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AMPLIFICATION OF THE GENES FOR BOTH COMPONENTS OF RIBONUCLEOTIDE REDUCTASE IN HYDROXYUREA RESISTANT MAMMALIAN CELLS

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Ribonucleotide reductase catalyzes the formation of deoxyribonucleotides from ribonucleoside diphosphate precursors, and is a rate-limiting step in the synthesis of DNA. The enzyme consists of two dissimilar subunits usually called M1 and M2. The antitumor agent, hydroxyurea, is a specific inhibitor of DNA synthesis and acts by destroying the tyrosyl free radical of the M2 subunit of ribonucleotide reductase. Two highly drug resistant cell lines designated H^R-15 and H^R-30 were isolated by exposing a population of mouse L cells to increasing concentrations of hydroxyurea. H^R-15 and H^R-30 cells contained elevated levels of ribonucleotide reductase activity, and were 68 and 103 times, respectively, more resistant than wild type to the cytotoxic effects of hydroxyurea. Northern and Southern blot analysis indicated that the two drug resistant lines contained elevated levels of M2 mRNA and M2 gene copy numbers. Similar studies with M1 specific cDNA demonstrated that H^R-15 and H^R-30 cell lines also contained increased M1 message levels, and showed M1 gene amplification. Mutant cell lines altered in expression and copy numbers for both the M1 and M2 genes are useful for obtaining information relevant to the regulation of ribonucleotide reductase, and its role in DNA synthesis and cell proliferation. © 1990 Academic Press, Inc.

The antitumor agent, hydroxyurea, is a specific inhibitor of DNA synthesis and arrests cell growth at S phase (1,2), making the drug a useful tool in cell synchrony studies (3). Hydroxyurea enters mammalian cells by a diffusion process (4), and has been used clinically in the treatment of a wide range of solid tumors, as well as acute and chronic leukemia (5,6). Furthermore, it has shown promise as a radiation potentiator (7), as a myelosuppressive agent in treating polycythemia vera (8), and in controlling the proliferation of psoriasis (9). The primary site of action for hydroxyurea is the highly regulated enzyme ribonucleotide reductase, which is responsible for the *de novo* conversion of ribonucleo-

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tides to deoxyribonucleotides (1,2), required for the synthesis of DNA. This reaction is rate-limiting for DNA synthesis, and therefore the enzyme plays an important role in the regulation of cell division (1,2). In mammalian cells, this enzyme contains two dissimilar components often called M1 and M2 (1,2,10). Protein M1 is a dimer with a molecular weight of 170,000 and possesses substrate and effector binding sites (11). Protein M2 is a dimer with a molecular weight of 88,000 and contains stoichiometric amounts of non-heme iron and a unique tyrosyl free radical required for activity (12,13). The mode of action of hydroxyurea has been shown both _in vivo_ and _in vitro_ to involve destruction of the tyrosyl free radical within the M2 protein (13,14).

Hydroxyurea has been very useful as a selective agent in cell culture for the isolation of drug resistant cell lines with modifications in ribonucleotide reductase (1,2). Drug resistance is a major clinical problem in the treatment of cancer (15), and the molecular processes that lead to the development of cell populations exhibiting drug resistant properties are of obvious interest (15-17). In this report we describe novel molecular alterations involving ribonucleotide reductase in mammalian cells directly selected for resistance to high concentrations of the antitumor agent, hydroxyurea.

MATERIALS AND METHODS

Cell lines and culture conditions. Cells were routinely cultured at 37°C on plastic tissue culture plates (Lux Scientific, Ltd.) in α-minimal essential medium (Flow Laboratories, Ltd.) supplemented with 10% fetal calf serum (Gibco, Ltd.) and antibiotics (18). The procedure used to isolate mouse cell lines with increasing drug resistance characteristics have been described (19). Starting with a wild type population of mouse L cells, the following hydroxyurea concentrations were used in the selections: 0.35 mM; 1.3 mM; 1.5 mM; 2.0 mM; 3.0 mM; 4.0 mM; 5.0 mM; 15.0 mM (H^R-15); 30 mM (H^R-30). Cells were frozen in the presence of growth medium containing 5% dimethyl sulfoxide at each selection step, and those selected between 0.35 mM and 5.0 mM drug have been characterized in detail (20). The drug resistance properties of the H^R-15 and H^R-30 cell lines presented in this report are stable and remained essentially unchanged during approximately 12 months of continuous culture in the absence of drug selection. The relative colony forming efficiency was defined as the ability to form colonies in the presence of hydroxyurea divided by that ability in the absence of drug (21). The D_{10} value is the drug concentration which reduces the relative colony forming efficiency to 10% (21).

Nucleic acid analysis. Genomic DNA was prepared from logarithmically growing cells by phenol-chloroform extraction (22), and total cellular RNA was isolated from logarithmically growing cells by the guanidium-cesium chloride method (23). Southern and Northern hybridizations were performed as previously described (e.g. 13,18,20), using a ^{32}P-labelled NcoI generated fragment containing the cDNA of clone 65 (M1) or the Pst 1 fragment of clone 10 (M2) (24). Loading of RNA was determined by probing with β-actin cDNA (20), and densitometric analysis was performed using a Beckman Dμ-8 gel scanning spectrophotometer.

Preparation and assay of ribonucleotide reductase. Enzyme preparations containing 1 to 4 mg of protein/ml were used to assay for ribonucleotide reductase by a modified method of Steeper and Stuart (25) using [^{14}C] CDP as substrate and snake venom phosphodiesterase to hydrolyze the nucleotides as we have previously described (19,20,26).

RESULTS AND DISCUSSION

Sensitivity to hydroxyurea. Phenotypically stable hydroxyurea resistant cell lines, H^R-15 and H^R-30 were isolated from a drug sensitive wild type mouse L cell population by step-wise selections in the presence of gradually increasing concentrations of hydroxyurea, as outlined in "Materials and Methods". The sensitivities of the wild type and the two variant lines to the cytotoxic effects of hydroxyurea were compared by assaying colony forming ability in the presence of various drug concentrations. The results of these studies are shown in Fig. 1. Both drug selected lines were very resistant to the cytotoxic effects of hydroxyurea, with the H^R-15 and H^R-30 populations exhibiting D_{10} values of 10.2 mM and 15.5 mM drug. The D_{10} value of the parental mouse L cells was only 0.15 mM hydroxyurea, indicating that the H^R-15 and H^R-30 cell lines were 68- and 103-fold less sensitive to hydroxyurea than the wild type line.

Ribonucleotide reductase activity. We have shown that cell lines selected for resistance to hydroxyurea and related drugs frequently possess elevated levels of ribonucleotide reductase (1,2). In agreement with previous

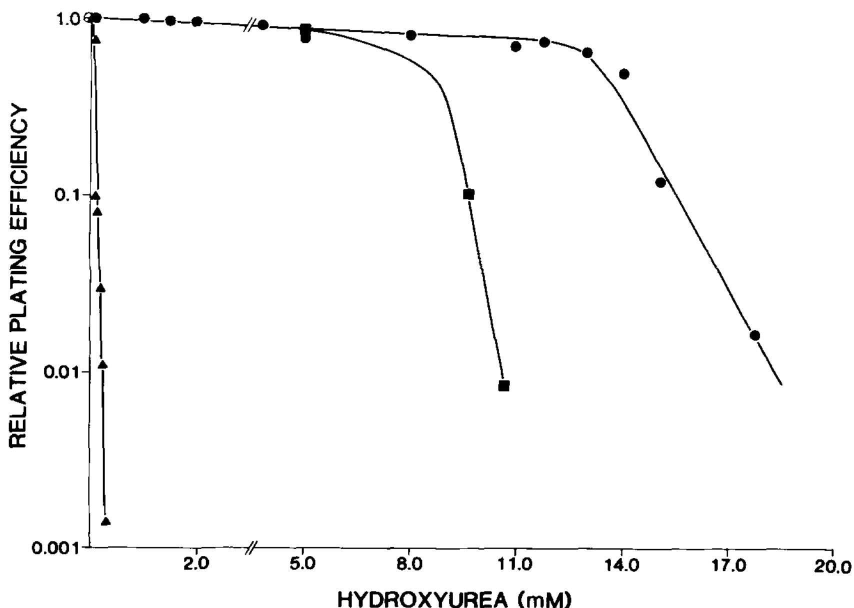

Fig. 1. Relative colony forming abilities in the absence and presence of various concentrations of hydroxyurea of wild type (▲), H^R-15 (■) and H^R-30 (●) cell lines.

Table 1: Ribonucleotide Reductase Activity

Cell Lines	Enzyme Activity (nmole CDP reduced/hr/mg)	Fold Increase
Wild type	0.98	-
H^R-15	3.79	3.87
H^R-30	22.6	23.1

observations Table 1 shows that both hydroxyurea resistant cell lines exhibited increased CDP reductase activity, when compared to parental wild type mouse L cells. H^R-15 cells showed approximately a 4-fold increase in enzyme activity, whereas the more resistant H^R-30 line contained about a 23-fold elevation in activity.

Ribonucleotide reductase message levels. The cDNA clones encoding the M1 and M2 components of ribonucleotide reductase were used to analyze the relative amounts of the M1 and M2 transcripts in wild type and drug resistant cell lines. Northern blot analysis of M2 mRNA levels (Fig. 2A) showed that there were considerable differences in the levels of M2 message present in the three cell lines. Densitometric measurements estimated M2 mRNA increases of 125- and 150-fold above wild type levels in H^R-15 and H^R-30 cell lines, respectively. Northern blot analysis, using M1 specific cDNA as hybridization probe was also interesting (Fig. 2B), and showed that there were significant elevations of M1 message in the drug resistant lines when compared to the wild type situation. Densitometric measurements showed a 7- and 10-fold increase in M1 message concentration in H^R-15 and H^R-30 cells, respectively.

Ribonucleotide reductase genes. A Southern blot analysis of wild type, H^R-15 and H^R-30 genomic DNA, digested with the Hind III restriction endonuclease, and probed with M2 cDNA is shown in Fig. 3. The wild type and the mutant cell lines showed similar banding pattern, with clear amplification with both H^R-15 and H^R-30 DNA, when compared to the wild type situation. Densitometric scans of bands amplified in drug resistant cells gave estimates of approximately 12 to 14-fold M2 gene amplification in the H^R-15 and H^R-30 cell lines. A Southern blot probed with M1 cDNA is shown in Fig. 4A and B. When compared to the mouse wild type line, it was obvious that the M1 gene was amplified in both H^R-15 and H^R-30 cells. Densitometric analysis indicated that the M1 gene copy number was increased in H^R-15 and H^R-30 cells by approximately 3- and 4-fold, respectively. Therefore, resistance to very high concentrations of hydroxyurea in mouse L

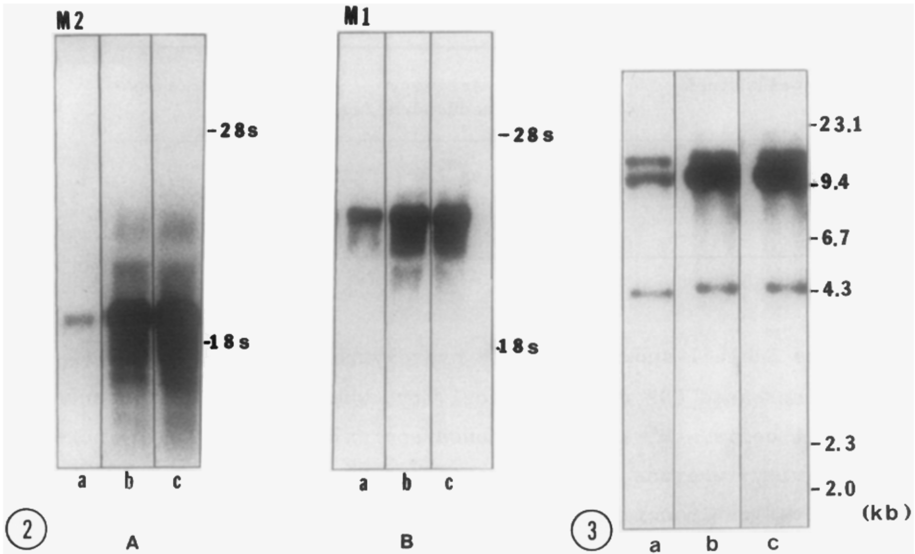

Fig. 2. Northern blots of M2 and M1 mRNA in the wild type and drug resistant cell lines. For Northern blots, 20 μg of total cellular RNA isolated from wild type and hydroxyurea resistant variants were run on 1% agarose formaldehyde gels as indicated in Materials and Methods. Loading of RNA was determined by reprobing with β-actin cDNA as described. (A): Northern blot of M2 mRNA: (a) wild type, (b) H^R-15 and (c) H^R-30. (B): Northern blot of M1 mRNA: (a) wild type, (b) H^R-15 and (c) H^R-30. The positions of 28s and 18s rRNA are indicated. Autoradiograms were exposed for 24 hours at -70°C with intensifying screens.

Fig. 3. Southern blot analysis of M2 genes in genomic DNA of the wild type and drug resistant cell lines. Twenty μg of high molecular weight DNA were digested to completion with Hind III. Blots were hybridized with a ^{32}P-labelled Pst 1 fragment of the M2 cDNA as described in Materials and Methods. Autoradiograms were exposed for 48 hours at -70°C with intensifying screens. The lanes are: (a) wild type; (b) H^R-15 and (c) H^R-30.

cells was accompanied by obvious amplifications of both genes of ribonucleotide reductase.

Conclusions. Understanding the regulation of mammalian ribonucleotide reductase is of much importance because this enzyme activity is a rate-limiting step in DNA synthesis and cell proliferation (1,2). Alterations in ribonucleotide reductase activity can have profound effects upon the biology of the cell (1,26), and its key role in the synthesis of DNA makes it a logical target for designing drugs with potential chemotherapeutic activity (27-29). Elevated M2 mRNA levels and M2 gene amplification frequently accompany resistance to the antitumor agent, hydroxyurea, but changes in M1 gene expression occur relatively rarely in drug-resistant cells (1,2). The present study clearly shows that modifications in M1 gene expression through M1 gene amplification can be achieved at very high drug resistance, by selection in the presence of step-wise increases in

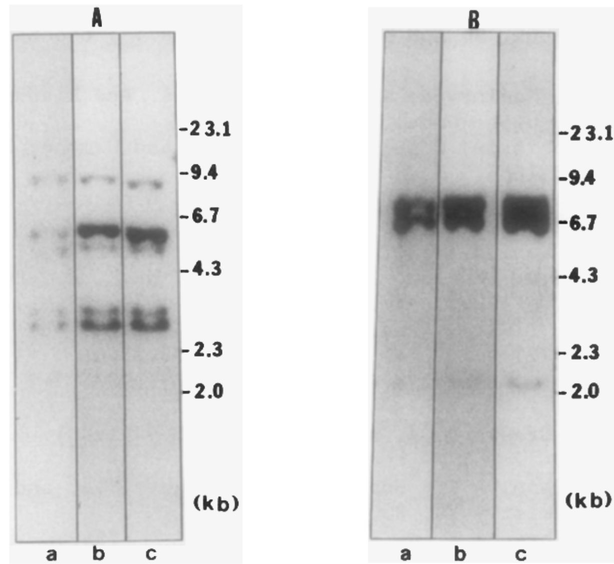

Fig. 4. Southern blot analysis of M1 genes in genomic DNA of the wild type and drug-resistant cell lines. Twenty μg of high molecular weight DNA were digested to completion with EcoR1 (A) or Hind III (B). Blots were hybridized with a ^{32}P-labelled Nco1 fragment of the M1 cDNA as described in Materials and Methods. Autoradiograms were exposed for 72 hours at -70°C with intensifying screens. The lanes are: (A): (a) wild type, (b) H^R-15 and (c) H^R-30 and (B): (a) wild type, (b) H^R-15 and (c) H^R-30.

hydroxyurea concentrations. This indicates for the first time that drug resistance involving M1 gene amplification can occur by direct drug selection without further genetic manipulation (30,31). Mutants of this type, altered in the expression of both the M1 and M2 genes provide a valuable approach for investigations of the complex regulation of mammalian ribonucleotide reductase and its key role in the synthesis of DNA (1,2,32).

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