	Carbon dioxide gas
cpm	Counts per minute
dADP	2'-Deoxyadenosine 5'-diphosphate
dATP	2'-Deoxyadenosine 5'-triphosphate
dCDP	2'-Deoxycytidine 5'-diphosphate
dCTP	2'-Deoxycytidine 5'-triphosphate
dGDP	2'-Deoxyguanosine 5'-diphosphate
dGTP	2'-Deoxyguanosine 5'-triphosphate
D-MEM	Dulbecco's modified Eagles's medium
dNTP	2'-Deoxyribonucleotide 5'-triphosphate
dTTP	2'-Deoxythymidine 5'-triphosphate

dUDP	2'-Deoxyuridine 5'-diphosphate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP-lysine	ε-Dinitrophenyl-lysine
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetate
EPR	Electron paramagnetic resonance
FACS	Fluorescent-activated cell sorting
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine 5'- diphosphate
HCl	Hydrogen chloride
HIV-1	Human immunodificiency virus type 1
IgG	Immunoglobin G
MAP	Mitogen-activated protein
$MgCl_2$	Magnesium chloride
MOPS	3-(*N*-morpholino)Propanesulfonic acid
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide

Na_2HPO_4	Disodium hydrogen phosphate
NP-40	Nonidet P-40
PBS	Phosphate bufered saline
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TGF-β_1	Transforming growth factor β_1
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TPCK	*N*-tosyl-L-phenylalanine chloromethyl ketone
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UDP	Uridine 5'-diphosphate

I. LITERATURE REVIEW

Ribonucleotide Reductase and Hydroxyurea Resistance

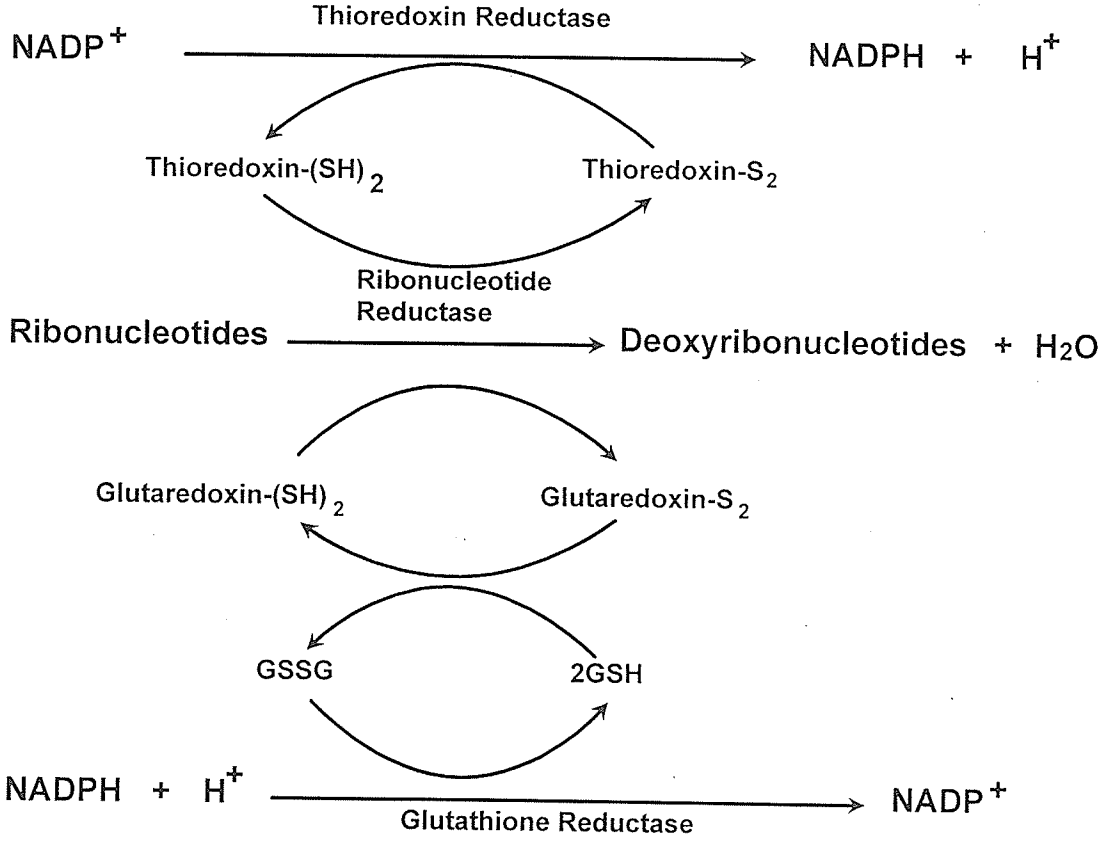
1. Ribonucleotide Reductase

1.1 Background

Ribonucleotide reductase (EC 1.17.4.1) is a highly regulated enzyme that catalyzes the direct reduction of ribonucleotides to the corresponding deoxyribonucleotides at the 2'-carbon position of the ribose moiety, providing a balanced supply of precursors for DNA synthesis (Thelander and Reichard, 1979; Wright, 1989; Wright *et al.*, 1990a). In mammalian cells, this reaction occurs at the diphosphate level. The reduction reaction is shown in Figure 1. Two small proteins, thioredoxin in the thioredoxin reductase system, and glutaredoxin in the glutathione and glutathionine reductase system, containing sulfhydryl groups are oxidized to disulfide during the reaction, and act as intermediate hydrogen carriers. The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) provides the reduction potential for converting these two proteins back to their reduced forms (Wright, 1989). Recent studies using thioredoxin/glutaredoxin double mutants suggest the possible existence of a new glutathione-dependent hydrogen donor for ribonucleotide reductase (Aslund *et al.*, 1994; Miranda Vizuete *et al.*, 1994).

Since ribonucleotide reductase is the only enzyme responsible for the conversion of ribonucleotides to deoxyribonucleotides, it plays a crucial role in DNA synthesis, cell proliferation and also DNA repair (Wright *et al.*, 1990a; Hurta and Wright, 1992a).

Figure 1. Reduction of ribonucleotides to deoxyribonucleotides by mammalian ribonucleotide reductase. Figure adapted from Wright. 1989.


NADP+
Thioredoxin Reductase
NADPH + H+
Thioredoxin-(SH)2
Thioredoxin-S2
Ribonucleotide Reductase
Ribonucleotides
Deoxyribonucleotides + H2O
Glutaredoxin-(SH)2
Glutaredoxin-S2
GSSG
2GSH
NADPH + H+
Glutathione Reductase
NADP+

Furthermore, alterations in ribonucleotide reductase activity and R1 and/or R2 gene expression have been observed under a variety of conditions. Due to its significant role in the biology of the cell, numerous studies have been conducted by various groups in the past 20 years, all aimed to obtain a better understanding of this unique enzyme.

1.2 Classes of Ribonucleotide Reductase

Three classes of ribonucleotide reductases have been described (Reichard, 1993), and enzymes from all three classes use the same chemical mechanism in spite of their differences in primary protein structure (Eliasson *et al.*, 1994,1995). A protein radical is used for the abstraction of a hydrogen atom from the C-3' of the ribose moiety. This facilitates the removal of the hydroxyl group from the C-2', which is subsequently reduced by the dithiol functionality of a small protein (Holmgren, 1989).

Class I ribonucleotide reductases are found in some prokaryotes and in all higher organisms, and the enzyme isolated from aerobically grown *E. coli* is the prototype of this class. It consists of two different proteins, called R1 and R2, and the functional enzyme has a $\alpha_2\beta_2$ protein structure. Enzymes in this class contain a diferric oxygen-linked iron center and a stable tyrosyl-free radical which generates a transient cysteinyl radical by intramolecular electron transfer during the catalytic reaction (Mao *et al.*, 1992; Uhlin and Eklund, 1994), and oxygen is required for the generation of the tyrosyl-free radical. Ribonucleotide reductase isolated from *Lactobacillus leichmannii* is the prototype of class II enzymes, which use adenosyl cobalamin as the radical generator (Blakley and Barker, 1964). It can be found in both aerobic and anaerobic microorganisms. The enzyme is a

monomer with size comparable to that of one of the protomers of *E. coli* R1. Class III enzymes only operate during anaerobiosis, for which the anaerobic *E. coli* reductase is the prototype (Mulliez *et al.*, 1993). S-adenosylmethionine is used as radical generator. It is a homodimer, and in its active form, it contains an iron-sulfur center and an oxygen sensitive glycyl radical (Sun *et al.*, 1993; Mulliez *et al.*, 1993).

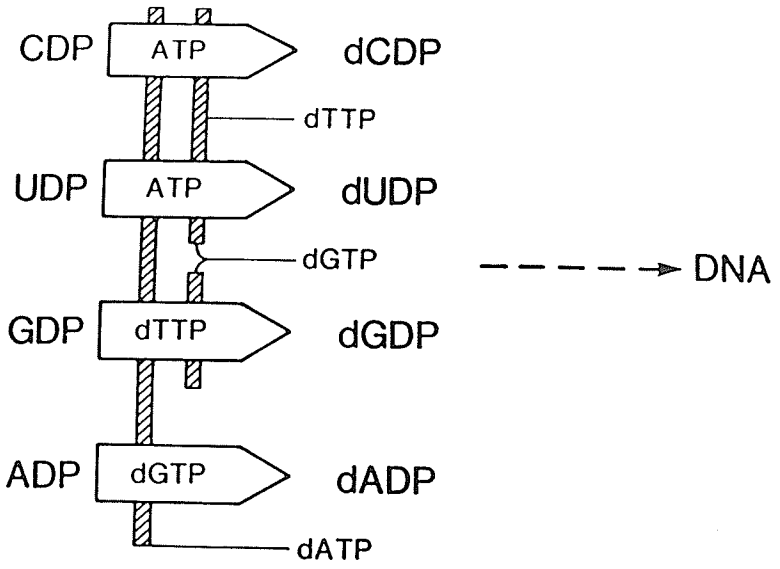
Additional reductases have been described which do not fit into any of the above categories (Willing *et al.*, 1988; Sze *et al.*, 1992), and yet further investigation is needed before assigning them into new classes.

1.3 Mammalian Ribonucleotide Reductase

1.3.1 Allosteric Regulation of Mammalian Ribonucleotide Reductase

Since ribonucleotide reductase is solely responsible for the *de novo* reduction of ribonucleotides to their corresponding deoxyribonucleotides, it requires rigid cellular control of its activity for the balanced production of all four deoxyribonucleotides. The enzyme substrate specificity and activity is strictly regulated in a complex manner by nucleoside triphosphate effectors (Figure 2). The reduction of CDP to dCDP and UDP to dUDP requires the presence of an ATP-activated enzyme. The reduction of GDP to dGDP requires dTTP, and dGTP is needed for the reduction of ADP to dADP. A decline in DNA synthesis leads to the accumulation of dATP, which in turn acts as an overall negative effector of all four ribonucleotide reductions. In addition to the above, dTTP is an inhibitor of pyrimidine reduction and dGTP is a negative feedback inhibitor of GDP reduction and pyrimidine reduction. This regulatory scheme suggests that ribonucleotide

Figure 2. Allosteric regulation of mammalian ribonucleotide reductase. (see Wright, 1989)


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

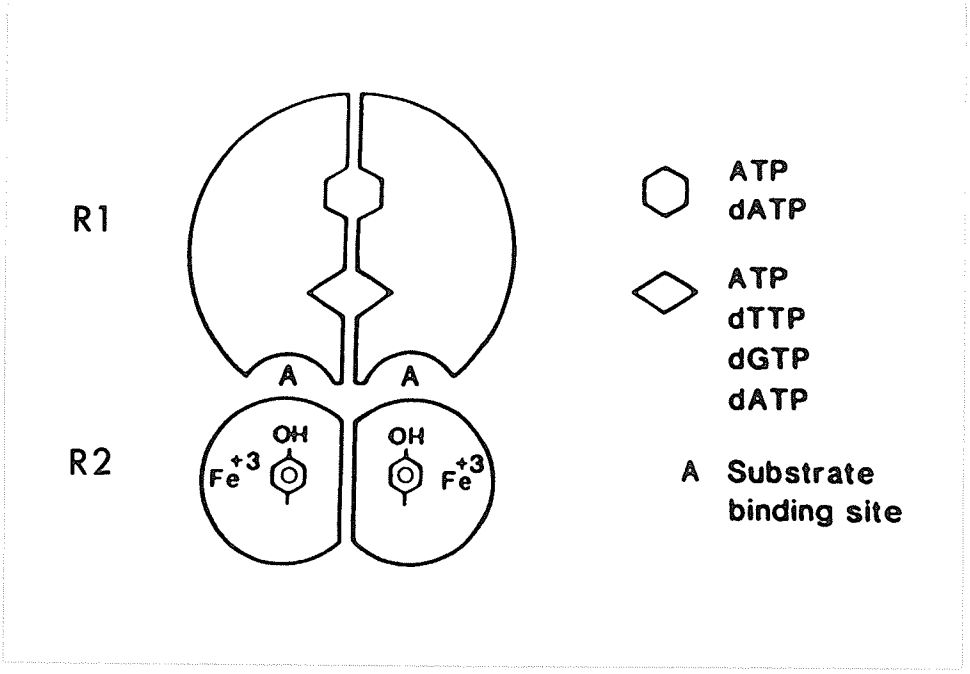
reduction may begin with a reduction of CDP and UDP by an ATP-activated enzyme, proceeds to GDP reduction via a dTTP-regulated activity, and finally reaches ADP reduction by a dGTP-activated enzyme (Wright, 1989; Wright *et al.*, 1990a). Furthermore, ribonucleotide reductase enzyme activity is modulated by metals. Iron and copper are found to stimulate enzyme activity whereas zinc has an inhibitory effect (Oblender and Carpentieri, 1990, 1991). There is also a suggestion that there may be a separate reductase activity in the mitochondria, which is responsible for maintaining pools of deoxyribonucleotides that is different from the much larger whole-cell pools (Young *et al.*, 1994).

1.3.2 Structural Features of Mammalian Ribonucleotide Reductase

As described earlier, mammalian ribonucleotide reductase is composed of two dissimilar protein components that are referred to as R1 and R2 (Figure 3), which are coded by different genes. The gene encoding R1 is located on human chromosome 11 (Engstrom and Francke, 1985), whereas the R2 gene is located on human chromosome 2. There are also inactive R2-related sequences (pseudogenes) on human chromosomes 1 and X (Yang-Feng *et al.*, 1987).

R1 protein exists as a homodimer with a molecular weight of 170,000 (Thelander et al., 1980). The dimeric molecule is about 110Å long, thin in the middle around the molecular two-fold axis (Uhlin *et al.*, 1993), and contains the binding sites for substrates and allosteric effectors. Circular dichroism spectroscopy shows that the R1 protein has an α-helical content of 50% (Davis *et al.*, 1994). R1 is not phosphorylated *in vivo* (Chan

Figure 3. Model of mammalian ribonucleotide reductase showing the R1 and R2 components. Protein R1 forms a homodimer, which contains the binding sites responsible for regulating overall enzyme activity (○) and substrate specificity (◇). Protein R2 also forms a homodimer, and the dimeric R2 protein contains non-heme iron centers which help stabilize the tyrosyl free radical. Note that the iron content per R2 component is higher than that shown in the figure. Figure adapted from Wright, 1989; Wright *et al.*, 1990a.


R1
R2
A
A
OH
OH
Fe^{+3}
Fe^{+3}
ATP
dATP
ATP
dTTP
dGTP
dATP
A Substrate binding site

et al., 1993), and it is still under debate whether or not it is a glycoprotein (Sikorska *et al.*, 1990; Davis *et al.*, 1994).

R2 protein is also a homodimer, with a molecular weight of 88,000 (Thelander *et al.*, 1985; McClarty *et al.*, 1987), and the α-helical content is about 50% in mouse protein (Mann *et al.*, 1991). It has been shown to be a phosphoprotein *in vivo* without the ability to autophosphorylate (Chan *et al.*, 1993). The phosphorylation site is believed to be at a serine residue within 20 amino acids of the N-terminal end. The dimeric R2 protein contains two non-heme iron centers that are about 25Å apart, with each center formed by two ferric ions coordinated by four carboxylates and two histidine ligands (Aberg *et al.*, 1993). The non-heme iron centers are required for the stabilization of a unique tyrosyl-free radical at position 122 (Nordlund *et al.*, 1990). This organic free radical is necessary for enzyme activity (McClarty *et al.*, 1987, 1990), and is located 5Å from the closest iron atom (Nordlund *et al.*, 1990).

A functional enzyme requires the formation of a tetramer which consists of both R1 and R2 dimers, and dimerization involves 7 residues in the mobile carboxyl terminus of R2 protein interacting with a region close to the carboxyl terminus of the R1 protein (Davis *et al.*, 1994; Laplante *et al.*, 1994). The catalytic center is located at the interface between the two subunits, with R1 contributing the redox active dithiols (Thelander, 1974) and R2 contributing the tyrosyl radical and non-heme iron centers. The redox activity of R1 involves 2 cysteines at positions 225 and 462, which are held close by their location on two adjacent anti-parallel strands in a ten-strand α/β-barrel (Uhlin and Eklund, 1994).

A recent site-directed mutagenesis study also provides evidence for a long-range electron pathway between the active site and the tyrosyl-free radical involving tryptophan and aspartic acid residues in the R2 protein (Rova *et al.*, 1995).

1.4 Biological Features of Ribonucleotide Reductase

1.4.1 Role in DNA Synthesis

Reduction of ribonucleotides is a rate-limiting step in the synthesis of DNA (Wright, 1989), and a tight relationship between the presence of ribonucleotide reductase and DNA synthesis has been observed (Lewis *et al.*, 1978; Weber, 1983). The specific activity of the enzyme increases dramatically just prior to and during DNA synthesis (S-phase), and returns to basal levels near the end of S-phase (Lewis *et al.*, 1978; Lewis and Wright, 1979). Moreover, ribonucleotide reductase has been described as a mutator gene. Alterations in enzyme activity lead to an imbalance of deoxyribonucleotide pools, the substrates for DNA polymerase, which significantly modify spontaneous mutation rates of cells (Weinberg *et al.*, 1981).

1.4.2 Role in Cell Proliferation

The close correlation between reductase activity and DNA synthesis implies the significance of its role in cell proliferation. Enhanced levels of ribonucleotide reductase are characteristic of rapidly proliferating tissues such as the thymus, bone marrow, spleen and intestine, whereas the enzyme level is low or undetectable in non-dividing cells (Millard, 1972; Elford, 1972; Takeda and Weber, 1981). In actively proliferating cells,

S phase seems to be dependent upon the synthesis of R2 protein, which is rate-limiting for ribonucleotide reduction (Wright *et al.*, 1990a; Bjorklund *et al.*, 1990). Studies done in budding yeast, *Sacchromyces cerevisiae*, and in fission yeast, *Schizosacchromyces pombe*, also demonstrate that mutations in the yeast analogue of R1 and/or R2 can either interfere with normal cell cycle progression (Laman *et al.*, 1995), or it can be lethal (Fernandez Sarabia *et al.*, 1993).

1.4.3 Role in DNA Repair

In addition to its role in DNA replication, ribonucleotide reductase activity is also required for repairing damaged DNA. In fact, in the yeast *Saccaromyces cerevisiae* encode a second large subunit gene of ribonucleotide reductase that is expressed only in the presence of DNA damage (Elledge and Davis, 1990; Elledge *et al.*, 1992). When enzyme activity is inhibited, it leads to an inhibition of DNA repair in cells (Hongslo *et al.*, 1993; Rannug *et al.*, 1995). Furthermore, alterations in reductase activity and regulation have been observed both in yeast and in mouse cells following exposure to DNA damaging agents at cytotoxic concentrations (Elledge and Davis, 1989; Hurta and Wright, 1992a). These include transient increases in enzyme activity, mRNA and protein levels of both R1 and R2, as well as changes in the transcriptional process of both R1 and R2 genes. The induction of reductase activity may occur outside of S-phase in mammalian cells since no unusual shift of the cell population into S-phase has been detected (Hurta and Wright, 1992a).

1.4.4 Biological Significance of Altered Enzyme Activity

Alterations in ribonucleotide reductase are associated with major changes in the biological properties of cells (Wright, 1989). For example, elevated ribonucleotide reductase activity is tightly linked with malignant transformation and tumor cell growth (Elford, 1972; Weber, 1983; Hurta *et al.*, 1991; Lassmann *et. al.*, 1991; Jensen *et al.*, 1994). Although the activity of reductase is high in rapidly growing normal cells, it increases even further in neoplastic cells with similar growth rates (Takeda and Weber, 1981; Weber *et al.*, 1981; Weber 1983). Furthermore, DNA tumor virus-transformed cells show an elevated reductase activity and a loss of the cell-cycle dependent enzyme regulation as compared to their normal untransformed counterparts (Tagger and Wright, 1984; Hengstschlager *et al.*, 1994). It has also been reported that ribonucleotide reductase may be involved in certain immunodeficiency diseases in human (Ullman *et al.*, 1979). These findings have made ribonucleotide reductase a target for the development of chemotherapeutic compounds (Weber, 1983; Wright *et al.*, 1990a; Matsumoto *et al.*, 1990; Cory *et al.*, 1994; Potsch *et al.*, 1994; Szekeres *et al.*, 1994; Bianchi *et al.*, 1994).

1.5 Regulation of Ribonucleotide Reductase

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 G_1 cells (Lewis *et al.*, 1978). In proliferating cells, the R1 protein levels are constant and in excess throughout the cell cycle with a half-life of around 20 hours (Engstrom *et al.*, 1985; Mann *et al.*, 1988), whereas the R2 protein levels are limiting. R2 proteins determine enzyme activity via an

S-phase specific *de novo* synthesis followed by subsequent breakdown, with a half-life of only 3 hours (Eriksson *et al.*, 1984; Choy *et al.*, 1988). Both subunits decrease to a very low level in quiescent or terminally differentiated cells (Eriksson *et al.*, 1984; Engstrom *et al.*, 1985).

Studies of the R1 protein showed that it can be modified by ubiquitination, and the level of ubiquitinated R1 increases in parallel to the DNA-synthetic activity of cells, suggesting that ubiquitination plays an important role in regulating enzyme activity during the cell cycle (Sikorska *et al.*, 1992). On the other hand, the ability of $p34^{cdc2}$ or CDK2 kinases to phosphorylate R2 protein also indicates that R2 phosphorylation may play an important role in the regulation of ribonucleotide reduction, DNA synthesis and cell cycle progression, and suggests a potentially important $p34^{cdc2}$ and/or CDK2 regulation point in DNA replication (Chan *et al.*, 1993).

At the molecular level, both R1 and R2 transcript levels are low to undetectable in G_0/G_1 cells, show a pronounced increase as cells progress into S-phase, and then drop when cells progress into G_2 and M phases. The observed constant level of R1 protein during the cell cycle is obviously a result of its long half-life (Bjorklund *et al.*, 1990). This cell cycle dependent expression of R1 and R2 genes is regulated both transcriptionally and post-transcriptionally through cis-trans element interactions (Chen *et al.*, 1993, 1994a; Amara *et al.*, 1993, 1994, 1995a; Bjorklund *et al.*, 1993; Filatov and Thelander, 1995; Parker *et al.*, 1995). For example, there is a proximal promoter element in the R2 gene that specifically binds transcription factor NF-Y that is required for continuous activity of the R2 promoter through the S-phase (Filatov and Thelander, 1995). In addition, there

are sequences within the 3'-untranslated regions of R1 and R2 mRNAs that can bind cytoplasmic proteins, and participate in a mechanism that leads to the regulation of ribonucleotide reductase message stability (Chen *et al.*, 1993; Amara *et al.*, 1993).

Interestingly, ribonucleotide reductase expression can be induced by growth factors such as transforming growth factor-β_1 (TGF-β_1), tumor promoters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and DNA damaging agents such as chlorambucil, and all these inductions occur outside of S-phase (Choy *et al.*, 1989; Hurta and Wright, 1992a, 1992b, 1995). This S-phase independent regulation of ribonucleotide reductase may exist as a means of responding to DNA damaging and DNA repair requirements (Hurta and Wright, 1992a). Furthermore, many studies have indicated that ribonucleotide reductase gene expression can be modulated by signal transduction pathways involving protein kinase C (Choy *et al.*, 1989; Hurta and Wright, 1992b; Chen *et al.*, 1994b, 1994c) and protein kinase A (Boyton and Whitfield, 1983; Hurta and Wright, 1994). These two pathways can cooperate to regulate ribonucleotide reduction and this can occur in a synergistic fashion (Hurta and Wright, 1994).

2. Hydroxyurea

2.1 General Comments

Since ribonucleotide reductase plays such a key role in the process of cell growth, alterations in its activity can have profound effects upon the biology of the cell (Wright,

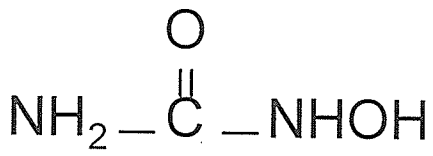
1989). As a result, drugs that can control intracellular reductase activity are of particular interest, with hydroxyurea being one of the most widely studied.

Hydroxyurea was first synthesized in Germany in 1869 (Dressler and Stein, 1869), but its potential biologic significance was not recognized until 1928 when Rosenthal *et al.* (1928) noted that it produced leukopenia and anemia with megaloblastosis in experimental animals. Hydroxyurea is a simple analogue of urea in which a hydrogen atom is replaced by a hydroxyl group (Figure 4). Hydroxyurea directly inhibits DNA synthesis and arrests cell proliferation at S-phase (Wright, 1989; Wright *et al.*, 1989) without affecting RNA or protein synthesis (Yarbro *et al.*, 1965a, 1965b). It enters animal cells by a diffusion process (Tagger *et al.*, 1987), and it is used clinically in the treatment of a wide variety of solid tumors, as well as acute and chronic leukemia (Bolin *et al.*, 1982; Engstrom *et al.*, 1984). In addition, it has shown promise as a myelosuppressive agent in treating polycythemia vera (Donovan *et al.*, 1984), as a radiation potentiator (Piver *et al.*, 1983), and in controlling the proliferation of psoriasis (McDonald, 1981). Recent studies also suggest hydroxyurea as a possible candidate for acquired immunodeficiency syndrome (AIDS) therapy since it inhibits human immunodeficiency virus type 1 (HIV-1) replication (Lori *et al.*, 1994; Goulaouic *et al.*, 1994; Gao *et al.*, 1994, 1995a, 1995b).

2.2 Effects of Hydroxyurea on Ribonucleotide Reductase

Ribonucleotide reductase has been identified as the site of action for hydroxyurea in many different cell types (Frenkel *et al.*, 1964; Elford, 1968; Lewis and Wright, 1974, 1979; Kuzik and Wright, 1980; Dick and Wright, 1980; Hards and Wright, 1981). The

Figure 4. Structure of hydroxyurea.



mode of action of hydroxyurea has been shown both *in vivo* and *in vitro* to involve destruction of the tyrosyl-free radical within the R2 protein (Graslund *et al.*, 1982; McClarty *et al.*, 1987). To further understand the reaction of the tyrosyl-free radical in the R2 protein with hydroxyurea, electron paramagnetic resonance (EPR) stopped flow techniques have been used. Transient nitroxide-like radicals from hydroxyurea have been detected, indicating that the dominating mechanism in the inhibitor reaction involves 1-electron transfer from hydroxyurea to the tyrosyl-free radical (Lassmann *et al.*, 1992). In mammalian cells, the iron center in the R2 protein is also reduced by hydroxyurea. This reduced form of the protein-bound iron is much more labile than its native diferric oxo-bridged form (Nyholm *et al.*, 1993), and this facilitates the loss of iron from the protein (McClarty *et al.*, 1990).

2.3 Mammalian Cell Mutants Altered in Ribonucleotide Reductase

Since drug resistance is a major clinical problem in cancer treatment (Stark, 1986), a great deal of research have been focused on obtaining a better understanding of this process. Hydroxyurea has been useful as a selective agent in cell culture for the isolation of drug-resistant cell lines with specific alterations in ribonucleotide reductase (Lewis and Wright, 1974, Wright and Lewis, 1974, Wright *et al.*, 1980, 1981; Wright, 1989). Investigations have shown that drug resistance may occur by many different mechanisms and all of these mutant cell lines contain elevated levels of reductase activity (Wright, 1989).

Low to intermediate resistance to hydroxyurea can occur by a straightforward mechanism involving elevations in R2 mRNA and protein levels without any changes in R1 gene expression, and these R2 alterations are usually accompanied by R2 gene amplification (Wright, 1989; Wright *et al.*, 1987, 1990a; Choy *et al.*, 1988; Tagger and Wright, 1988). These findings support the concept that the R2 protein subunit is limiting for ribonucleotide reduction in proliferating cells and the levels of R2 protein are important for DNA synthesis and cell proliferation. However, high to very high drug resistance requires alterations in both R1 and R2 gene expression (McClarty *et al.*, 1987; Choy *et al.*, 1988; Hurta and Wright, 1990a, 1990b). Moreover, reductase enzyme activity and the levels of both R1 and R2 proteins increase even further without any change in their corresponding mRNAs when these hydroxyurea-resistant cell lines are cultured in the presence of the drug (McClarty *et al.*, 1988).

In general, when cells become more resistant to the cytotoxic effects of hydroxyurea, the alterations in R2 gene expression and eventually R1 gene expression become progressively more complex. The underlying mechanisms leading to drug resistance may include R1 and R2 gene amplifications, modifications in gene transcription, alterations in message and protein half-lives, and changes in translational efficiencies (McClarty *et al.*, 1988; Wright, 1989; Amara *et al.*, 1995b).

II. MATERIALS AND METHODS

1. Cells and Culture Conditions

1.1 Culture Conditions

All Cell lines were routinely cultured on plastic tissue culture plates (Lux Scientific, Ltd.) in α-minimal essential medium (α-MEM; Gibco, Grand Island, New York) supplemented with penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml) (Sigma Chemical Co.) and 10% (v/v) fetal bovine serum (FBS; Fetal Clone III, Hyclone Laboratories). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Whenever dialysed serum was required, FBS was dialysed against four serial renewals of a 10-fold volume of 0.8% (w/v) sodium chloride solution.

1.2 Cell Line

BALB/c 3T3 cells were used throughout the entire course of this research. This cell line was purchased from the American Type Culture Collection, Rockville, Maryland, and it was originally isolated from BALB/c mouse embryos (Aronson and Todaro, 1968). This is an immortal cell line with the following properties: non-tumorigenic, highly sensitive to contact inhibition and grows only at high dilutions.

From this wild type population, a hydroxyurea resistant mutant, H^R3T3, was isolated for the ability to grow in the presence of 1 mM hydroxyurea. The selection was done in a step-wise manner. Non-mutagenized BALB/c 3T3 cells were first grown in the presence of 0.5 mM hydroxyurea for several weeks. At the end, the concentration of hydroxyurea was increased to a final concentration of 1 mM to select for mutants used in this study.

1.3 Routine Culture Procedures

1.3.1 Cell Removal with Trypsin Solution

Sterile 0.3% trypsin (Difco Laboratories) solution was prepared in phosphate buffered saline (PBS; 137 mM sodium chloride, 2.68 mM potassium chloride, 1.62 mM potassium phosphate monobasic, 8.1 mM sodium phosphate dibasic, pH 7.3) and 4 mM ethylenediaminetetraacetate (EDTA), and it was stored at 4°C. To remove a monolayer of cells from the surface of tissue culture plates, the growth medium was removed and the plates were washed twice with PBS. One to two ml of 0.3% trypsin-EDTA solution was added to the plates and incubated for three to five minutes. Afterwards, two to five ml of medium containing 10% FBS was added to the plates to inhibit the trypsin and remove the cells. The cell suspension was then centrifuged to remove the medium, resuspended in fresh growth medium supplemented with 10% FBS, and dispensed as needed.

1.3.2 Subculture

Cells that were about 70% confluent were subcultured. Cells were trypsinized and removed from the plates as described above. The cells were then resuspended in growth medium supplemented with 10% FBS, counted, and aliquots of 3 $x10^5$ cells were transferred to fresh 150 mm tissue culture plates containing 20 ml of fresh growth medium supplemented with 10% FBS.

1.3.3 Long-term Cell Storage

To store cells for a long period of time, cells in culture were removed with a trypsin-EDTA solution and centrifuged as described above. Cells were then suspended between 10^6-10^7 cells in 2 ml of FBS containing 10% dimethyl sulfoxide (DMSO). The cell suspensions were placed in 2 ml cryotube vials (Nunc) and frozen to -80°C. To recover these frozen cells, the vial was thawed rapidly by placing it into a water bath at 37-40°C. The cell suspension was then added to a sterile 15 ml Falcon plastic tube (Becton Dickinson Labware) containing 10ml of growth medium. The cells were pelleted by centrifugation, resuspended in growth medium, and added to tissue culture plates.

1.3.4 Cell Counting

Cells were removed from tissue culture plates as described above. Aliquots of cells were counted either before centrifugation, or after the cell pellets were resuspended in growth medium. The cell suspensions were counted using a hemacytometer (American Optic) and the average of at least five independent counts were used.

2. Determination of Colony-forming Ability and Growth Rate

2.1 Determination of Colony-forming Ability

The colony-forming ability of both wild type and mutant BALB/c 3T3 cells were determined as previously described (Hards and Wright, 1981). Exponentially growing cells were trypsinized, pelleted and resuspended in growth medium as described earlier. To determine plating efficiencies, cells were added to culture plates containing 10 ml of

α-MEM supplemented with 10% (v/v) FBS at cell numbers ranging from 10^2 to 10^6 cells/100 mm plate. After an incubation period of about 10-12 days at 37°C, viable cells were visualized by staining with a saturated solution of methylene blue (Sigma Chemical Co.) in 50% ethanol for 10 minutes. The plates were then rinsed with water and stained colonies containing more than about 40 cells were counted. The plating efficiency was defined as the number of colonies divided by the number of cells plated on each plate.

To study the effect of different hydroxyurea concentrations on the colony-forming ability of cells, their relative plating efficiencies were determined. 10^2 to 10^6 cells/100 mm plate were grown in α-MEM supplemented with 10% (v/v) FBS with increasing concentrations of hydroxyurea, and also in the absence of hydroxyurea as a control. The relative plating efficiency was defined as the plating efficiency in the presence of hydroxyurea divided by that in the absence of the drug (Hards and Wright, 1981). The concentrations of the drug which reduced colony-forming abilities by 50% (LD_{50} values) were extrapolated from a semilogarithmic plot of the dose response (Rittberg and Wright, 1989).

2.2 Determination of Growth Rate

Cells were removed from culture plates and counted as described above. They were added to 60 mm plates containing 5 ml of α-MEM supplemented with 10% FBS at a density of 5 $x10^4$ cells/plate. After an initial incubation period of 24 hours at 37°C, cells were collected and counted at different time points for up to five days.

To study the growth rate of cells in the presence of hydroxyurea, the drug was added to the plates at a final concentration of 0.33 mM after the first 24 hours of incubation at 37°C. Cells were counted at the same time points as those growing in the absence of hydroxyurea.

3. Determination of DNA, RNA and Protein Concentrations

3.1 Quantitation of the amount of DNA and RNA

The concentration of DNA or RNA in solution was determined spectrophotometrically as described in Manniatis *et al.* (1989).

3.2 Determination of Protein Concentrations in Cytoplasmic Cell Lysate

The Bio-Rad protein assay kit (Bio-Rad Laboratories) was used to measure the concentration of protein in cytoplasmic cell lysates. Protein concentrations were determined using the Standard Assay Procedure as described in the user's manual, and purified serum albumin fraction V (Sigma Chemical Co.) was used to generate the standard curve.

4. Southern Blot Analysis

4.1 Genomic DNA Isolation

Genomic DNA was extracted from logarithmically growing cells according to the procedure of Blin and Stafford (1976) with modification. Two to three 150 mm plates of cells were harvested, centrifuged and washed with PBS once. They were suspended in cold DNA isolation buffer (10 mM Tris, 10 mM EDTA, 10 mM NaCl, pH 8.0) at a concentration of 5 x10^6 cells/ml. Sodium dodecyl sulfate (SDS) was then added at a final concentration of 0.5% (w/v). To the suspension, 100 μg/ml of DNase-free RNase was added and allowed to incubate at room temperature for 2 hours or longer. The sample was then treated with 100 μg/ml of proteinase K at 55-60°C for at least another 3 hours. Afterwards, the DNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25/24/1) three times, and chloroform/isoamylalcohol (24/1) once, dialysed against 1 x TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) overnight with 2 changes of buffer, and stored at 4°C until needed.

4.2 Southern Blot Analysis

Twenty μg of genomic DNA was digested to completion with the desired restriction endonuclease at a concentration of 4 units/μg DNA at 37°C overnight. The sample was then concentrated to a volume of 50 μl by using a speed-vac concentrator (Savant), and mixed in with 1/5 volume of the 6 x gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). The mixture was slowly loaded onto a 0.7% agarose 0.5 x TBE (1 x TBE is 90 mM Tris-HCl, 90 mM boric acid, 20 mM EDTA) gel containing 0.5 μg/ml ethidium bromide. The gel was electrophoresed overnight at 30 volts in 0.5 x TBE. Molecular weight markers mixed with gel loading buffer was loaded onto separate

lanes. Gels were visualized under ultraviolet light after electrophoresis to check for sample loading and also the distance of migration.

Fractionated DNA was then blotted onto a nylon membrane (Zeta-Probe blotting membranes, Bio-Rad Laboratories) using the alkaline blotting method as described in the instruction manual. The gel was soaked in 0.25 M HCl for 10 minutes to depurinate the DNA, which facilitates the efficient transfer of high molecular weight DNA onto the membrane. The gel was then rinsed with water and the DNA samples were subsequently transferred to a Zeta-Probe blotting membrane in 0.4 M NaOH for 16-24 hours. After transfer, the membrane was rinsed in 2 x SSC (1 x SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) twice, air-dried, and baked at 80°C for 2 hours in a vacuum gel drier for fixing the DNA to the membrane.

Hybridization of ^{32}P-labeled probe to the membrane was done using the formamide protocol described in the Zeta-Probe blotting membranes instruction manual (Bio-Rad Laboratories). All blots were prehybridized at 42°C for 5 minutes in 50% formamide, 0.25 M Na_2HPO_4, pH 7.2, 0.25 M NaCl, 7% (w/v) SDS and 1 mM EDTA. Hybridization was performed in the same solution for 16-24 hours with (1-2) x10^6 c.p.m./ml of denatured ^{32}P-labeled probe. Blots were washed 3 times for 15 minutes, each in 0.1% SDS plus 2 x SSC, 0.5 x SSC and 0.1 x SSC, respectively. The first and second washes were done at room temperature, and the last one was done at 65°C. The membranes were then autoradiographed at -70°C using Kodak X-Omat AR film and Cronex Lightning Plus intensifying screen (Dupont). Autoradiograms were scanned densitometrically on a Bio-Rad model 620 video densitometer coupled to a printer and the

peaks corresponding to the bands were measured by computer integration. Optionally, the membranes were exposed to Phosphorscreens, which were then scanned by the PhosphoImager (ImageQuant).

5. Northern Blot Analysis

5.1 Cytoplasmic RNA Isolation

A rapid RNA extraction method was used to prepare total cytoplasmic RNA (Gough; 1988). One to three plates of cells were harvested from 150 mm tissue culture plates, pelleted in a 1.5 ml microfuge tube, and resuspended gently in 200 μl of cold 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1.5 mM $MgCl_2$, and 0.65% Nonidet P-40 (NP-40, Sigma Chemical Co.) in order to break up the cytoplasmic membrane, and leave the nuclear membrane intact. The nuclei were removed by centrifugation and the cytoplasmic lysates were added to microfuge tubes containing 200 μl 7M urea, 1% SDS, 0.35 M NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.5) along with 400 μl phenol/chloroform/isoamylalcohol (25/24/1 by volume). The RNA was then extracted for a second time with 300 μl of chloroform/isoamylalcohol (24/1 by volume), and recovered by chilling at -20°C overnight in 1 ml of 95% ethanol. After centrifugation, the pellet containing total cytoplasmic RNA was resuspended in 50-100 μl of sterile diethyl pyrocarbonate (DEPC)-treated water and stored at -70°C.

5.2 Northern Blot Analysis

This method was adapted from those of Lehrach *et al.* (1977), Goldberg (1980), and Seed (1982). Twenty μg of total cytoplasmic RNA was added to a denaturing buffer containing 1 μl 1 x MOPS buffer (0.02 M morpholinopropane sulfonic acid, pH 7.0, 8 mM sodium acetate, 1 mM EDTA), 1 μl DEPC-treated water, 3.5 μl formaldehyde and 10 μl of formamide. The samples were incubated at 65°C for 15 minutes. 3 μl of 6 x RNA gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 1 mM EDTA, 50% glycerol, pH 8.0) was added to each sample, and the mixture was then loaded onto 1% formaldehyde-agarose gel containing 18% formaldehyde and 1 x MOPS. The gel was electrophoresed at 30 volts overnight in 1 x MOPS buffer. An extra RNA sample containing ribosomal RNA was loaded into a separate lane as molecular weight marker. After electrophoresis, the marker lane was cut from the gel, stained with 0.5 μg/ml ethidium bromide for 30-45 minutes, and visualized under ultraviolet light. The migration distance of the 18s and 28s ribosomal RNA bands were measured.

The rest of the gel was blotted onto a nylon membrane (Zeta-Probe, Bio-Rad Laboratories) in 10 x SSC. After the transfer, the blot was bake-dried, prehybridized and hybridized using the same procedures as described for Southern blots.

6. DNA and RNA Slot Blot Analysis

6.1 DNA Slot Blot Analysis

Whole cells were used for the quantitative analysis of gene copy numbers (McIntyre and Stark, 1988; Wright *et al.*, 1990b). Logarithmically growing cells were

harvested from tissue culture plates, centrifuged and washed once with PBS. 250 μl of 0.4 M NaOH was added to resuspend each cell pellet with 10^3 to 10^5 cells in a screw-cap tube. The cell suspension was heated at 80°C for 10 minutes to hydrolyse the cells. After brief cooling, the sample was centrifuged and 100 μl of the supernatant was applied to a Zeta-Probe membrane (Bio-Rad Laboratories) prewetted with 0.4 M NaOH using the Bio-Rad Slot Blot apparatus (Bio-Rad Laboratories). Each well was rinsed with 100 μl of 0.4 M NaOH. After the blot was disassembled from the apparatus, it was rinsed twice for 5 minutes with 250 ml of 2 x SSC to neutralize the NaOH, air-dried and baked at 80°C for 30 minutes. Blots were prehybridized and hybridized as described above for Southerns (Reed and Mann, 1985).

6.2 RNA Slot Blot Analysis

To perform RNA slot blot analysis, cells were trypsinized from tissue culture plates, collected by centrifugation and washed once with PBS. Cell pellets with 2 x10^5 cells were resuspended gently in 100 μl of 0.65% NP-40 prepared in TE buffer (pH 7.4) for 5 minutes on ice. Nuclei were removed by centrifugation and 90 μl of supernatant was added to another tube containing 60 μl 20 x SSC and 40 μl formaldehyde. The mixture was incubated at 65°C for 15 minutes to denature the RNA. Any insoluble materials in the sample were removed by centrifugation. 150 μl of the supernatant was mixed with 350 μl of cold 20 x SSC and applied to a prewetted Zeta-Probe membrane using the Bio-Rad Slot Blot apparatus (Bio-Rad Laboratories). After removal from the apparatus, each blot was treated, prehybridized and hybridized as described above for DNA slot blot.

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

The cDNA probes were labeled by the hexanucleotide method of Feinberg and Vogelstein (1983) to specific activities between 5 x10^8 to 1 x10^9 c.p.m./μg using α-^{32}P-dCTP (specific activity 3000 Ci/mmole) (Amersham). This method was used to generate probes from denatured, linear, double-stranded DNA. Approximately 500 ng of an appropriate probe in a volume of 30 μl was boiled for 3 minutes and then quenched on ice immediately to denature the DNA. The single-stranded probe was labeled using an oligolabeling kit (Pharmacia LKB Biotechnology Inc.) and α-^{32}P-dCTP. 10 μl of Reagent mix, 5 μl of dATP, dGTP and dTTP in concentrated buffer, 7 μl of α-^{32}P-dCTP and 1 μl of Klenow fragment of *E. coli* DNA polymerase I were added to the probe and incubated at 37°C for 30-60 minutes. The radiolabeled probe was then separated from the unincorporated dNTPs by centrifugation through a 1 ml Sephadex G-25 (Pharmacia) column and eluted with 100 μl TE buffer. The specific activity of the purified radiolabeled probe was determined by counting 3 μl of the probe using a ^{32}P Cerenekov scintillation counter program (Beckman, model LS7800). All radiolabeled probes were boiled for 3 minutes and quenched on ice to denature the DNA before they were used for hybridization.

An alternative way of labeling probes was nick translation. The Nick-Translation System (Gibco BRL) was used to label linear or circular double-stranded DNA. To 500 ng of probe in a volume of 25 μl, 5 μl dATP/dGTP/dTTP mixture, 5 μl DNA polymerase/ DNAase I (100 units) and 7 μl α-^{32}P-dCTP (Amersham) were added. The reaction was

allowed to take place at 15-16°C for 1 hour. The radiolabeled probe was purified and counted as described above.

8. Sources of cDNA Probes and Plasmids

Plasmids containing murine R1 cDNA of clone 65 (pCD-R1) and R2 cDNA of clone 10 (pCD-R2) were used to prepare probes for studying amplification and expression of the ribonucleotide reductase R1 and R2 genes (Thelander and Berg, 1986). Plasmids containing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (pM-GAP) were used to prepare probes for determining loading of RNA in Northern blot analysis (Edwards *et al.*, 1985). The NcoI-generated 2 kb fragment from pCD-R1 containing R1 cDNA and PstI-generated 1.5 kb fragment from pCD-R2 containing R2 cDNA were used for making ^{32}P-labeled probes (Thelander and Berg, 1986).

9. Western Blot Analysis

Cytoplasmic lysates were prepared by resuspending pelleted cells in hypotonic buffer (25 mM Tris-HCl, pH 7.9, 0.5 mM EDTA) followed by repetitive cycles of freeze-thaw (Amara, *et al.*, 1993). Protein concentration was measured, and a predetermined amount of protein was heated at 100°C for 3 minutes in SDS loading buffer and then loaded onto a 10% linear SDS-polyacrylamide gel in a Bio-Rad mini-gel apparatus (Bio-Rad Laboratories). Molecular weight markers were loaded into separate lanes, and the gel

was electrophoresed at a constant current of 30 mA in 1 x Tris-glycine-SDS buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) for 60-90 minutes. Proteins were then transferred to PVDF membranes (Bio-Rad) at 70 volts for 1.5 hours in Tris-glycine transfer buffer (25 mM Tris-HCl, pH 8.3, 25 mM glycine) (Towbin *et al.*, 1979).

After transfer, membranes were baked at 65°C for 30 minutes in a vacuum gel drier, and stained with 0.3% India ink in TTBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5%(v/v) Tween 20) for 1 hour. Non-specific protein binding was reduced by blocking potential binding sites on the membrane with 7.5% nonfat dried milk in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 hour at room temperature with agitation (Johnson *et al.*, 1984). The membrane was then incubated with either AD203 anti-R1 mouse monoclonal antibody obtained from InRo Biomedtek (Umea, Sweden), or anti-R2 rabbit antiserum (Chan *et al.*, 1993) for 1 hour and washed 3-5 times for 10 minutes each in TTBS. The membranes were then incubated with the appropriate secondary antibody for 30 minutes. Sheep anti-mouse IgG conjugated with horseradish peroxidase was used for detecting R1 protein and donkey anti-rabbit IgG conjugated with horseradish peroxidase was used for R2 protein detection. The bound antibodies were detected using the enhanced chemiluminescence (ECL) detection system (Amersham).

10. Phosphopeptide Mapping Analysis of *In Vivo* and *In Vitro* Phosphorylated R2 Protein

10.1 *In Vitro* Phosphorylation of R2 Protein

Mouse recombinant R2 protein was prepared from *E. coli* containing the R2 expression plasmid (Mann *et al.*, 1991; Chan *et al.*, 1993), and *in vitro* phosphorylation of the protein was performed as described by Haystead *et al.* (1990). The different kinases used were, mitogen-activated protein (MAP) kinase, *lyn* kinase, *lck* kinase , *fyn* kinase, human recombinant c-*src* kinase (the above five kinases were obtained from Upstate Biotechnology Incorporated), cAMP-dependent protein kinase (Sigma Chemical Co.) and $p34^{cdc2}$ (obtained from Dr. D. Litchfield, Department of Biochemistry and Molecular Biology, University of Manitoba). One unit of enzyme activity equals the amount of enzyme that transfers 1 nmol of phosphate from γ-^{32}P-ATP to the substrate per minute. In each kinase assay, 1-3 units of kinase were used to phosphorylate 3 μg recombinant R2 protein in the presence of 25 mM β-glycerophosphate, pH 7.3, 0.5 mM dithiothreitol, 1.25 mM EGTA, 0.15 mM sodium vanadate, 10 μM calmidazolium, 0.2 mM γ-^{32}P-ATP (specific activity 167 c.p.m./nmole) (Amersham), 10 mM $MgCl_2$, 1 mg/ml bovine serum albumin (BSA) and 1 μM okadaic acid in a final volume of 24 μl. The reaction mix was incubated at 30°C for 20-30 minutes.

10.2 *In Vivo* Labeling of Cells with ^{32}P-orthophosphate

Cells growing in α-MEM supplemented with 10% FBS on 150 mm plates were washed twice with phosphate-free Dulbecco's modified Eagles's medium (D-MEM) (Gibco), and labeled with ^{32}P-orthophosphate in phosphate-free D-MEM supplemented with the appropriate amount of FBS. The cells were then incubated at 37°C for 3 hours in a humidified atmosphere containing 5% CO_2 (Chan *et al.*, 1993).

10.3 Immunoprecipitation of R2 Protein

Immunoprecipitation of R2 protein was carried out by using saturating amounts of rabbit anti-R2 polyclonal antibody according to the method of Firestone *et al.* (1982) with some modifications. Following *in vivo* cell labeling in 150 mm plates, the labeling medium was aspirated, and the cells were washed 3 times with cold PBS. 200 μl of solubilizing buffer (SB150) (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 1 mM PMSF) was added to each plate to lyse the cells at room temperature. The cell extract was then clarified by centrifugation, and solubilizing buffer (SB250) (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl, 1% Triton X-100, and 0.5% sodium deoxycholate) was added to give a final concentration of approximately 2 x10^7 cells/ml. Forty μl of formalin-fixed *Staphylococcus aureus* cells (Pansorbin) purchased from Calbiochem in a 10% suspension were added to the cell extract to preclear any non-specific immune complexes. The mixture was incubated at room temperature for 10 minutes with occasional mixing, and the precleared extract was recovered by centrifugation. Five μl of rabbit anti-R2 antiserum and 170 μl of 50 mg/ml BSA dissolved in SB250 were added to the extract, and incubated at room temperature for 15 minutes. The antigen-antibody complexes were precipitated by incubating with 60 μl of preloaded *S. aureus* IgG immune complexes in a 10% suspension for 15 minutes, and recovered by centrifugation. The labeled R2 protein could then be electrophoresed in a SDS-polyacrylamide gel.

10.4 Two-Dimensional Phosphopeptide Mapping Analysis

Labeled proteins were extracted from dried SDS-polyacrylamide gel into 1.2 ml of extraction buffer containing 50 mM ammonium bicarbonate, pH 7.8, 0.1% SDS and 1% mercaptoethanol in 1.5 ml screw-cap microfuge tubes (Beemon and Hunter, 1978; Kazlauskas and Cooper, 1989). The samples were briefly mixed, boiled for 3-5 minutes, and incubated at room temperature for 2 hours with shaking. After brief centrifugation, the supernatant was transferred to a fresh tube, and the gel bits were extracted with a second volume of extraction buffer overnight at room temperature with shaking. Ten μg of carrier protein (RNase) (Sigma Chemical Co.) and 0.2 ml of 100% trichloroacetic acid (TCA) was added to the supernatant on ice for at least 1 hour to concentrate the labeled protein and remove any SDS. The precipitated proteins were collected by centrifugation, washed twice with 0.5 ml ethanol/ether (1/1 by volume), and air-dried. Next, the pellet was dissolved in 20 μl performic acid (10 parts 88% formic acid and 1 part 30% hydrogen peroxide), and incubated at 0°C for exactly 1 hour. As soon as the oxidation step was finished, the sample was diluted in 0.5 ml water, frozen, and lyophilized to completion in a speedvac concentrator (Savant). The oxidized protein pellet was resuspended in 25 μl of 50 mM ammonium bicarbonate (pH 7.8) and digested with 10 μg TPCK-treated trypsin at 37°C overnight. The next day, 5 μg of additional protease was added to the sample, which was then incubated for at least 2 more hours. After digestion was complete, the ammonium bicarbonate in the sample was removed by 4 repeated lyophilizations.

Peptides resulting from tryptic digestion were resuspended in 5 μl of water and spotted onto 10 cm x 10 cm cellulose thin layer chromatography (TLC) plates (Merck). The first dimension of separation was electrophoresis at 1000 volts for approximately 20

minutes in a pH 1.9 buffer (15% glacial acetic acid, 5% formic acid) (Boyle *et al.*, 1991). This was carried out at 7°C with a Pharmacia FBE 3000 apparatus. A small aliquot of basic fuschin dye was applied to each plate as a marker. After the run was complete, the plates were air-dried completely in the fume hood. The second dimension was ascending thin layer chromatography for 4 hours at room temperature in a buffer containing isobutyric acid, pyridine, acetic acid, butanol, and water in a ratio of 65:5:3:2:29 by volume, respectively (Scheidtmann *et al.*, 1982; Chan *et al.*, 1993). A spot of DNP-lysine was applied to the original origin as a dye marker. Phosphopeptides on the dried plates were visualized by autoradiography (Chan *et al.*, 1993).

11. Cell Synchronization at G_0/ early G_1

11.1 Cell Synchronization by Serum Starvation

Synchronization of BALB/c 3T3 cells at G_0 or early G_1 was achieved by serum starvation (Bjorklund *et al.*, 1990; Feldherr and Akin, 1991; Zalvide *et al.*, 1992). Cells were allowed to grow to confluence for 2 days in α-MEM supplemented with 10% dialysed FBS. To obtain quiescent population, confluent cells were incubated in medium containing 0.5% dialysed FBS for another 2-3 days (Zalvide *et al.*, 1992). To initiate synchronous entry into the cell cycle, α-MEM containing 20% dialysed FBS was added (Gupta and Sirover, 1980).

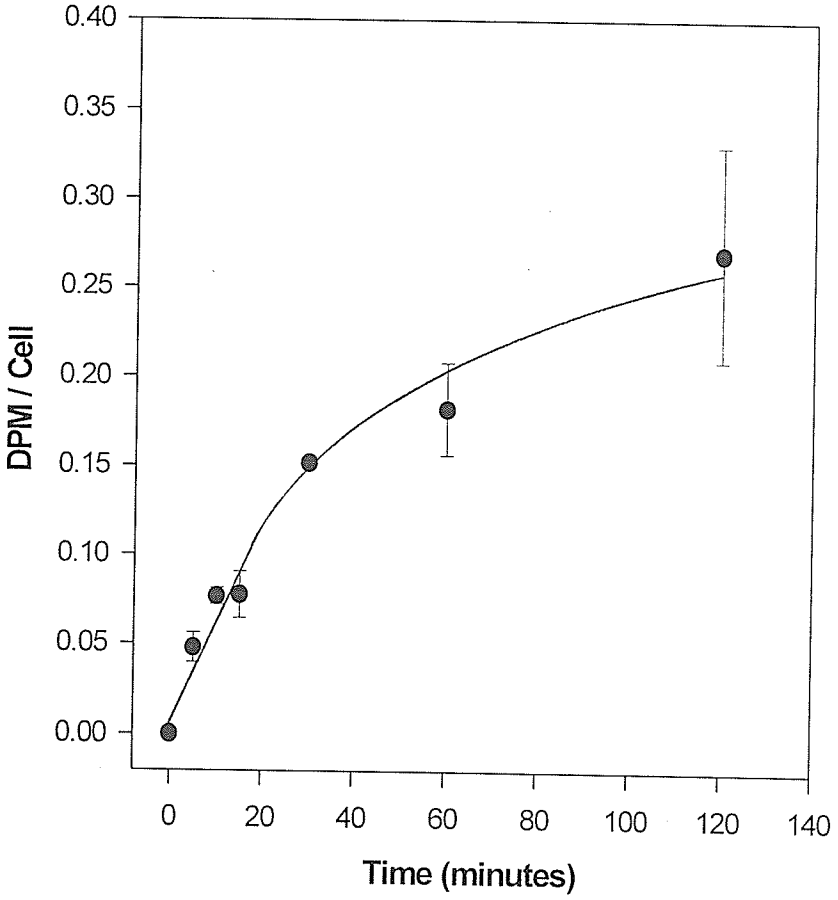
11.2 Assays for DNA Synthesis

Incorporation of [CH_3-^{3}H] thymidine (specific activity 25 Ci/mmole) (Amersham) into logarithmically growing or quiescent cells was performed as described by Hards and Wright (1983). Cells were incubated with 0.3 μM labeled nucleosides in medium supplemented with the appropriate amount of dialysed FBS at 37°C for 15 minutes. The length of incubation was determined from a calibration curve (Figure 5), within a region where a linear relationship between incorporated radioactivity and duration of incubation was observed. As for control, labeled nucleosides were added to cells placed on ice, and immediately after the addition, the medium was removed. After incubation, cells were washed 3 times with cold PBS and removed with 0.3% trypsin-EDTA solution. Ice-cold TCA was added to a final concentration of 5% for 30 minutes at 4°C. Cellular material was filtered through glass microfiber filters (Whatman) presoaked in ice-cold 10% TCA. The filters were then washed twice with 10 ml of ice-cold 10% TCA, and once with 10 ml of ice-cold 95% ethanol. The filters were oven-dried and placed into 6 ml of BetaMax scintillation fluid (ICN Biomedicals, Inc.). Radioactivity was determined by liquid scintillation spectroscopy.

11.3 Flow Cytometric Analysis

The proportion of cells in the different cell cycle phases was determined by fluorescent-activated cell sorting (FACS) as described by Blosmanis *et al.* (1987). After the cells were washed, removed with a solution of trypsin-EDTA and centrifuged, the cell

Figure 5. Calibration curve of incorporated radioactivity versus duration of incubation in exponentially growing BALB/c 3T3 cells. Error bars indicate standard errors from 3 independent determinations.


0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
DPM / Cell
0
20
40
60
80
100
120
140
Time (minutes)

pellets were washed once with PBS, and resuspended in 1 ml of PBS. The cells were then fixed in 5 ml of 95% ethanol for 30 minutes at room temperature, resuspended in 1 mg/ml RNase (Sigma) in PBS, and incubated at 37°C for 2 hours. DNA in the cells was stained by a brief incubation at room temperature in PBS containing 1 μg/ml ethidium bromide. Aliquots of cell suspension were sent to Dr. E. Rector at the Department of Immunology of the University of Manitoba for analysis using an EPICS flow cytometer (Coulter Electronics). Cell cycle phase distribution was estimated from the area of histograms, assuming a Gaussian function of the G_1 and G_2 plus M maxima and attributing the remaining part of the DNA histogram to cells in S phase (Blosmanis *et al.*, 1987; Bjorklund *et al.*, 1990). The length of each phase of the cell cycle is approximately equal to the fraction of cells in that phase at any instant multiplied by the doubling time of the logarithmic cells (Alberts *et al.*, 1989).

III. RESULTS

1. Molecular Mechanisms of Drug Resistance

1.1 Hydroxyurea Sensitivity of Wild Type and Mutant Cell Lines

Hydroxyurea resistant H^R3T3 cells were selected from the parental wild type BALB/c 3T3 cells in the presence of 1 mM hydroxyurea, as described in Materials and Methods. Colony-forming abilities in the presence of hydroxyurea for both wild type and mutant cell lines are shown in Figure 6. The H^R3T3 cell line was clearly less sensitive to the cytotoxic effects of hydroxyurea when compared to the parental cell line. The LD_{50} value of BALB/c 3T3 cells was 0.025 mM, whereas H^R3T3 cells exhibited a LD_{50} value of 0.82 mM. These results indicated that H^R3T3 cells were 33-fold less sensitive to hydroxyurea than the wild type cells.

1.2 Growth Rates of Wild Type and Mutant Cell Lines

The growth rates of wild type and hydroxyurea resistant 3T3 cells were determined both in the presence and in the absence of 0.33 mM hydroxyurea. After an initial incubation period of 24 hours, the number of cells per plate was determined at approximately 1-day intervals, and the results are shown in Figure 7. Wild type BALB/c 3T3 cells had a doubling time of approximately 16 hours when they were grown in growth medium without hydroxyurea. In keeping with their high sensitivity to hydroxyurea, these wild type cells were dead within 48 hours after the addition of the drug. On the other hand, H^R3T3 cells had a doubling time of approximately 19 hours in the absence of hydroxyurea. When they were grown in the presence of the drug, they doubled every 28

Figure 6. Relative colony-forming ability of wild type (□), and H^R3T3 (■) cells in the presence of increasing concentrations of hydroxyurea. Experiments were performed as described in Materials and Methods. Bars represent standard errors from 3 determinations.

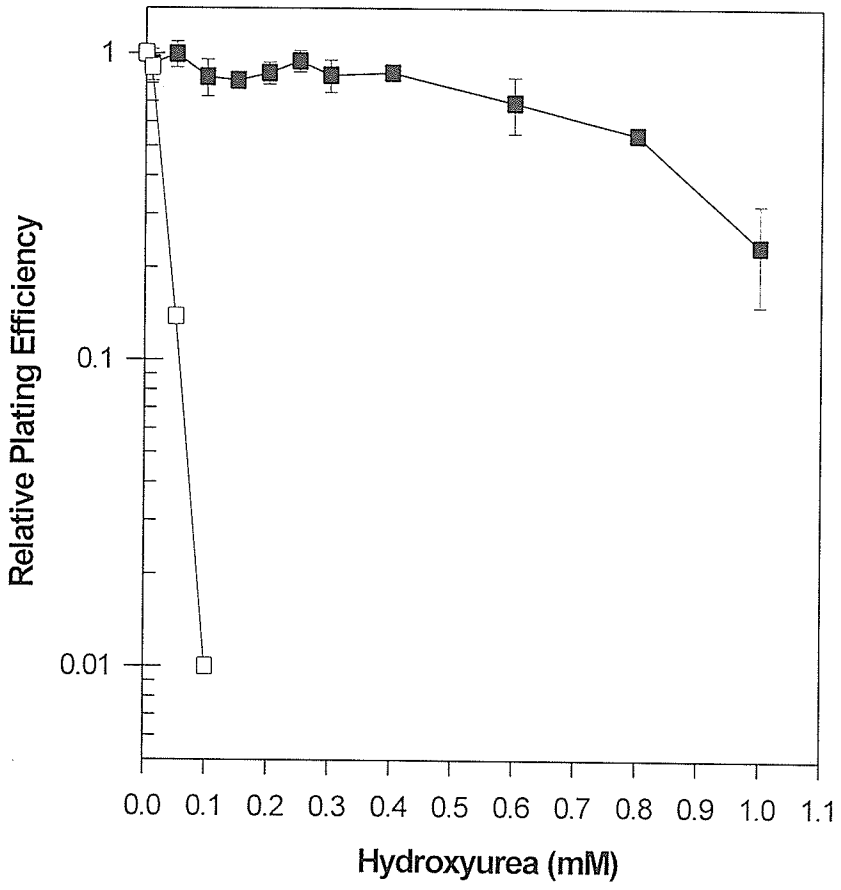
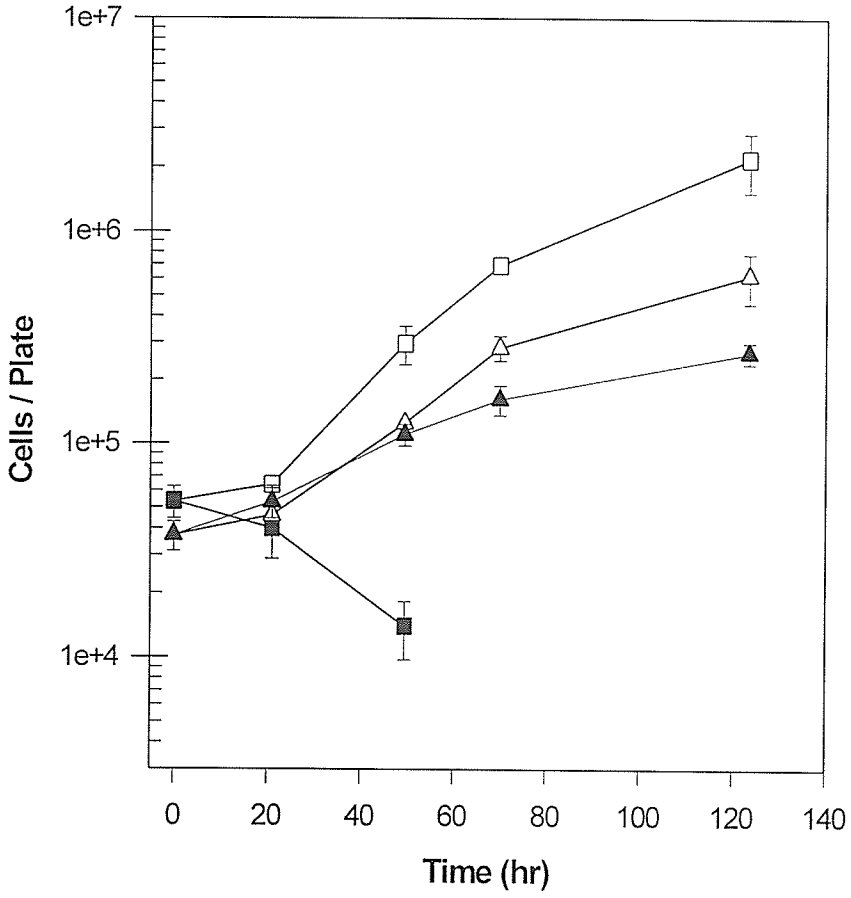

Relative Plating Efficiency
1
0.1
0.01
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1
Hydroxyurea (mM)

Figure 7. Growth rates of wild type BALB/c 3T3 cells (■,□),and mutant H^R3T3 cells(▲,△). Cells were plated in the absence (hollow symbols) or presence (solid symbols) of 0.33 mM hydroxyurea for up to five days. Bars represent standard errors from three determinations.

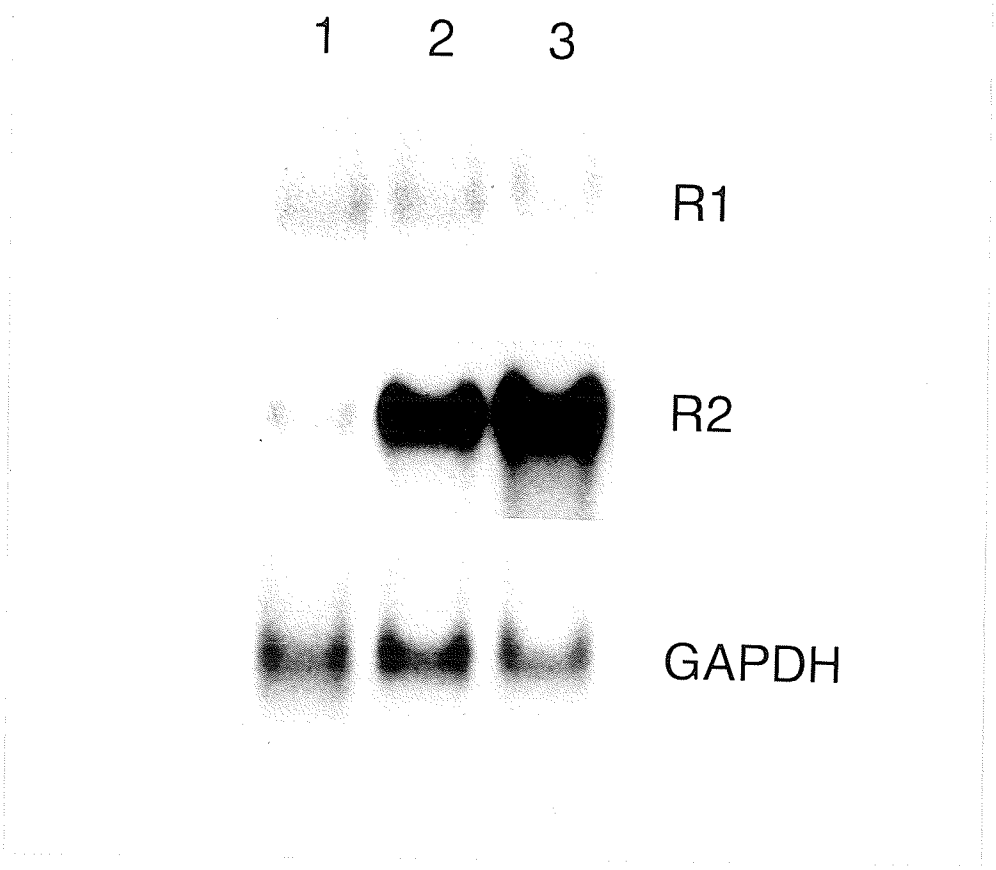

1e+7
1e+6
1e+5
1e+4
Cells / Plate
0
20
40
60
80
100
120
140
Time (hr)

hours. These results suggested that there was only a slight difference between the parental and the drug resistant 3T3 cells in terms of their growth rate in normal growth medium. However, when cultured in the presence of 0.33 mM hydroxyurea only the H^R3T3 grew, although at a slower rate than in the absence of the drug, and this is in agreement with previous observation obtained from hydroxyurea resistant mouse L cells (McClarty *et al.*, 1988).

1.3 Analysis of R1 and R2 mRNA Levels in Wild Type and Mutant Cell Lines

The cDNA clones encoding the R1 and R2 components of ribonucleotide reductase were used to analyze the relative amounts of R1 and R2 mRNAs in wild type and hydroxyurea resistant 3T3 cells, as described in Materials and Methods. Results of Northern blot analysis of R1 and R2 message levels are shown in Figure 8. No difference in concentration of R1 mRNA between H^R3T3 and the parental cells was observed. On the other hand, R2 mRNA was elevated in H^R3T3 cells as compared to the wild type cells when they were grown in the absence of hydroxyurea. Densitometric measurements revealed that R2 message was elevated 8.5-fold in H^R3T3 cells. These results were consistent with some previous studies conducted in our laboratory as well as others, which demonstrated that low to intermediate hydroxyurea resistance involved alterations in R2 gene expression without changes in R1 (Wright, 1989; Wright *et al.*, 1987; Choy *et al.*, 1988; Tagger and Wright, 1988; Bjorklund *et al.*, 1990). To study the effect of hydroxyurea on R1 and R2 mRNA levels, H^R3T3 cells previously grown in the absence of drug were cultured in the presence of 0.33 mM hydroxyurea for 2 days. Results of

Figure 8. Northern blots of R1 and R2 mRNA in wild type and H^R3T3 cells. 20 μg of total RNA isolated from wild type BALB/c 3T3 cells (*lane 1*), H^R3T3 cells (*lane 2*), and H^R3T3 cells in the presence of 0.33 mM hydroxyurea (*lane 3*) was denatured then run on a 1% formaldehyde-agarose gel followed by transfer to a nylon membrane as described in Materials and Methods. The blots was probed for R1 and R2 mRNAs on the same membrane. Loading of RNA was determined by reprobing with GAPDH cDNA as described in Materials and Methods. Autoradiographs of the Northern blots were quantitated by densitometry.


1
2
3
R1
R2
GAPDH

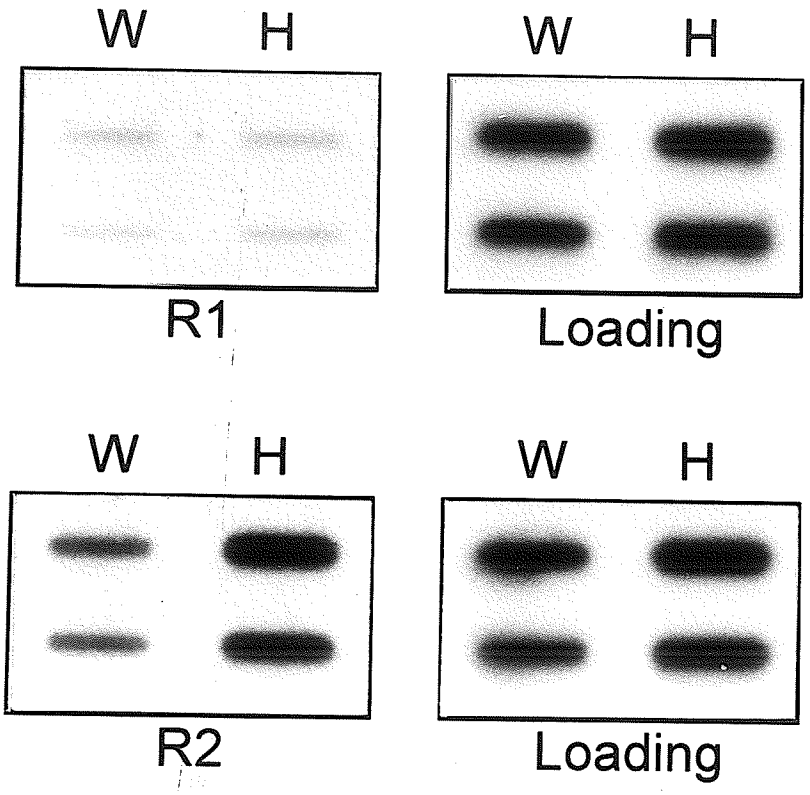
Northern blot analysis showed no change in the level of R1 message, whereas there was a 18-fold elevation in R2 mRNA level as compared to the wild type BALB/c 3T3 cells growing in the absence of hydroxyurea. As a loading control for each lane, the Northerns were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The level of GAPDH messages remained relatively constant (Figure 8), suggesting equal loading of RNA.

1.4 Analysis of R1 and R2 DNA from Wild Type and Mutant Cell Lines

Stable overexpression of mRNA in drug resistant cells is often associated with amplification of the gene (Stark *et al.*, 1989). To determine whether the increase in R2 mRNA observed in H^R3T3 cells was due to amplification of the R2 gene, the copy numbers of both R1 and R2 genes were analyzed by DNA slot blot techniques using whole cells, as described in Materials and Methods. The blots were probed with either R1 or R2 cDNA, and normalization for loading was done by hybridizing the same membrane with ^{32}P-labeled total genomic DNA probes randomly primed from 100 ng of high-molecular-weight genomic DNA, as described by McIntyre and Stark (1988).

The results of this analysis are shown in Figure 9. There was no indication of R1 gene amplification in the hydroxyurea resistant H^R3T3 cells, since the intensities of the bands were approximately the same in both wild type and mutant cells. However, H^R3T3 cells showed an obvious increase in R2 gene copy number as compared to the wild type cells. Densitometric scans of bands amplified in hydroxyurea resistant cells estimated a 4-fold R2 gene amplification in these cells. Furthermore, Southern blot analysis of wild

Figure 9. Slot blot analysis of R1 and R2 gene copy numbers in wild type and H^R3T3 cells. Wild type BALB/c 3T3 (W) or hydroxyurea resistant H^R3T3 (H) cells were treated as described in Materials and Methods. Samples were loaded in 1.5-fold dilutions, and the DNA was hybridized with ^{32}P-labeled R1(*top left*) or R2 (*bottom left*). Normalizations for loading are illustrated on the right panels using ^{32}P-labeled total genomic DNA probes.


W
H
R1
W
H
Loading
W
H
R2
W
H
Loading

type and H^R3T3 cells was used to check for possible R2 gene rearrangement. Twenty μg of genomic DNA from BALB/c 3T3 or H^R3T3 cells were digested to completion with EcoRI or BamHI endonucleases, and Southern blots of the digested mixtures were hybridized with ^{32}P-labeled PstI fragment of the R2 cDNA (Figure 10). Both wild type and mutant cell lines showed similar banding pattern, indicating that no gross R2 gene rearrangement has occurred. H^R3T3 cells showed a 4-fold increase in R2 gene copy number as estimated by densitometry, and this agreed with results obtained from slot blot analysis.

1.5 Analysis of the R1 and R2 Proteins in Wild Type and Mutant Cell Lines

The increase in R2 mRNA levels in hydroxyurea resistant H^R3T3 cells suggested that these cells contained elevated levels of the R2 protein component. The relative amount of both R1 and R2 proteins in drug resistant cells and their parental wild type cells was determined by Western blot analysis, as described in Materials and Methods. Twenty μg of cell extract protein prepared from BALB/c 3T3 and H^R3T3 cells, as well as H^R3T3 cells that had been growing in 0.33 mM hydroxyurea for 2 days, were subjected to 10% SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membrane, and stained with India ink. Anti-R1 mouse monoclonal antibody was used for the detection of R1 protein, and the results are shown in Figure 11. The anti-R1 antibody detected a high molecular weight band of about 88,000, corresponding to protein R1. The levels of R1 protein in H^R3T3 cells showed increases of approximately 6-fold and 7.7-fold over the wild type cell line when the cells were growing in the absence and in the presence of 0.33

Figure 10. Southern blot analysis of R2 DNA from wild type (*lanes 1 and 3*) and H^R3T3 cells (*lanes 2 and 4*). Twenty μg of genomic DNA was digested to completion with EcoRI (*lanes 1 and 2*) or PstI (*lanes 3 and 4*). The DNA size markers were HindIII digested lambda DNA (Boehringer, Mannheim, FRG).

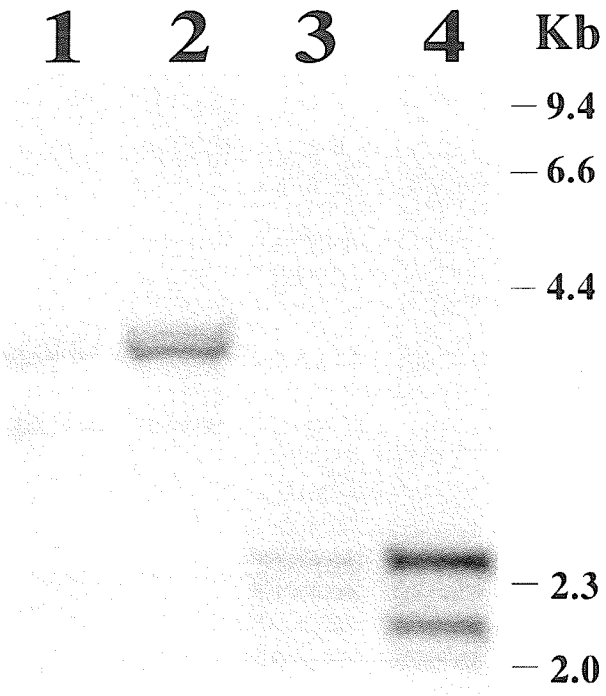
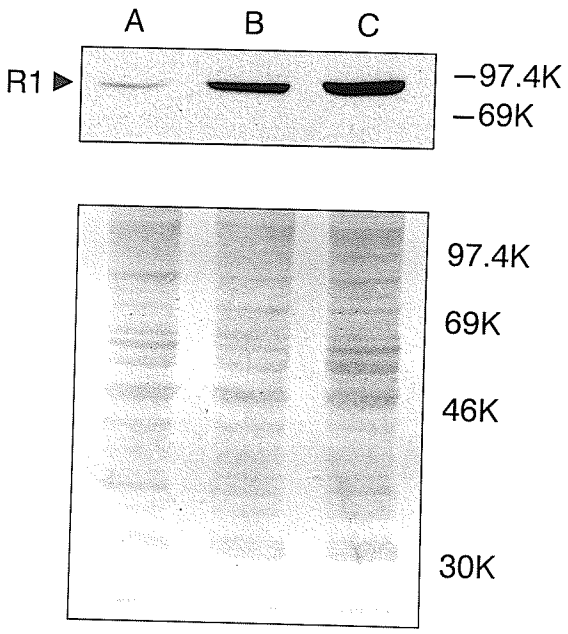

1
2
3
4
Kb
9.4
6.6
4.4
2.3
2.0

Figure 11. Western blot analysis for the R1 subunit in wild type and H^R3T3 cells. The cell-free extract was prepared from BALB/c 3T3 cells (A), and H^R3T3 cells in the absence (B), and presence (C) of 0.33 mM hydroxyurea. Twenty μg of cell extract protein was resolved on 10% SDS-polyacrylamide gel. *Top*: Protein on the blot was detected by anti-R1 mouse monoclonal antibody as described in Materials and Methods. *Bottom*: India ink-stained pattern of total cytoplasmic protein transferred to the membrane.


A
B
C
R1
−97.4K
−69K
97.4K
69K
46K
30K

mM hydroxyurea, respectively. Total cytoplasmic protein on the membrane was stained with India ink, and the result suggested that each lane contained an equal amount of total cytoplasmic protein. Therefore, the observed difference in R1 protein level was specific. The difference in R1 protein levels between wild type and mutant cells, given similar levels of R1 mRNA, suggested that changes in R1 protein occurred by a post-transcriptional mechanism, such as an increase in protein half-life (McClarty *et al.*, 1988), or through an increase in the biosynthetic rate of R1 protein in drug resistant cells (Choy *et al.*, 1988; McClarty *et al.*, 1988).

For the detection of the R2 protein, anti-R2 rabbit antiserum was used to stained the blots immunochemically. The antibody detected a single band with a molecular weight of 44,000, corresponding to the R2 component of ribonucleotide reductase, as shown in Figure 12. H^R3T3 cells exhibited 3-fold and 5-fold elevations in R2 protein levels in the absence and in the presence of hydroxyurea, respectively, as compared to the wild type cells. These changes were specific, since the level of total cytoplasmic protein remained constant, as demonstrated by the India ink-stained pattern on the membrane.

2. Phosphorylation of Ribonucleotide Reductase R2 Protein

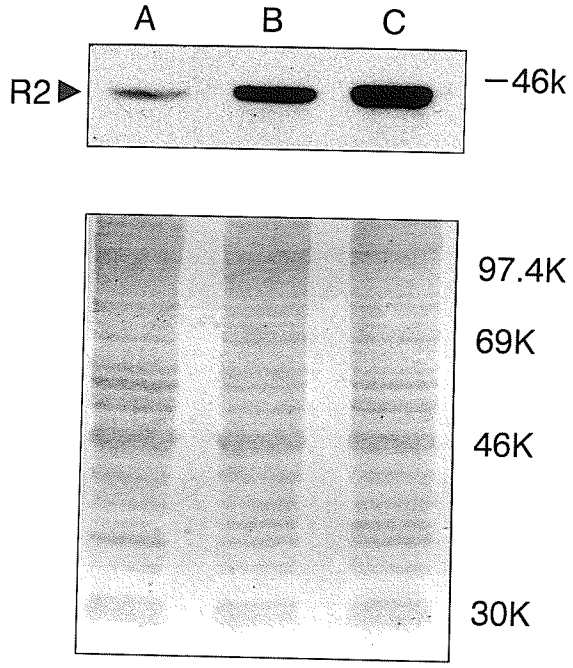
2.1 Phosphopeptide Mapping Analysis of *in Vivo* ^{32}P-Orthophosphate- labeled R2 Protein in Wild Type and Mutant Cells

Protein phosphorylation plays an important role in the regulation of many cellular functions. For example, activation of cyclin-dependent kinases (CDKs) requires

Figure 12. Western blot analysis for the R2 subunit in wild type and hydroxyurea resistant cells. The cell-free extract was prepared from BALB/c 3T3 cells (A), and H^R3T3 cells in the absence (B). and presence (C) of 0.33 mM hydroxyurea.

Top : The membrane was immunochemically stained with anti-R2 rabbit antiserum for the detection of R2 protein.

Bottom : Total cytoplasmic protein on the membrane was stained with India ink.


A
B
C
R2
46k
97.4K
69K
46K
30K

phosphorylation and dephosphorylation of the protein at different sites (Morgan, 1995). Recent studies in our laboratory have shown that the R2 subunit of ribonucleotide reductase in mouse L cells is a phosphoprotein that gives a characteristic phosphorylation mapping pattern upon tryptic digestion, and the phosphorylation site is at serine-20 (Chan *et al.*, 1993). To examine the *in vivo* phosphorylation state of the R2 protein in hydroxyurea resistant H^R3T3 cells, and their parental BALB/c 3T3 cells, cells were labeled and two-dimensional phosphopeptide mapping was performed as described in Materials and Methods. Figure 13 shows the phosphopeptide map of protein R2 immunoprecipitated from labeled BALB/c 3T3 cells. Two spots with different intensities were detected, which could be due to the inability of trypsin to function efficiently as an exopeptidase as suggested by Chan *et al.* (1993), and the pattern appeared to be identical to what was obtained in the previous study with mouse L cells (Chan *et al.*, 1993).

Since hydroxyurea resistant H^R3T3 cells had elevated level of R2 protein as compared to BALB/c 3T3 cells (Figure 12), it is possible that the phosphorylation status or the phosphorylation site of the R2 protein in H^R3T3 cells may be different from that of the wild type cells. However, the pattern on the phosphopeptide map of R2 protein isolated from H^R3T3 cells (Figure 14) was very similar to the one obtained with BALB/c 3T3 cells, showing two spots with uneven intensities. Therefore, the increase in R2 protein level observed in hydroxyurea resistant cells was not due to a change in the phosphorylation status or site of R2 protein in these cells.

Figure 13. Phosphopeptide mapping analysis of the R2 subunit in BALB/c 3T3 cells. *In vivo* phosphorylated R2 protein was proteolytically digested with TPCK-trypsin, and analyzed by electrophoresis (horizontal dimension, with -ve electrode on right), followed by ascending chromatography (vertical dimension). The position of the origin is marked with the arrow.

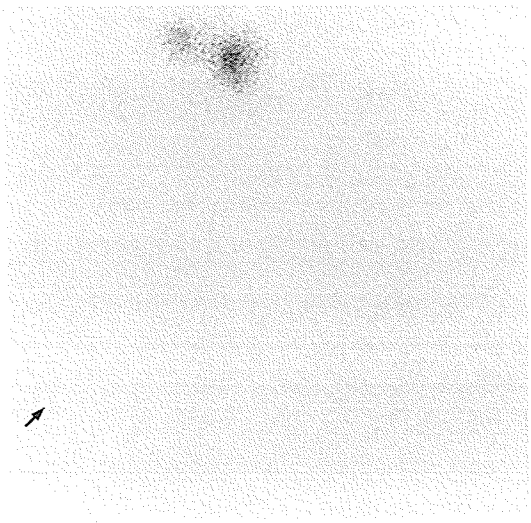
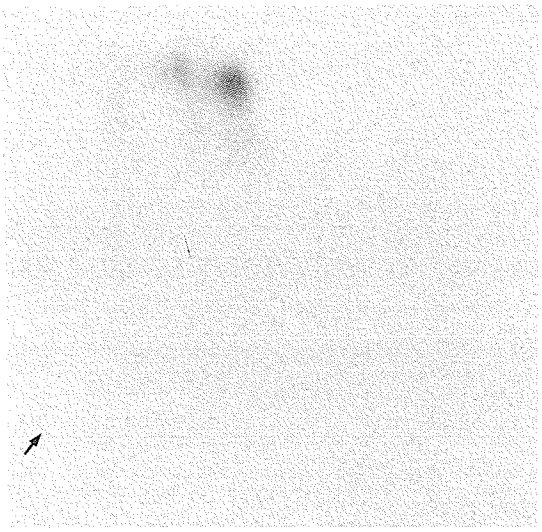


Figure 14. Phosphopeptide mapping analysis of the R2 subunit in hydroxyurea resistant H^R3T3 cells. Tryptin digested peptides were subjected to electrophoresis (horizontal dimension with -ve electrode on right) and ascending chromatography (vertical dimension). The arrow indicates the origin.

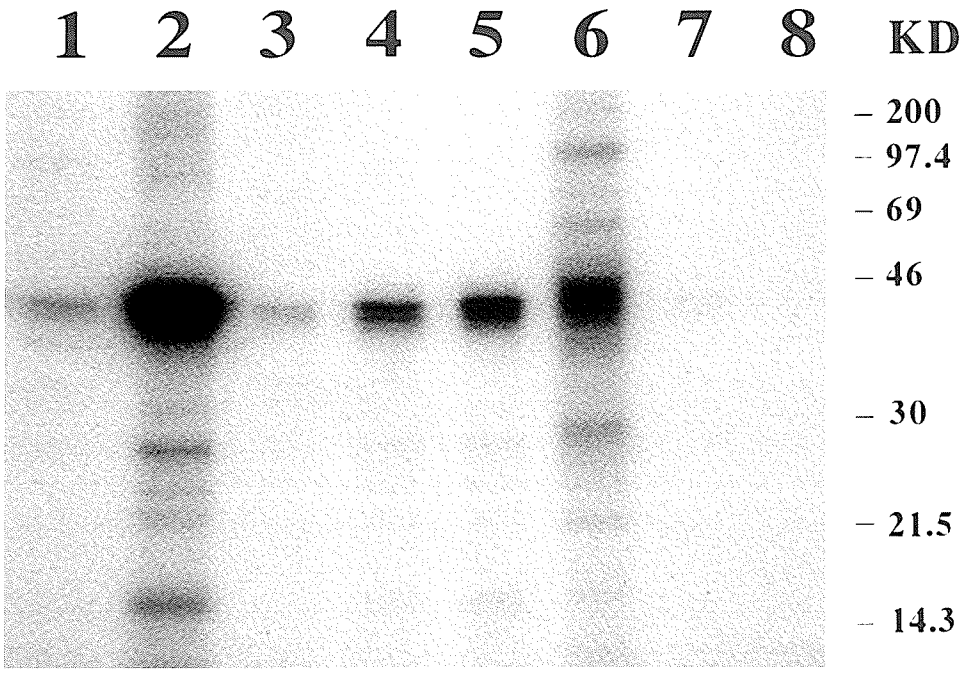


2.2 Phosphorylation of Recombinant R2 Protein *in Vitro* by Purified Kinases

The R2 protein has been found to be phosphorylated *in vivo* at a serine residue near the N-terminus of the polypeptide during logarithmic cell growth, suggesting the possible regulatory role of serine kinases on ribonucleotide reductase inside the cell. In fact, it has been shown that $p34^{cdc2}$ and CDK2 are two potential candidates that may be involved in R2 phosphorylation *in vivo* (Chan *et al.*, 1993). However, it is still possible that other kinase activities may have some other role in ribonucleotide reductase regulation, such as during cellular differentiation or DNA repair. One example is cAMP-dependent kinase, which has been shown to be involved in reductase regulation in S49T lymphoblasts (Albert and Nodzenski, 1989), and in altered mechanisms of malignant cell proliferation (Hurta and Wright, 1994).

To further identify protein kinases responsible for R2 phosphorylation *in vivo*, recombinant R2 protein was used as an *in vitro* substrate for a number of different kinases, including cAMP-dependent protein kinase, $p34^{cdc2}$, MAP kinase, *lyn* kinase, *fyn* kinase, human recombinant c-*src* kinase, and *lck* kinase. Except for the first three kinases, which are serine/threonine kinases, all the others are protein tyrosine kinases (Ray and Sturgill, 1987; Pearson and Kemp, 1991; Cheng *et al.*, 1992). cAMP-dependent protein kinase and $p34^{cdc2}$ were included as positive controls (Chan *et al.*, 1993). Phosphorylated R2 was isolated from the reaction mixtures by immunoprecipitation. Results of the kinase assays are shown in Figure 15. All kinases except *lck* kinase had the ability to phosphorylate the

Figure 15. Phosphorylation of R2 protein by different protein kinases. Recombinant R2 protein was used as an *in vitro* substrate for various purified protein kinases, including cyclic-AMP dependent protein kinase (*lane 1*), $p34^{cdc2}$ (*lane 2*), MAP kinase (*lane 3*), *lyn* kinase (*lane 4*), *fyn* kinase (*lane 5*), human recombinant c-*src* kinase (*lane 6*), and *lck* kinase (*lane 7*). An extra reaction containing all the reactants but without the addition of any protein kinase was included as a negative control (*lane 8*).


1 2 3 4 5 6 7 8
KD
– 200
– 97.4
– 69
– 46
– 30
– 21.5
– 14.3

R2 subunit *in vitro*. It also suggested that R2 protein was incapable of autophosphorylation, as demonstrated by the negative control reaction.

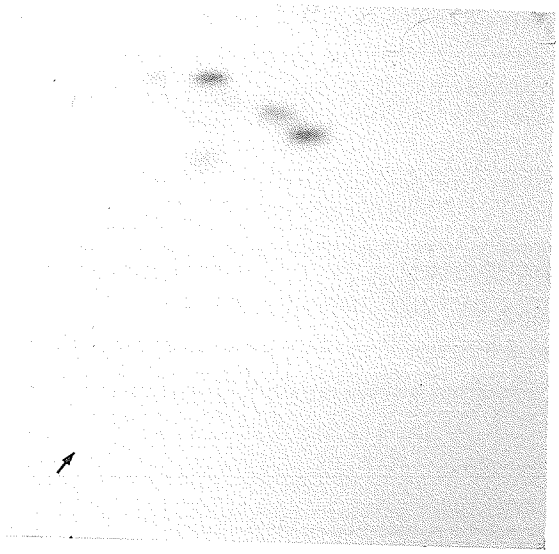
Since MAP kinase was a serine/threonine kinase that can phosphorylate R2 protein *in vitro*, its ability to phosphorylate peptides that are phosphorylated in logarithmically growing cells was tested by comparative phosphopeptide mapping study. R2 protein was phosphorylated by MAK kinase *in vitro*, immunoprecipitated, and subjected to proteolytic digestion by TPCK-trypsin. The phosphopeptide mapping pattern of the products is shown in Figure 16. By comparison, this map did not resemble the map obtained from R2 that had been phosphorylated *in vivo* (Figures 13 and 14), suggesting that MAP kinase was unlikely to be important in directly regulating the enzyme during logarithmic growth.

3. Regulation of Ribonucleotide Reductase in G_0/early G_1 Arrested Cells

3.1 Synchronization of Wild Type and Mutant 3T3 Cells at G_0/early G_1

When a cell moves into a non-proliferating quiescent and/or terminally differentiated G_0 phase from G_1, it clearly enters a different metabolic state. Cellular DNA synthesis is shut-off completely, and protein synthesis is significantly reduced (Pardee *et al.*, 1978; Baserga, 1985). Since the cell no longer requires to go through DNA replication, which is believed to be the major biological event where ribonucleotide reductase activity is required, the regulation of ribonucleotide reductase in the G_0 phase of the cell cycle was studied using growth-arrested cells. Both wild type BALB/c 3T3 and hydroxyurea resistant H^R3T3 cells were synchronized by serum-starvation, as described in Materials and Methods. Cell were harvested, fixed, stained, and the cell cycle

Figure 16. Phosphopeptide map of R2 protein phosphorylated by purified MAP kinase *in vitro*. Tryptic-digested phosphopeptides were separated by electrophoresis (horizontal dimension with -ve electrode on right) and ascending chromatography (vertical dimension). Origin is marked with a arrow.



distribution of growth-arrested cells was determined by flow cytometric analysis as described earlier (Blosmanis *et al.*, 1987). The DNA histograms of wild type BALB/c 3T3 cells are shown in Figure 17. When the cells were in logarithmic growth, 56% of the cells were in G_0/G_1 phase, 24% in S-phase, and 20% in G_2/M. A significant shift in cell cycle distribution was observed in cells that had been serum-starved by growing in 0.5%dialysed FBS for 72 hours : 89% of cells were in G_0/G_1 phase, and only 5% and 6% were in S and G_2/M phases, respectively.

Similarly, the flow cytometry profiles of hydroxyurea resistant H^R3T3 cells are shown in Figure 18. The percentage of cells in G_0/G_1 phase changed from about 70% in asynchronized exponentially growing cells to about 90% in cells that had been serum-starved for 48 to 72 hours. These results indicated that the synchronising effect of serum-depletion was obvious in both wild type and hydroxyurea resistant cells.

In addition, when growth arrested cells were stimulated to proliferate by the addition of 20% FBS as described in Materials and Methods, synchronous resumption of DNA synthesis was observed (Figure 19). For BALB/c 3T3 cells, DNA synthesis began to resume at around 12 hours after serum readdition, and reached a peak after 20-25 hours. This is in good agreement with results obtained by other groups (Bjorklund *et al.*, 1990; Lecka-Czernik *et al.*, 1995). These results support the view that cells were arrested in the G_0 phase during serum starvation, since re-entering the cell cycle requires high levels of protein synthesis, which is reflected by the long lag period between the time of serum readdition and the onset of DNA synthesis. Another possibility is that cells were arrested in very early G_1 phase. However, this seems less likely since the length of G_1 phase

Figure 17. Flow cytometry profiles of BALB/c 3T3 cells before and after growth-arrest at G_0/early G_1. Cells were synchronized as described in Materials and Methods. The top panel shows the cell cycle distribution of asynchronized logarithmically growing cells. The bottom panel shows the distribution of cells that had been serum-starved for 72 hours. The number of cells is shown on the *y-axis*, and the amount of DNA in ethidium bromide-stained cells (DNA fluorescence) is shown on the *x-axis*.

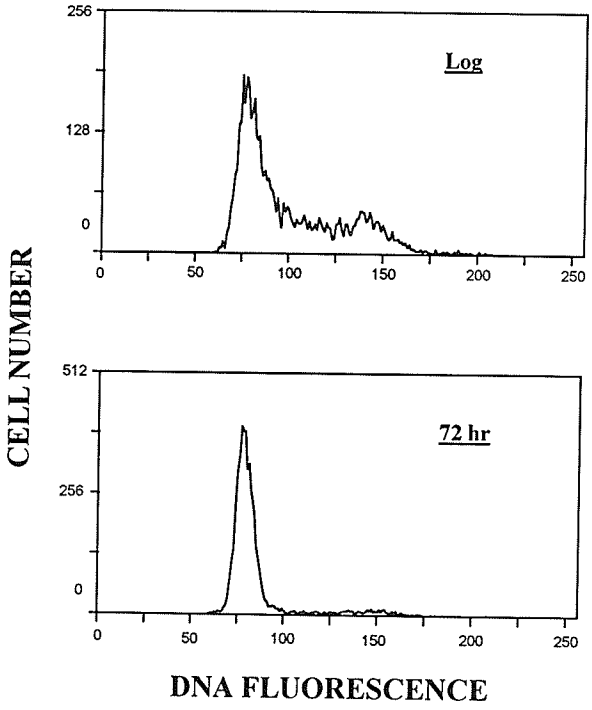
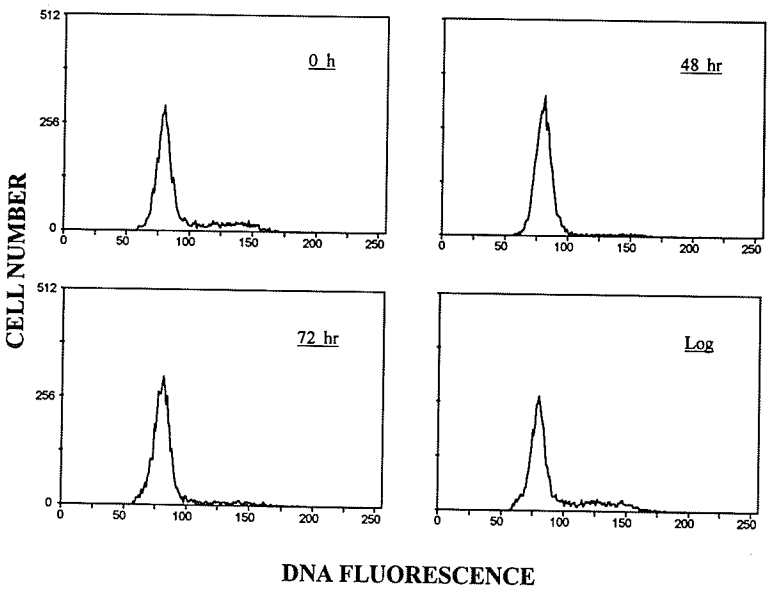

256
128
0
Log
0
50
100
150
200
250
512
256
0
72 hr
0
50
100
150
200
250
CELL NUMBER
DNA FLUORESCENCE

Figure 18. Flow cytometry profiles of H^R3T3 cells during the process of cell synchronization. *Top left* : Confluent cells just before the addition of 0.5% dialysed FBS. *Top right* : Cells that had been growing in 0.5% dialysed FBS for 48 hours. *Bottom left* : Cells that had been growing in 0.5% dialysed FBS for 72 hours. *Bottom right* : Asynchronized logarithmically growing cells.


0 h
48 hr
72 hr
Log
512
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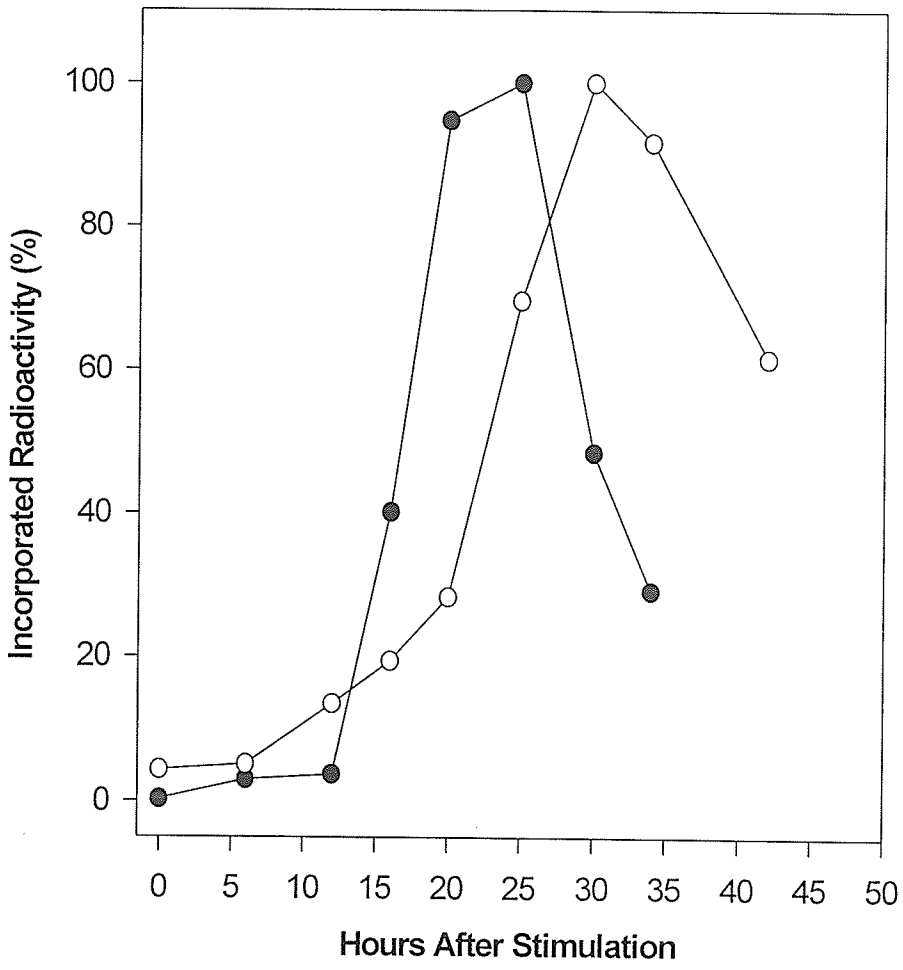
200
250
CELL NUMBER
DNA FLUORESCENCE

Figure 19. Analysis of ^{3}H-thymidine incorporation into DNA in cells synchronized by serum starvation. Growth arrested BALB/c 3T3 (●) and H^{R}3T3 cells (○) were stimulated to enter DNA synthesis by the addition of medium containing 20% FBS. The rate of DNA synthesis was determined by labeling the cells with ^{3}H-thymidine, and the amount of incorporated radioactivity was determined by TCA precipitation, as described in Materials and Methods. The highest amount of incorporated radioactivity was taken as 100%. Points represent means of 4-6 determinations.

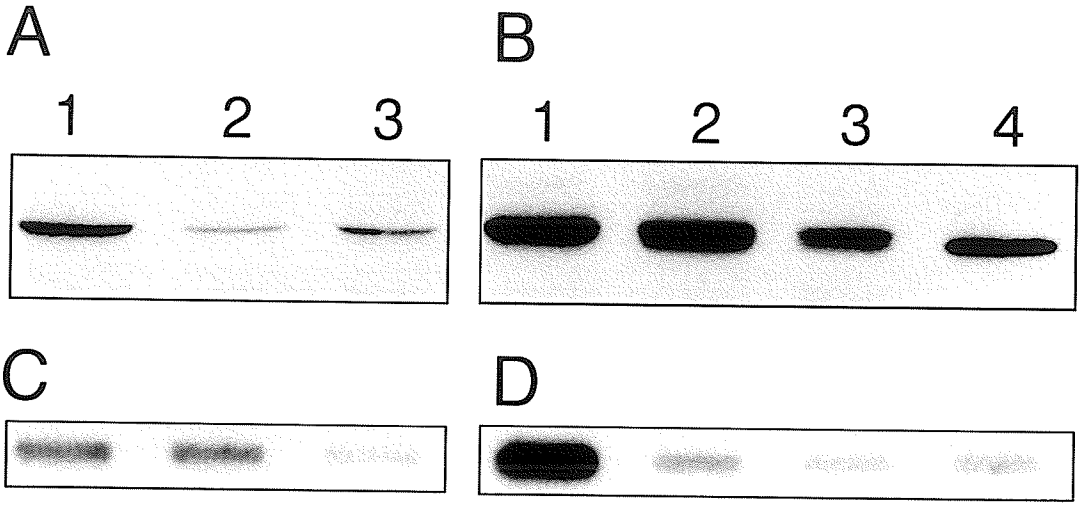

Incorporated Radioactivity (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50
Hours After Stimulation

in BALB/c 3T3 cells was only 8-9 hours when calculated from the doubling time and the proportion of G_1 population in exponentially growing cells, as described in Materials and Methods. Similarly, results obtained from H^R3T3 cells also revealed a lag period of roughly 12 hours, and a peak of DNA synthesis at around 30 hours after serum repletion. Although there was good evidence that both cell types were arrested in the G_0 phase, the possibility of G_1-arrest must also be taken into consideration, since there is no unique way to distinguish the G_0 phase from G_1 (Prescott, 1987).

3.2 Protein and mRNA Levels of R1 and R2 in G_0/early G_1 Phase Arrested Cells

Cultures of wild type BALB/c 3T3 and hydroxyurea resistant H^R3T3 cells were arrested at G_0/early G_1 by serum starvation for 72 hours as described earlier, and the levels of protein and mRNA for both R1 and R2 were examined using Western blot and RNA slot blot techniques, respectively. In G_0/early G_1 arrested wild type cells, the level of R1 protein was 4 times lower than in asynchronized exponentially growing cells (Figure 20A), and R1 mRNA level had dropped to approximately 35% of its original level (Figure 20C). As for H^R3T3 cells, both protein (Figure 20B) and mRNA (Figure 20D) levels of R1 had dropped 3-fold in growth-arrested cells as compared to asynchronized logarithmically growing cells. These results suggested that maybe R1 proteins were still being synthesized from R1 message in growth-arrested cells, but at a much lower level when compared to proliferating cells, or R1 proteins were being turned over at a different rate in quiescent cells. Similar results were obtained for R2. The R2 protein level in G_0/early G_1 arrested

Figure 20. Protein and mRNA levels of R1 in G_0/early G_1 arrested cells. H^R3T3 and the parental BALB/c 3T3 cells were synchronized as described in Materials and Methods. (*A*) The relative amount of R1 protein in BALB/c 3T3 cells was determined by Western blot analysis and densitometric measurements. 40 μg of cytoplasmic protein isolated from exponentially growing cells (*lane 1*), confluent cells (*lane 2*), and 72-hour serum-starved cells (*lane 3*) were used. (*B*) Western blot analysis of R1 protein in H^R3T3 cells. 20 μg of cytoplasmic protein isolated from exponentially growing cells (*lane 1*), confluent cells (*lane 2*), 48-hour (*lane 3*) and 72-hour (*lane 4*) serum-starved cells were analyzed. (*C*) Levels of R1 mRNA in growth-arrested BALB/c 3T3 cells was determined by RNA slot blot analysis, as described in Materials and Methods. Refer to panel *A* for sample in each lane. (*D*) Slot blot analysis of R1 mRNA in growth-arrested H^R3T3 cells. Refer to panel *B* for sample in each lane.


A
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2
3
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2
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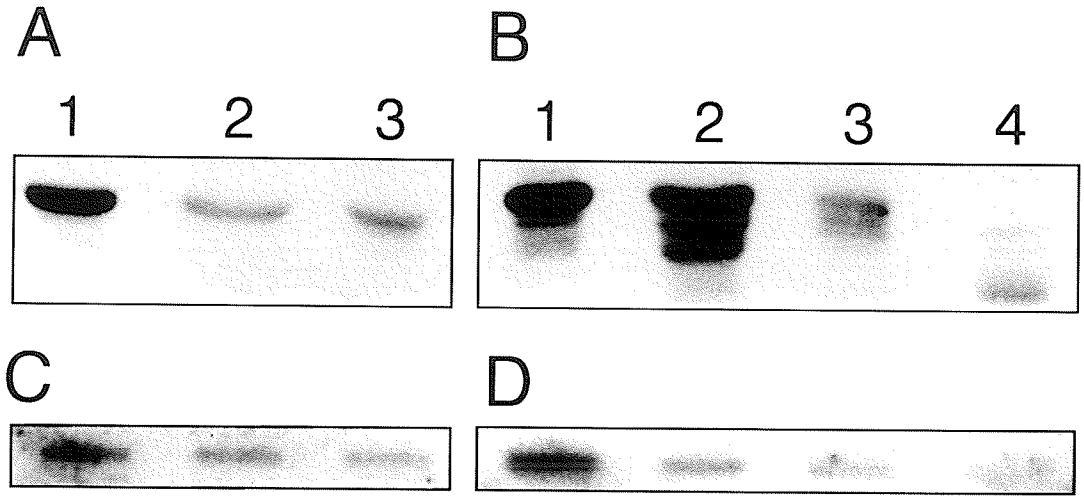
C
D

BALB/c 3T3 cells showed a 4-fold decrease as compared to asynchronized cells (Figure 21A), which was accompanied by a 3.5-fold decrease in message level (Figure 21C). In the case of H^R3T3 cells, the level of R2 protein had dropped 4-fold after 48 hours of serum-starvation as compared to asynchronized cells (Figure 21B). Interestingly, by the end of the 72-hour incubation under reduced serum conditions, the level of R2 protein in these cells was almost undetectable. Instead, a protein with a much lower molecular weight was detected, and this could be a degradation product of R2 protein. As for R2 mRNA level, there was a 10-fold decrease in arrested mutant cells as compared to logarithmic cells (Figure 21D). These observations suggested that when cells were arrested in G_0/early G_1 phase, a low level of both R1 and R2 subunits of ribonucleotide reductase were still present in the cell. An exception to this was observed in H^R3T3 cells, where R2 proteins were degraded during the process of growth arrest, and no new R2 protein appeared to be synthesized in arrested cells.

3.3 Phosphopeptide Mapping of *in Vivo* Labeled R2 Protein in G_0/early G_1 Arrested Cells

Cells in the quiescent state do not require ribonucleotide reductase activity to generate precursors for DNA synthesis, and yet both R1 and R2 proteins could still be detected in growth-arrested cells (Figures 20 and 21). Since it is known that the phosphorylation state of a protein can play an important role in enzyme activation/inactivation (Morgan, 1995), the phosphorylation status of the R2 protein

Figure 21. Protein and mRNA levels of R2 in G_0/early G_1 arrested cells. (*A*) The relative amount of R2 protein in BALB/c 3T3 cells was determined by Western blot analysis. 40 μg of cytoplasmic protein isolated from exponentially growing cells (*lane 1*), confluent cells (*lane 2*), and 72-hour serum-starved cells (*lane 3*) were analyzed. (*B*) Western blot analysis of R2 protein in H^R3T3 cells. 20 μg of cytoplasmic protein isolated from exponentially growing cells (*lane 1*), confluent cells (*lane 2*), 48-hour (*lane 3*) and 72-hour (*lane 4*) serum-starved cells were analyzed. (*C*) RNA slot blot analysis of R2 mRNA in growth-arrested BALB/c 3T3 cells. Refer to panel *A* for sample in each lane. (*D*) RNA slot blot analysis of R2 mRNA in growth-arrested H^R3T3 cells. Refer to panel *B* for sample in each lane.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subunit in these G_0/early G_1 arrested cells was examined. Cells were labeled with ^{32}P-orthophosphate and phosphopeptide mapping analysis of labeled R2 proteins was performed. The phosphopeptide map of R2 in growth-arrested BALB/c 3T3 cells (Figure 22) showed two spots with uneven intensities, which was very similar to the pattern obtained from asynchronized cells in logarithmic growth (Figure 13). Since the level of R2 protein in these cells was very low, the signal from the phosphopeptide map was very difficult to detect. To solve this problem, R2-overexpressing H^R3T3 cells were used. However, most of the R2 proteins in H^R3T3 cells were degraded after 72 hours of serum-starvation (Figure 21B). It had been demonstrated that after 48 hours of serum starvation, 90% of H^R3T3 cells were already arrested at G_0/early G_1 (Figure 18). Therefore, 48-hour serum-starved H^R3T3 cells were used in phosphopeptide mapping analysis instead of 72-hour serum-starved cells. The phosphopeptide map of *in vivo* labeled R2 protein isolated from these cells (Figure 23) had the same pattern as asynchronized logarithmic H^R3T3 cells, as well as both asynchronized logarithmic and arrested BALB/c 3T3 cells. These results suggested that the phosphorylation status or phosphorylation site of the R2 subunit of ribonucleotide reductase was not modified in G_0/early G_1 arrested cell.

Figure 22. Phosphopeptide mapping analysis of *in vivo* labeled R2 protein isolated from G_0/early G_1 arrested BALB/c 3T3 cells. The experiment was performed as described in Materials and Methods. Horizontal dimension was electrophoresis, with the -ve electrode on the right, and the vertical dimension was ascending chromatography. The arrow marks the origin.

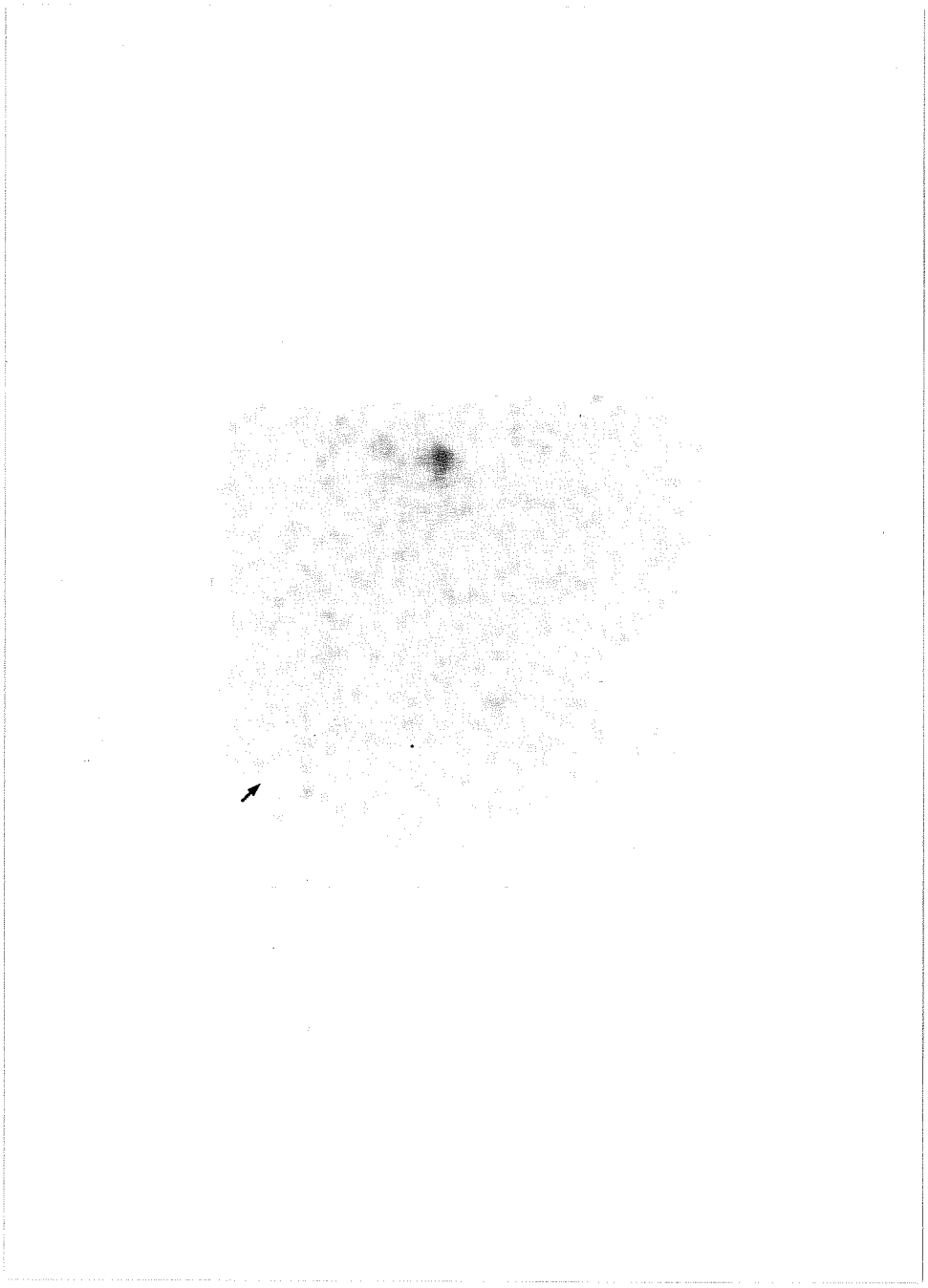
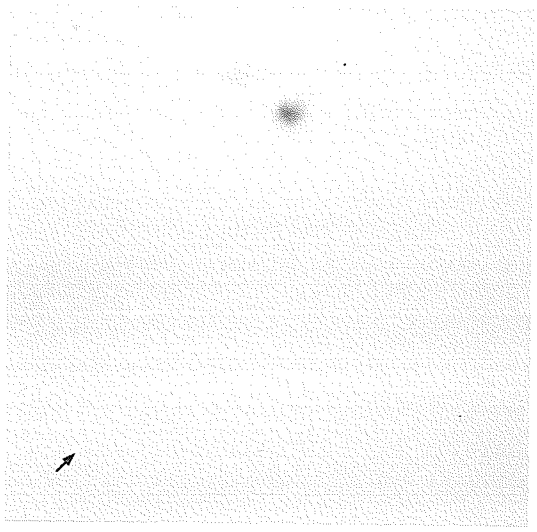


Figure 23. Phosphopeptide mapping analysis of *in vivo* labeled R2 protein isolated from G_0/early G_1 arrested H^R3T3 cells. The experiment was performed as described in Materials and Methods. The horizontal dimension was electrophoresis, with the -ve electrode on the right, and the vertical dimension was ascending chromatography. The arrow indicates the position of the origin.



IV. DISCUSSION

1. Molecular Mechanisms of Drug Resistance

Mammalian ribonucleotide reductase is the enzyme responsible for the *de novo* conversion of the four ribonucleotides into deoxyribonucleotides, and thereby providing a continuous and balanced supply of precursors for DNA synthesis (Thelander and Reichard, 1979; Wright, 1989). It consists of two protein subunits, R1 and R2. R1 protein exists as a *M*r 170000 homodimer with binding sites for substrates and allosteric effectors. R2 protein is also a homodimer, with a molecular weight of 88000, and it contains a non-heme iron center and a tyrosyl free radical that are needed for enzyme activity (Wright *et al.*, 1990a).

To analyze the complex structural, regulatory, and functional properties of this enzyme, cytotoxic drugs whose intracellular target is ribonucleotide reductase, have been used as selective agents in cell culture to isolate drug-resistant mutant cell lines with alterations in ribonucleotide reductase (Wright, 1989). The most widely used drug is hydroxyurea, an antitumor agent, which acts specifically at the R2 subunit to destroy the tyrosyl free radical required for enzyme activity through destabilization of the iron center (McClarty *et al.*, 1990).

Many different hydroxyurea resistant cell lines have been isolated in our laboratory in the past, and results from these studies demonstrate that mutants that are resistant to hydroxyurea at cytotoxic concentrations contain elevated levels of reductase activity, and drug resistance may occur by a number of different mechanisms (Wright, 1989; Wright *et al.*, 1990a). Interestingly, over-production of R2 protein and mRNA is always detected, which supports the notion that the R2 subunit of ribonucleotide reductase is limiting for

enzyme activity (Eriksson *et al.*, 1984; Choy *et al.*, 1988). Alterations in R1 expression, on the other hand, is only found in mutants with a very high resistance to hydroxyurea. Such high levels of R2 and/or R1 expression make hydroxyurea resistant mutants very useful tools for studying this enzyme.

In this present study, a non-tumorigenic BALB/c 3T3 cell line was used for the selection of a hydroxyurea resistant mutant, H^R3T3, with the ability to proliferate in the presence of 1 mM hydroxyurea. Differences in sensitivity to the cytotoxic effects of hydroxyurea was determined by comparing colony-forming abilities in the presence of increasing concentrations of hydroxyurea. These results demonstrated that hydroxyurea resistant cells exhibited a decreased sensitivity to hydroxyurea inhibition (Figure 6), which is commonly observed in many different hydroxyurea resistant cell lines (Wright and Lewis, 1974; Hards and Wright, 1981; Tagger and Wright, 1988; Rittberg and Wright, 1989; Hurta and Wright, 1991).

Results from Western blot analysis using anti-R1 mouse monoclonal antibody and anti-R2 rabbit antiserum revealed that the cellular levels of both R1 and R2 proteins are elevated in these mutant H^R3T3 cells (Figures 11 and 12). A significant increase in R2 mRNA levels was also observed (Figure 8). Furthermore, the R2 gene is amplified 4-fold in these hydroxyurea resistant cells, and no gross R2 gene rearrangement was observed in Southern blot analysis when total genomic DNA isolated from these cells was digested to completion with EcoRI or BamHI endonucleases (Figure 10).

The increase in R2 mRNA levels was higher than the increase in gene copy number, indicating that in addition to gene amplification, other mechanisms may be

involved, such as elevated transcription rate (McClarty *et al.*, 1987). A recent study also provided evidence for increased R2 message stability in hydroxyurea resistant mouse L cells (Amara *et al.*, 1995b).

On the other hand, the message levels of R1 are unchanged in hydroxyurea resistant cells as compared to wild type cells (Figure 8). This result suggests that no R1 gene amplification should be found, and in fact results from DNA slot blot assays showed this to be the case (Figure 9). These observations are in agreement with the concept that low to intermediate hydroxyurea resistance only involves changes in R2 gene expression, without altering R1 gene expression (Wright *et al.*, 1990a). Furthermore, these results suggest that the elevated levels of R1 protein observed in these mutant cells is the result of post-transcriptional modifications, such as increased protein half-life (McClarty *et al.*, 1988) or increased translational efficiency (Choy *et al.*, 1988).

It is evident that the observed changes in R2 gene expression occur independent of R1 in these hydroxyurea resistant cells, and this is in keeping with the fact that mammalian R1 and R2 genes are located on different chromosomes (Engstrom and Francke, 1985; Tonin *et al.*, 1987; Yang-Feng *et al.*, 1987), and the levels of these two protein components are regulated by different mechanisms during cell growth (Eriksson and Martin, 1981; Choy *et al.*, 1988). This is in contrast to the *E. coli* system where both R1 and R2 genes are found in a single operon and their synthesis are coordinately controlled (Hanke and Fuchs, 1983; Carlson *et al.*, 1984).

In the past, it has been shown that ribonucleotide reductase activity is further elevated in hydroxyurea resistant cells when they are cultured in the presence of the

selective drug (Lewis and Wright, 1979; Wright *et al.*, 1980,1981; Hards and Wright, 1983; McClarty *et al.*, 1986, 1987). To determine whether such an increase is a result of elevations in protein R1 and/or R2, H^R3T3 cells previously grown in the absence of hydroxyurea were cultured in the presence of the drug for 2 days. Results from Western blot analysis indicate that there was only a very slight increase in the cellular level of R1 protein in these mutants when they were grown in the presence of hydroxyurea, as compared to growth in the absence of the drug (Figure 11). R2 protein levels, on the other hand, showed a much larger increase (Figure 12). The drug-induced elevation in R1 protein levels was not accompanied by any increase in the corresponding R1 mRNA (Figure 8), which is consistent with a previous study showing that hydroxyurea is capable of modifying ribonucleotide reductase expression post-transcriptionally (McClarty *et al.*, 1988).

Such drug-induced R1 and R2 protein elevations could be the result of increased protein half-lives and biosynthetic rates (McClarty *et al.*, 1988). It could also be due to decreased susceptibility of these proteins to proteolytic degradation in the presence of hydroxyurea, since it has been found that the degradation rate of the enzyme dihydrofolate reductase is affected by its inhibitors (Cowan *et al.*, 1986). These mechanisms seem to be important in allowing the cells to survive in normally cytotoxic concentrations of hydroxyurea. Surprisingly, hydroxyurea did induce an increase in R2 mRNA levels, which is in contrast to what was observed previously (McClarty *et al.*, 1988). This observation indicates a significant difference in regulation of R2 mRNA when compared to the previously described hydroxyurea mutants.

When hydroxyurea resistant H^R3T3 cells were grown in the presence of 10% FBS without the presence of hydroxyurea, they demonstrated a growth rate that is only slightly slower than that of the wild type cells. However, these mutant cells showed a significant increase in their doubling time when they were cultured in the presence of hydroxyurea (Figure 7). According to a recent report, overexpression of ribonucleotide reductase R1 and/or R2 messages and proteins can lead to a decrease in growth rate, with R2 overexpression having a more prominent effect than R1 (Zhou *et al.*, 1995). Therefore, the decrease in growth rate observed in H^R3T3 cells, especially in the presence of hydroxyurea, could be in part due to the significant increase in R2 mRNA levels. This explanation is also supported by the fact that elevated R1 and R2 expression in hydroxyurea resistant cells can lead to pertubations of deoxyribonucleotide pools (Aakerblam *et al.*, 1981; Dick and Wright, 1984; Creasey and Wright, 1984), which may in turn affect cell cycle progression (Amellem *et al.*, 1994).

2. Phosphorylation of Ribonucleotide Reductase R2 Protein

Phosphorylation and dephosphorylation reactions are one of the dynamic mechanisms through which eukaryotes modulate intracellular processes in response to environmental stimuli. It has been estimated that approximately one third of the proteins in a typical eukaryotic cell may undergo phosphorylation in response to diverse extracellular stimuli, reflecting the huge number of distinct protein kinases and their broad specificity (Hunter, 1987).

It is evident that protein phosphorylation mechanisms play a potentially important role in the regulation of ribonucleotide reductase. Previous studies carried out in our laboratory have demonstrated that ribonucleotide reductase gene expression and enzyme activity in cells can be altered by a wide variety of external agents, including DNA-damaging agents like chlorambucil (Hurta and Wright, 1992a), tumor promoters such as TPA (Chen *et al.*, 1993) or okadaic acid (Hurta and Wright, 1992b), transforming growth factor-β_1 (Hurta *et al.*, 1991, 1992), and anti-tumor agents such as hydroxyurea (McClarty *et al.*, 1988). Many of these agents can modulate ribonucleotide reductase expression within a very short period of time after addition, without shifting the cell population into S-phase (McClarty *et al.*, 1988; Hurta *et al.*, 1991; Hurta and Wright, 1992a, 1992b; Amara *et al.*, 1993; Chen *et al.*, 1993). However, the signal transduction pathway that links these external stimuli to the elevation in ribonucleotide reductase expression remains unclear. Rudimentary evidence suggests that pathways involving both protein kinase C and c-AMP dependent protein kinase may play a role in reductase regulation (Choy *et al.*, 1989; Hurta and Wright, 1992b, 1994; Chen *et al.*, 1994b, 1994c).

The regulation of mammalian ribonucleotide reductase at the level of direct subunit phosphorylation has not been studied until the 1990's. Direct evidence of potential ribonucleotide reductase regulation through protein phosphorylation was provided by Chan *et al.* (1993) using mouse L cells. Those results show that the R2 subunit of ribonucleotide reductase is phosphorylated *in vivo*. The proposed phosphorylation site is serine-20 near the N-terminal end, within the sequence Gln_{18}-Leu_{19}-Ser_{20}-Pro_{21}-Leu_{22}-Lys_{23}-Arg_{24}-Leu_{25}. In this study, the phosphorylation status of BALB/c 3T3 cells during

logarithmic growth was examined using the same two-dimensional phosphopeptide mapping technique that was used by Chan *et al.*(1993). The close resemblance of the pattern on the two-dimensional phosphopeptide map (Figure 13), to that obtained from mouse L cells indicates that the observed phosphopeptide map pattern of R2 protein is not cell-type specific, and the phosphorylation site is not the same in both cell lines.

Drug resistance can cause both quantitative and qualitative changes in the phosphorylation status of the drug target, as demonstrated by Ritke *et al.* (1995), showing that human leukemia K562 cells that are resistant to a topoisomerase II inhibitor results in altered topoisomerase II phosphorylation. To examine the possible connection between ribonucleotide reductase R2 protein phosphorylation and hydroxyurea resistance, *in vivo* ^{32}P-labeled R2 proteins isolated from H^R3T3 cells were analyzed by two-dimensional phosphopeptide mapping technique (Figure 14). Since no difference was observed in the phosphopeptide patterns between H^R3T3 cells and the parental wild type cells, it is unlikely that the hydroxyurea resistant phenotype in these mutants involves any changes in the phosphorylation of R2 subunit.

In general, the effect of R2 phosphorylation on ribonucleotide reduction remains an open question. It can only be answered by generating cells that express mutant R2 protein that does not have the putative phosphorylation site, and look for any changes in reductase regulation in these cells. Current efforts are underway to construct plasmids containing mutated R2 cDNA that gives a different amino acid residue at the putative phosphorylation site, using site-directed mutagenesis techniques.

It has been postulated that cell cycle regulatory serine/threonine kinases $p34^{cdc2}$ and CDK2 may play a role in the direct phosphorylation of R2 protein (Chan *et al.*, 1993). However, other protein kinases may still have some role in ribonucleotide reductase regulation, as demonstrated by Albert and Nodzenski (1988), Elledge *et al.* (1993), and Hurta and Wright (1994). To further identify other enzymes that are capable of phosphorylating R2 protein, protein kinase assays were carried out using recombinant R2 proteins as *in vitro* substrates.

There was no kinase activity associated with the R2 protein itself, since the R2 protein did not undergo autophosphorylation. The finding that MAP kinase, another serine/threonine kinase, can phosphorylate R2 protein *in vitro* lead to the investigation of its potential role *in vivo*. Before two-dimensional phosphopeptide mapping analysis of *in vitro* MAP kinase phosphorylated R2 was performed, there were two major reasons to believe MAP kinase may be physiologically important in direct R2 phosphorylation. The first reason was related to the consensus phosphorylation site sequence on the substrate that MAP kinase recognizes. In the case of serine/threonine-specific protein kinases, specificity is achieved through recognition of distinct amino acid residues in the vicinity of the phosphorylation site (Kennelly and Krebs, 1991). Once such a consensus sequence for phosphorylation has been established, the putative substrates for the kinase can be predicted based upon the appearance of this sequence in the protein. Both $p34^{cdc2}$ and MAP kinases can phosphorylate a serine/threonine residue within the Ser/Thr-Pro consensus sequence (Kennelly and Krebs, 1991), and therefore we postulated that MAP kinase may also be able to phosphorylate the same peptides on R2 that are phosphorylated

in actively growing cells, just like what was observed for $p34^{cdc2}$ (Chan *et al.*, 1993). The second reason takes into consideration the crucial role MAP kinase plays in signal transduction (Blenis, 1991), and the ablilty for various external stimuli to regulate ribonucleotide reductase expression (Hurta and Wright, 1992b, 1994; Hurta *et al.*, 1991, 1992; Chen *et al.*, 1994b, 1994c). However, the two-dimensional phosphopeptide map of MAP kinase *in vitro* phosphorylated recombinant R2 protein showed a complex pattern with multiple phosphopeptide spots, indicating phosphorylation at multiple sites. This pattern did not resemble the maps obtained from *in vivo* phosphorylated R2 proteins isolated from ^{32}P-labeled cells. Therefore, MAP kinase is unlikely to have a role in the direct phosphorylation of R2 protein *in vivo* in proliferating cells. Nevertheless, this does not rule out the possibility of ribonucleotide reductase regulation by MAP kinase in the signal transduction pathway. It has been shown that phorbol esters can modulate ribonucleotide reductase gene expression in cells by signal transduction pathways involving protein kinase C (Choy *et al.*, 1989; Hurta and Wright, 1992b; Chen *et al.*, 1994b, 1994c), and activation of protein kinase C by phorbol esters can lead to activation of MAP kinase (Li *et al.*, 1995; Yamaguchi *et al.*, 1995).

Surprisingly, a series of tyrosine kinases also have the ability to phosphorylate recombinant R2 protein *in vitro* (Figure 15), whereas tyrosine phosphorylation is not observed in cells during logarithmic growth (Chan *et al.*, 1993). In attempt to explain this, two possibilities exist. First, the obtained results could be artifacts resulting from *in vitro* experimental conditions, and therefore bear no biological significance. A much more

interesting possibility that can be implied from these results is as follows: provided that these results reflect what happens *in vivo*, there are sites within the R2 protein that can be recognized and phosphorylated by protein tyrosine kinases. Under normal growth conditions without any external stimuli, phosphorylation at tyrosine residue(s) will not take place, and therefore only the phosphorylation at serine-20 is observed. However, when the cells are growing under different conditions, such as after exposure to external stimuli, or when DNA damage is induced in the cell, alterations in the phosphorylation status of R2 protein will follow, involving phosphorylation of the R2 protein at tyrosine residue(s) by protein tyrosine kinases. This hypothesized involvement of protein tyrosine kinase in R2 phosphorylation remains to be explored.

3. Regulation of Ribonucleotide Reductase in G_0/early G_1 Arrested Cells

When cells enter the non-proliferating quiescent G_0 phase, they enter a very different metabolic state (Stevenson *et al.*, 1995). During quiescence, mammalian cells economize by reducing their content of many enzymes in the pathways of nucleotide metabolism and DNA replication, since these processes are not required in non-proliferating cells (Kit *et al.*, 1965; Groudine and Casimir, 1984; DeGregori *et al.*, 1995; Tuusa *et al.*, 1995). Upon reinitiation of growth and DNA synthesis in response to appropriate external stimuli, cells elevate nucleotide precursor pools, and increase their capacity to incorporate nucleotides into DNA. Ribonucleotide reductase is an example of one of these growth-regulated enzymes.

There is a close correlation between ribonucleotide reductase activity and the rate of DNA synthesis. It shows a periodic increase in activity during transit through the cell cycle in exponentially growing cells. In resting cells, no enzyme activity can detected. Just before cells enter S-phase, ribonucleotide reductase activity begins to appear, and it shows a maxima during S-phase (Nordenskjold *et al.*, 1970; Lewis *et al.*, 1978). Interestingly, this cell cycle/proliferation specific regulation of enzyme activity is not controlled by enzyme activation/inactivation, but instead at the level of gene expression. The R1 protein levels in proliferating cells are approximately constant and in excess throughout the cell cycle (Engstrom *et al.*, 1985; Mann *et al.*, 1988). On the other hand, R2 protein levels are limiting, and the increase in holoenzyme activity during S-phase correlates with its S-phase specific *de novo* synthesis (Eriksson *et al.*, 1984; Choy *et al.*, 1988).

To study the regulation of ribonucleotide reductase at the molecular level during quiescence, both BALB/c 3T3 and H^R3T3 cells were arrested at G_0/early G_1 phase by the widely used serum-starvation method (Bjorklund *et al.*, 1990; Feldherr and Akin, 1991; Zalvide *et al.*, 1992). Cell cycle distributions of these growth-arrested cells were analyzed by the fluorescence activated cell sorting (FACS) technique. These cell cycle arrest experiments were successful, since significant shifts in cell cycle distribution were observed. About 90% of the cells arrest in the G_0/G_1 phase, for both wild type and drug-resistant cells, as demonstrated by the flow cytometric profiles (Figures 17 and 18). Due to the lack of adequate methods for distinguishing the G_0 period from G_1, these growth-arrested cells were considered to be arrested at G_0/early G_1, as discussed under the Results

section.

In the past, it has been demonstrated that the amount of both R1 and R2 protein subunits decrease to very low levels in quiescent or terminally differentiated cells, and both transcripts are almost undetectable in these cells (Eriksson *et al.*, 1984; Engstrom *et al.*, 1985). Interestingly, results from this study show that both R1 and R2 mRNAs are still present in quiescent cells, even though they exist at much lower levels than asynchronized proliferating cells. It has been shown that the promoter of the R2 gene is inactive in quiescent cells (Bjorklund *et al.*, 1992), and therefore the detected R2 message may indicate post-transcriptional regulation, possibly at the level of mRNA stability (Luscher *et al.*, 1985; Lieberman *et al.*, 1988; Chang *et al.*, 1990; Feder *et al.*, 1990; Amara *et al.*, 1995b).

Growth-arrested BALB/c 3T3 cells have been shown to have an overall decrease in signal-mediated nuclear transport, which is the result of a decrease in the functional diameter of nuclear pores in the nuclear envelope (Feldherr and Akin, 1991). Although the functional diameter of these transport channels in quiescent cells is sufficiently large to accommodate most proteins that normally enter the nucleus, the export of precursor RNA is restricted (Feldherr and Akin, 1991). This piece of information suggests that R1 and R2 mRNAs detected in growth-arrested cells are likely the left-over products from the period before the cells become quiescent. It also strengthens the hypothesis that there is a change in the half-life for both R1 and R2 mRNAs in these growth-arrested cells, since both R1 and R2 mRNAs have a half-life of 1.5 hours in proliferating cells (Chen *et al.*,

1993; Amara *et al.*, 1993), which is too short to account for the levels of mRNAs detected in these quiescent cells.

Reduced levels of both R1 and R2 proteins were observed in quiescent BALB/c 3T3 cells when compared to asynchronized proliferating cells, and the observed extent of the decreases roughly paralleled the observed extent of decreases in mRNA levels. This parallel pattern suggests that maybe both R1 and R2 proteins are still being synthesized from their corresponding mRNAs at this stage, but at a much lower level due to the decrease in mRNA levels. Other possibilities exist. For example, *de novo* synthesis of R1 and R2 proteins from the corresponding long-lived mRNAs is halted, and the R1 and R2 proteins observed in quiescent cells is the result of carry-over from G_1 phase. However, if this is the case, the half-life of R2 protein would be modified from the original 3-hour in logarithmic cells (Eriksson *et al.*, 1984; Choy *et al.*, 1988). Further investigations are needed for us to understand the underlying mechanisms behind these observations.

However, G_0/early G_1 arrested H^R3T3 cells showed solid evidence of R2 protein degradation. The band at *M*r 44000, corresponding to the molecular weight of R2 protein, can hardly be detected in cellular protein extract isolated from quiescent H^R3T3 cells. It appears that the protein is being fragmented into polypeptides with different sizes. The explanation for the differential regulation of R2 protein in quiescent wild type and mutant 3T3 cells is not known. One way to explain this observation is to take into consideration the several "depths" of G_0 that exist. According to Augenlicht and Baserga (1974), the biochemical state of arrest changes as cells remain quiescent. As cells go "deeper" into

G_0, the capacity for protein synthesis as well as the level of cellular protein decrease, and it takes longer for the cells to re-enter DNA synthesis after growth stimulation (Augenlicht and Baserga, 1974; Miska and Bosmann, 1980). Hydroxyurea resistant H^R3T3 cells probably were arrested at a "deeper" G_0 state than the wild type cells due to differences in cellular behavior, thereby showing extensive degradation of the R2 protein. If this is true, it can also help to explain the difference in terms of the amount of time it takes for quiescent wild type and mutant 3T3 cells to enter S-phase after serum stimulation (Figure 19).

Studies have demonstrated that the phosphorylation status of many proteins is altered as a function of the cell cycle (Saijo *et al.*, 1992; Mayol *et al.*, 1995; Wells and Hickson, 1995). For instance, the retinoblastoma-related protein p130 exists in multiple phosphorylated forms. Specific phosphorylated forms of p130 are found at different times after serum re-stimulation, and two of these forms are only found in serum-starved cells (Mayol *et al.*, 1995).

The cell cycle regulation of mammalian ribonucleotide reductase R2 protein phosphorylation has not been studied before. By using the serum-starved cells, the phosphorylation status of ribonucleotide reductase R2 subunit in quiescence was studied. The experimental results suggest that the phosphorylation site on R2 protein is not altered as the cell enters quiescence (Figures 22 and 23). Moreover, as demonstrated by quiescent H^R3T3 cells, the degradation of R2 protein does not appear to be regulated by any change in the phosphorylation pattern of the protein.

4. Concluding Remarks

In this study, a hydroxyurea resistant mutant BALB/c 3T3 cell line has been isolated and characterized. It showed a decrease in sensitivity to hydroxyurea, which was accompanied by amplification of the R2 gene, elevated levels of R2 mRNA, and elevated R1 and R2 protein levels when compared to parental wild type cells. Its R2 protein over-producing property makes it a good model for studying R2 protein phosphorylation *in vivo*. Drug resistant cells did not show any change in the phosphorylation pattern of the R2 protein, suggesting that drug resistance does not involve an alteration of R2 phosphorylation. By using the technique of serum-starvation, the regulation of ribonucleotide reductase at the molecular level, especially at the level of protein phosphorylation, has been studied in quiescent cells. Decreases in protein and mRNA levels of both R1 and R2 were observed, and R2 protein degradation was particularly noticeable in quiescent drug resistant cells, which was not mediated by changes in phosphorylation pattern. However, this is only the starting point. The cell cycle phosphorylation characteristics of the R2 protein can be studied by arresting cells at different stages of the cell cycle. The study of R2 protein phosphorylation in this work was focused on qualitative aspects, and future studies may need to include quantitative comparison between the levels of R2 phosphorylation under different growth conditions.

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