Elevated Expression of M1 and M2 Components and Drug-Induced Posttranscriptional Modulation of Ribonucleotide Reductase in a Hydroxyurea-Resistant Mouse Cell Line†

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ABSTRACT: Ribonucleotide reductase, a rate-limiting enzyme in the synthesis of DNA, consists of two nonidentical subunits, proteins M1 and M2. Hydroxyurea, a specific inhibitor of DNA synthesis, acts by destroying the unique tyrosyl free radical of protein M2. In the past, we have described a mouse L cell line which exhibited a stable resistance to high concentrations of hydroxyurea [McClarty, G. A., Chan, A., & Wright, J. A. (1986) *Somat. Cell Mol. Genet. 12*, 121–131]. When this line was grown in the absence of hydroxyurea, the cells contained a modest but stable elevation in ribonucleotide reductase activity. However, the activity was further increased on the addition of drug to the culture medium. This was accompanied by an increase in protein M2 activity as shown by activity titration experiments. Likewise, removal of hydroxyurea resulted in a decrease in M2 activity. In the present study, we make use of recently isolated cDNAs and monoclonal antibodies for both the M1 and M2 proteins to further our understanding of the mechanism of hydroxyurea resistance at the molecular level in a subclone of this cell line. Our results indicated that protein M1 levels were elevated 2–3-fold and protein M2 levels were increased about 50-fold in the mutant cells when they were grown in the absence of hydroxyurea, compared to wild-type cells. These protein increases were accompanied by corresponding elevations in the levels of mRNAs for both subunits and increased rates of transcription of both genes. There was a 6-fold amplification in the gene copy number for protein M2. However, most interesting was the observation that both M1 and M2 protein levels were further elevated when mutant cells were cultured in the presence of hydroxyurea, and this elevation was not accompanied by increases in the corresponding mRNAs. These results indicate that hydroxyurea can modulate ribonucleotide reductase expression posttranscriptionally.

The first unique step leading to DNA synthesis is the conversion of ribonucleotides to their corresponding deoxyribonucleotides, a reaction catalyzed by ribonucleotide reductase (Lammers & Follman, 1983). In mammalian cells, the enzyme consists of the two nonidentical subunits often called M1 and M2, both of which have been purified to homogeneity (Thelander et al., 1980, 1985). Although once postulated to be a reaction that takes place in the nucleus (Prem veer Reddy & Pardee, 1980, 1982), recent immunocytochemical studies, using specific antibodies to both the M1 and M2 subunits, have demonstrated that ribonucleotide reductase is exclusively localized in the cytoplasm (Engstrom et al., 1984; Y. Engstrom, unpublished observations). The two ribonucleotide reductase subunits are differentially regulated during the cell cycle. The levels of the M1 subunit appear to be constant through the cell cycle (Engstrom et al., 1985; Mann et al., 1987). In contrast, there is an S-phase-correlated increase in protein M2 resulting from its de novo synthesis (Eriksson et al., 1984). Therefore, the activity of ribonucleotide reductase is controlled during the cell cycle by the synthesis and breakdown of protein M2 (Eriksson et al., 1984).

Hydroxyurea is an antitumor agent which inhibits ribonucleotide reductase activity by interacting with the M2 component specifically at the tyrosyl free radical site (Graslund et al., 1982). Mutant cells selected for resistance to hydroxyurea have been shown to overproduce ribonucleotide reductase activity mainly as a result of a specific overproduction of the M2 subunit (Akerblom et al., 1981; Lewis & Srinivason, 1983; Wright, 1983; Wright et al., 1980, 1987; McClarty et al., 1986a). In a recent report, Thelander and Berg (1986) used the cDNAs they isolated for both the M1 and M2 components of ribonucleotide reductase to examine the expression of both genes in a hydroxyurea-resistant mouse cell line. They reported a 50–100-fold excess of M2 mRNA in the hydroxyurea-resistant cell line compared to the parent cell line and a 10-fold excess of M1 mRNA (Thelander & Berg, 1986). Furthermore, their mutant cell line showed a 5-fold amplification of the M2 gene whereas the M1 gene was still a single copy (Thelander & Berg, 1986). In addition, Wright et al. (1987) have characterized a series of hamster, mouse, rat, and human cell lines resistant to the cytotoxic effects of hydroxyurea. All of these resistant lines contained increased levels of M2 mRNA, and five of the six lines showed M2 gene amplification (Wright et al., 1987). In contrast, there was no evidence of M1 gene amplification, and the levels of M1 mRNA were not markedly elevated in any of these cell lines (Wright et al., 1987). It is now evident that gene amplification is a common mechanism for achieving resistance to hydroxyurea.

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Recently, we have characterized a mouse L cell line selected for resistance to increasing cytotoxic concentrations of hydroxyurea (McClarty et al., 1986a). This cell line exhibited a stable resistance to high concentrations of drug (5 mM) as a result of increased ribonucleotide reductase activity. An additional increase in ribonucleotide reductase can occur when hydroxyurea-resistant cells are cultured in the presence of hydroxyurea (Lewis & Wright, 1979; Wright et al., 1980). This characteristic was particularly pronounced in the highly drug-resistant mouse L cell line, which overproduced enzyme activity in a drug concentration dependent manner (McClarty et al., 1986a). When cultured in the absence of hydroxyurea, the mutant cells contained a 5-fold increase in ribonucleotide reductase activity. However, when the cells were grown in the presence of 5 mM hydroxyurea, there was a further 4-fold increase in activity. Upon removal of hydroxyurea from the culture medium, ribonucleotide reductase activity rapidly returned to its uninduced level (McClarty et al., 1986a). Both the M1 and M2 subunits were elevated in the resistant cell line grown in the absence of hydroxyurea. Subunit M2 activity titration experiments indicated that there was a further increase in M2 activity when the mutant cells were cultured in the presence of hydroxyurea and these results lead us to suggest that overproduction of enzyme activity in the presence of hydroxyurea and decline in activity in the absence of drug were dependent upon the levels of the M2 component. The availability of monoclonal antibodies to both proteins M1 and M2 as well as cDNAs from recent clonings of both subunits of ribonucleotide reductase has enabled us to examine the effects of hydroxyurea on the expression of ribonucleotide reductase in this mutant cell line.

Experimental Procedures

Cell Lines and Culture Conditions. The mutant cell line (SC2) used in this study is a subclone of a mutant line (LHF) that we have previously characterized (McClarty et al., 1986a,b). The properties of the SC2 cell line appear to be identical with those previously described for the LHF line (McClarty et al., 1986a,b). Wild-type mouse L cells were routinely cultured at 37 °C on plastic tissue culture plates (Lux Scientific) in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (Gibco). SC2 mutant cells were cultured as described above in the presence (SC2+) or absence (SC2−) of 5 mM hydroxyurea. Unless otherwise stated, SC2+ and SC2− refer to drug-resistant cells that were cultured in the presence and absence of hydroxyurea, respectively. SC2− cells were routinely cultured in the absence of hydroxyurea for at least 2 weeks prior to experimentation.

Electron Paramagnetic Resonance (EPR) Spectroscopy and Flow Cytometry. EPR spectroscopy was carried out on whole cells and cellular extracts as previously described (Akerblom et al., 1981; Eriksson et al., 1984). Hydroxyurea was not present during the extraction of the cells. The porportion of cells in the different cell cycle phases (G_1, S, and G_2 + M) was determined by flow cytometric analysis as described (Eriksson et al., 1984).

Reactivation of Protein M2. To reactivate protein M2, the EPR tube containing extract prepared from cells grown in the presence of hydroxyurea was rapidly thawed. $FeCl_3$ and dithiothreitol (DTT) were then added to final concentrations of 11 μM and 11 mM, respectively. Following vigorous mixing and aeration, the extract was incubated at 37 °C for 10 min, frozen in liquid nitrogen, and stored in liquid nitrogen.

For in vivo reactivation of protein M2, hydroxyurea-containing medium was aspirated from plates, and the cell monolayer was washed 2 times with 37 °C tris(hydroxymethyl)aminomethane (Tris)/saline. Fresh medium (DMEM) containing 10% fetal calf serum and 10 mM dithiothreitol (final pH 8.0) was added to the plates, and then they were returned to the 37 °C incubator for 1 h. Following this incubation, cells were packed in an EPR tube and frozen in liquid nitrogen as described previously (Akerblom et al., 1981; Eriksson et al., 1984).

Northern and Southern Blot Analysis. Total cellular RNA was extracted from logarithmically growing cells by the guanidinium thiocyanate/cesium chloride method (Chirgwin et al., 1979), and poly(A+) RNA was recovered after one cycle of adsorption and elution from an oligo(dT)–cellulose column (Aviv & Leder, 1972). Ten micrograms of poly(A+) RNA was subjected to electrophoresis through 1% formaldehyde/agarose gels followed by transfer to nitrocellulose membranes. Genomic DNA was prepared from logarithmically growing cells by phenol/chloroform extraction (Blin & Stafford, 1976). For Southern analysis, 20 μg of genomic DNA was digested to completion with *Eco*RI, *Bam*HI, or *Hin*dIII endonucleases followed by fractionation on 0.75% agarose gels and subsequent transfer to nitrocellulose membranes. All blots were prehybridized at 42 °C for 18 h in 50% formamide, 5 × SCC (1 × SCC is 0.15 M NaCl/15 mM sodium citrate, pH 7.0), 7.5 × Denhardt's solution [1 × Denhardt's solution contains 20 mg each of Ficoll, poly(vinylpyrrolidone), and bovine serum albumin in 100 mL of water], 50 mM sodium phosphate, pH 7.0, 0.1% sodium dodecyl sulfate (SDS), and 100 μg/mL denatured salmon sperm DNA or 0.25 mg/mL tRNA and 1 μg/mL poly(A) RNA. Hybridization was performed in the same solution for 18 h with (1–2) × 10^6 cpm/mL of a ^{32}P-labeled *Nco*I-generated fragment containing the cDNA of clone 65 (M1 protein) or the *Pst*I fragment of clone 10 (M2 protein) (Thelander & Berg, 1986). cDNA probes were labeled by the hexadeoxyribonucleotide method of Feinberg and Vogelstein (1983) using [α-^{32}P]dCTP (specific activity 3000 Ci mmol^{-1}, Amersham) to a specific activity of 5 × 10^8 to 1 × 10^9 cpm/μg.

Blots were washed 4 times for 15 min each in 2 × SSC and 0.1% SDS at room temperature and then 2 times for 30 min in 0.1 × SSC and 0.1% SDS at 60 °C. Autoradiography was done at −70 °C using Kodak X-Omat AR film and Cronex high-speed intensifying screens.

Nuclei Isolation and Nuclear Run-off Transcription Assay. Nuclei isolation and nuclear run-off transcription analysis were performed essentially as described by Greenberg and Ziff (1984). Following isolation, nuclei (1 × 10^7) were resuspended in 100 μL of 50 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) and frozen in liquid nitrogen. The nuclei were thawed for the run-off transcription assay and mixed with an equal volume (100 μL) of reaction mix [10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM each of ATP, CTP, and GTP, and 100 μCi of [α-^{32}P]UTP (400 Ci mmol^{-1}, Amersham)] and incubated with vigorous shaking at 30 °C for 45 min. The ^{32}P-labeled RNA was isolated as described by Stallcup and Washington (1983). Following ethanol precipitation, the ^{32}P-labeled RNA pellet was resuspended in distilled water and heat denatured at 65 °C for 5 min, and then an equal number of cpm (2 × 10^6 cpm) was added to the hybridization mixture. Prehybridization and hybridization conditions were the same as described for Southern and Northern blots except hybridization was carried out for 72 h at 42 °C. Filters were washed, and autoradiography was carried out as described above for Southern and Northern blots.

For binding to nitrocellulose, plasmids were first linearized by restriction enzyme digestion, and the DNA was denatured by boiling in 0.1 M NaOH for 5 min followed by neutralization with 5 volumes of 6 × SSC. The denatured DNA was spotted onto nitrocellulose using the Bio-Rad dot blot apparatus. Three micrograms of DNA was applied per dot. Recombinant plasmids used were D 65 containing the complete coding sequence of the mouse M1 cDNA (Thelander & Berg, 1986), C 10 containing the complete coding sequence of the mouse M2 cDNA (Thelander & Berg, 1986), and, as a background control, the pUC 18 derivative of pBR322. Conditions were sufficient for complete hybridization, as demonstrated by the lack of detectable hybridization of run-off RNA to a second set of identical filters (data not shown).

Immunoblot Assay. For Western blot analysis, protein M1 was detected with anti-M1 mouse monoclonal antibody (Engstrom et al., 1984), and protein M2 was detected with anti-M2 rat monoclonal antibody (Y. Engstrom, unpublished observations). Following cell extract preparation, total cell extract protein content was determined, and then a given amount of protein was analyzed on a 10% linear SDS–polyacrylamide gel (Engstrom et al., 1979). Proteins were then transferred to nitrocellulose membranes by the method of Towbin et al. (1979). The transfer was carried out at 250 mA at 20 °C for 12–16 h. After transfer, membranes were blocked in 50 mM Tris-HCl (pH 7.6) in saline (TBS) containing 0.5% Tween 20 (TBS/Tween) plus 1% bovine serum albumin for 1 h. The membranes were then incubated with either AD 203 anti-M1 mouse monoclonal antibody or JB 4 anti-M2 rat monoclonal antibody for 3 h and washed 3 times, for 30 min each, in TBS/Tween followed by incubation with the appropriate second antibody for 3 h. Goat anti-mouse IgG conjugated with alkaline phosphotase (Sigma) was used for protein M1 detection, and rabbit anti-rat IgG conjugated with alkaline phosphatase (Sigma) was used for detecting protein M2. Following incubation with second antibodies, the blots were washed 3 times for 30 min each in TBS/Tween. Finally, the bound antibodies were detected by the development of the alkaline phosphatase reaction as described by Blake et al. (1984), except that veronal acetate buffer was replaced by 0.1 M ethanolamine chloride (pH 9.6).

Results

Protein M2 Tyrosyl Free Radical Concentrations in Wild-Type and Hydroxyurea-Resistant Cells. In order to obtain a more quantitative measure of M2 protein levels in our mutant cell line, we took advantage of the fact that the M2 protein tyrosyl free radical gives a characteristic EPR signal that can be quantified even when measured in whole cells (Graslund et al., 1982). The tyrosyl radical EPR signals measured in logarithmically grown wild-type and mutant cells continually grown in the absence of hydroxyurea (SC2–) and mutant cells continually grown in the presence of hydroxyurea (SC2+) are shown in Figure 1. Also shown in Figure 1 (inset) are the results of flow cytometric analysis indicating the proportion of cells in the different cell cycle phases in each of the three cell populations. The proportion of the population in S phase for wild-type, SC2–, and SC2+ cell lines is 46%, 49%, and 55%, respectively, and the concentration of the free radical in wild-type, SC2–, and SC2+ cell lines was estimated at 0.14, 7.3, and 0.56 μM, respectively, in this experiment. The dramatic decrease in free radical concentration in the mutant cell line grown in the presence of hydroxyurea, as compared to the same cell line grown in the absence of drug, is a result of the ability of hydroxyurea to destroy the M2 tyrosyl free radical, leading to loss of the EPR signal. Previously, it has been shown that it is possible to achieve partial (58%) regeneration of the protein M2 tyrosyl free radical in vivo simply by replacing hydroxyurea-containing medium with drug-free medium, followed by incubation at 37 °C for 35 min (Eriksson et al., 1984). In a separate experiment, incubation of SC2+ cells in the absence of hydroxyurea for 1 h at 37 °C led to a 7–8-fold increase in radical concentration; a further 2-fold increase in radical concentration in SC2+ cells occurred if dithiothreitol was added to the replacement medium (Table I). It is important to note that incubation with dithiothreitol

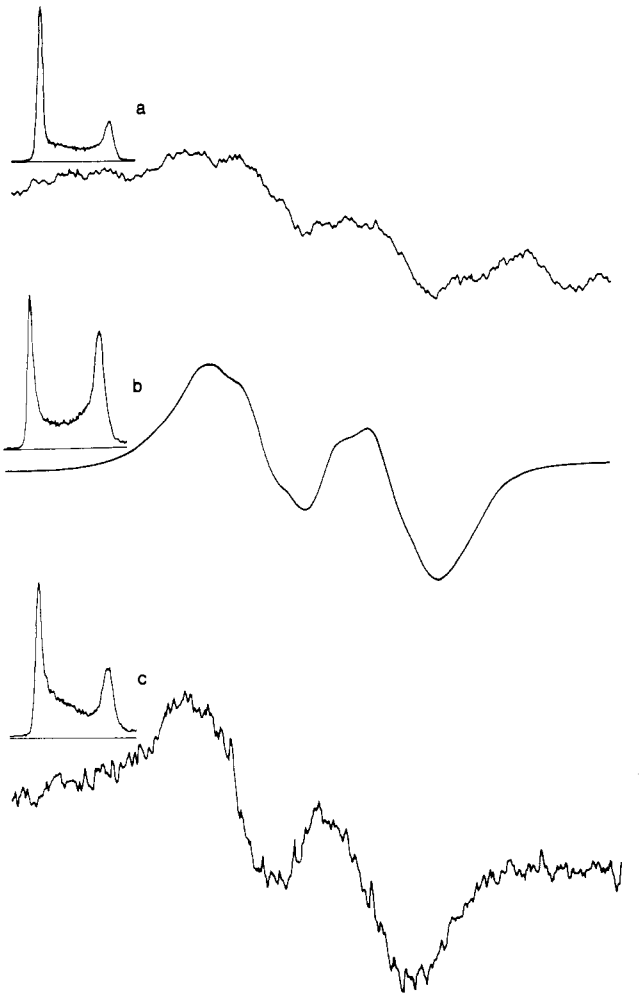

FIGURE 1: EPR spectra at 77 K of packed cells from wild-type (a), SC2– (b), and SC2+ cells (c). In the EPR measurements, the microwave power was 91.5 mW, and the modulations were 0.63, 0.25, and 0.63 mT for curves a, b, and c, respectively. For curve a, the spectrometer amplification was 10× and for curve c 8× that of curve b. Inset: DNA histograms of wild-type (a), SC2– (b), and SC2+ (c) cells. In the histograms, the ordinate gives the number of cells and the abscissa the relative fluorescence, which is proportional to DNA content with the peak on the left corresponding to G_1-phase cells.

Table I: EPR Measurements of the M2 Tyrosyl Free Radical in SC2 Cells and Cellular Extracts[a]

	tyrosyl free radical concn (μM)				
	whole cells			cell extracts	
cell line	untreated	new medium	new medium + 10 mM DTT	untreated	11 μM $FeCl_3$, 11 mM DTT
SC2–	3.8		4.0	0.17	0.24
SC2+	0.36	2.8	5.9	0.05	0.52

[a] Free radical concentrations were estimated by comparing EPR signals with a control signal as previously described (Wright et al., 1987).

has very little effect on the concentration of free radical in SC2- cells (Table 1), a result which supports previous findings that normally there is no pool of radical-free M2 protein present in cells (Eriksson et al., 1984). Following incubation in dithiothreitol-containing medium, we found that SC2+ cells contained approximately 1.5-fold more tyrosyl free radical than a similar number of SC2- cells. These results suggest that the inability of SC2+ cells to totally regenerate the tyrosyl free radical, in the absence of hydroxyurea, is a consequence of a limited supply of endogenous reducing power within the cell. Dithiothreitol has previously been shown to be a suitable source of reducing power for ribonucleotide reductase tyrosyl free radical regeneration in vitro (Graslund et al., 1982).

A similar set of radical-regenerating experiments was conducted using extracts prepared from SC2- and SC2+ cells. It is known that cell extracts treated with hdyroxyurea in vitro have their M2 radical signal destroyed; however, the signal can be regenerated if the drug-treated extracts are incubated in the presence of dithiothreitol, Fe^{2+}, and oxygen (Graslund et al., 1982; Thelander et al., 1983). The results in Table I extend these findings since extracts prepared from SC2 cells grown in the presence of hydroxyurea also lacked a significant tyrosyl free radical signal. Incubation of these extracts in the presence of dithiothreitol, Fe^{2+}, and oxygen regenerated a clearly detectable radical signal. In this instance, the free radical of protein M2 was destroyed by hydroxyurea in vivo and regenerated in vitro. There was a barely detectable radical signal in extracts prepared from SC2- cells, and this signal increased slightly following incubation with dithiothreitol, Fe^{2+}, and oxygen (Table I). This increase in radical concentration was probably a result of regeneration of free radical that had been inactivated during cell extract preparation. Results from these experiments support in vivo tyrosyl radical data in that following regeneration, SC2+ cell extracts contained approximately 2-fold more free radical than SC2- cell extracts.

Proteins M1 and M2 in Wild-Type and Hydroxyurea-Resistant Cell Lines. As mentioned above, the characteristic EPR signal of the protein M2 tyrosyl free radical provides a convenient means of determining active M2 levels in whole cells. However, mutant cells grown in the presence of hydroxyurea only showed a fraction of their radical signal unless the cells were incubated in a medium lacking drug and supplemented with a suitable reducing agent. The recent purification of protein M2 (Thelander et al., 1985) and subsequent development of specific monoclonal antibodies to it have enabled us to directly measure the levels of protein M2 in cell extracts, regardless of whether or not the protein contains the tyrosyl free radical. Cell extracts prepared from wild-type, SC2-, and SC2+ cells were subjected to SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose, and protein M2 was detected by using anti-M2 monoclonal antibody. The results clearly indicated that protein M2 was dramatically overproduced in the hydroxyurea-resistant SC2- cell line compared to the wild-type cell line (Figure 2). Furthermore, it was evident from these blots that the level of protein M2 in SC2+ cells was higher than in SC2- cells. These data support our findings with EPR measurements that mutant cells grown in the presence of hydroxyurea contain more protein M2 than the same cells grown in the absence of drug.

Protein M1 levels were compared in wild-type and SC2 cell lines by Western blot analysis using anti-M1 monoclonal antibody (Engstrom et al., 1984). The results indicated that SC2 cells grown in the absence of hydroxyurea contained approximately 2-3-fold more protein M1 than did wild-type cells (Figure 3). Also shown in Figure 3 are the results of a

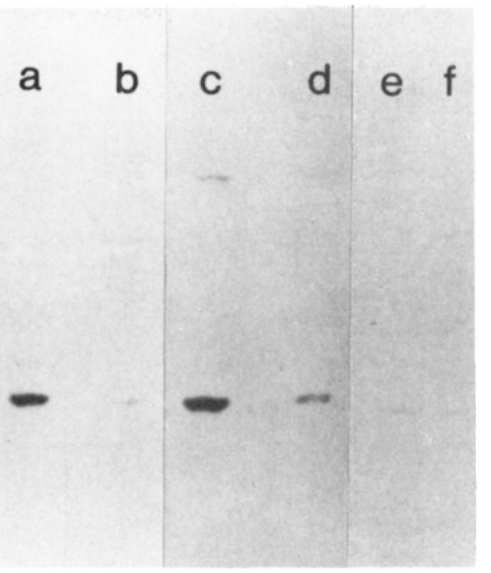

FIGURE 2: Western blot analysis for protein M2 in wild-type, SC2-, and SC2+ cell extracts. Cell extract preparation and the Western blot procedure were carried out as described under Experimental Procedures. (a) SC2- cell extract, 5 μg of protein; (b) SC2- cell extract, 1 μg of protein; (c) SC2+ cell extract, 5 μg of protein; (d) SC2+ cell extract, 1 μg of protein; (e) wild-type cell extract, 40 μg of protein; and (f) wild-type cell extract, 20 μg of protein. The high molecular weight band (about 88 000) in (c) may be due to the presence of M2 dimers.

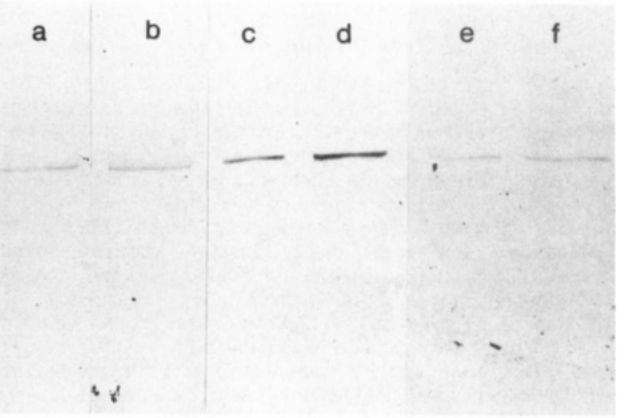

FIGURE 3: Western blot analysis for protein M1 in wild-type, SC2-, and SC2+ cell extracts. Cell extract preparation and the Western blot procedure were carried out as described under Experimental Procedures. (a) SC2- cell extract, 200 μg of protein; (b) SC2- cell extract, 400 μg of protein; (c) SC2+ cell extract, 25 μg of protein; (d) SC2+ cell extract, 50 μg of protein; (e) wild-type cell extract, 400 μg of protein; (f) wild-type cell extract, 800 μg of protein.

comparison of protein M1 levels in SC2 cells grown in the presence and absence of hydroxyurea. The data clearly indicate that there was a dramatic increase in subunit M1 levels in SC2+ cells compared to SC2- cells.

M1 and M2 mRNA Levels in Wild-Type and Hydroxyurea-Resistant Cell Lines. It is now evident that both the M1 and M2 subunits of ribonucleotide reductase are overproduced in the SC2 mutant cell line. Increased concentration of proteins in variant cell lines frequently results from overproduction of their corresponding mRNAs. In order to determine whether M1 and M2 mRNA levels were increased in our mutant cell line, and whether mRNA levels in the mutant cell line changed when the cells were grown in the presence of hydroxyurea compared to the absence of drug, both Northern blot and dot blot analyses were carried out. Results of Northern blot analysis using poly(A+) RNA extracted from wild-type, SC2-,

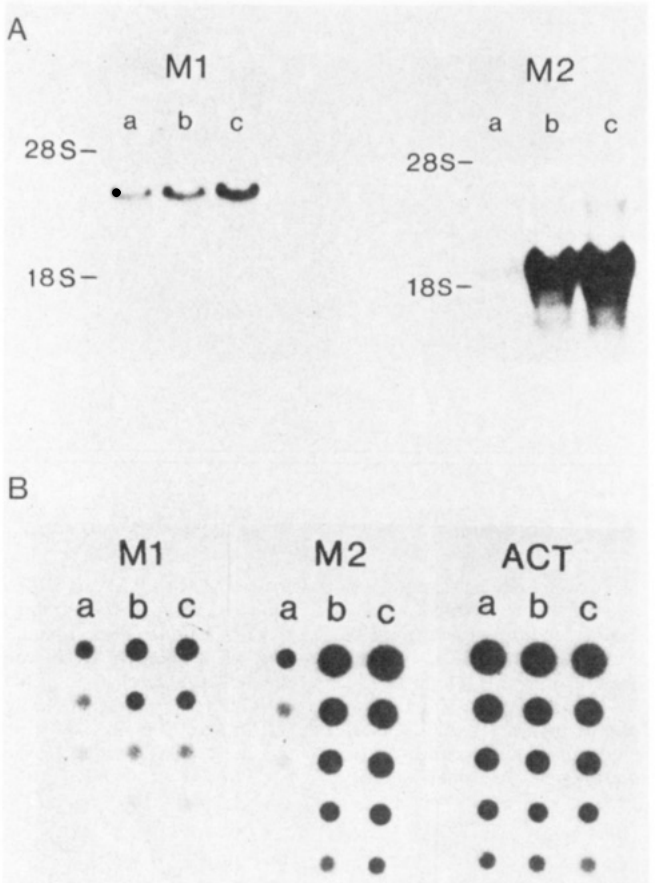

FIGURE 4: (A) Northern blot analysis of M1 and M2 mRNAs in wild-type, SC2–, and SC2+ cell lines. Ten micrograms of poly(A+) mRNA isolated from wild-type (a), SC2– (b), and SC2+ (c) cells was denatured and run on a 1% agarose/formaldehyde gel. After transfer to nitrocellulose, the filters were probed with a ^{32}P-labeled *Nco*I-generated fragment containing part of the cDNA for M1 or with a *Pst*I-generated fragment containing part of the cDNA for M2. The autoradiograms were exposed for 12 h at –70 °C with intensifying screens. The position of the 28S and 18S rRNA is indicated. (B) RNA dot blot analysis of M1, M2, and actin mRNAs in wild-type, SC2–, and SC2+ cell lines. Poly(A+) was extracted, spotted in serial 2-fold dilutions, and hybridized with either an M1 cDNA fragment, an M2 cDNA fragment, or a β-actin cDNA probe, to quantitate the relative amounts of M1 and M2 mRNAs in each preparation. The amount of RNA present in the least dilute spot is 2.0 μg each for wild-type, SC2–, and SC2+ for the M1 blot; 2.0 μg for wild-type and 1.0 μg for SC2– and SC2+ for the M2 blot; and 1.0 μg each for wild-type, SC2–, and SC2+ for the actin blot. The autoradiograms were exposed for 6 h for M1 and 2 h for both M2 and actin at –70 °C with intensifying screens.

and SC2+ cells are shown in Figure 4A. The M1-specific cDNA probe detected a single 3.1-kilobase (kb) mRNA species in both wild-type and mutant cell lines. The M2-specific cDNA probe also detected a single band at approximately 2.1 kb in wild-type and hydroxyurea-resistant cell lines. However, upon longer exposure of the filters, other bands appeared in both cell lines, the most predominant of which had a size of about 1.6 kb (data not shown). The detection of two M2-specific mRNA bands at 2.1 and 1.6 kb for mouse cell mRNA has previously been reported by Thelander and Berg (1986) and Wright et al. (1987). Clearly, the M2 mRNA transcript was dramatically overproduced in the SC2 cell line whereas M1 mRNA levels were only slightly elevated. In order to determine if the level of M1 and M2 mRNAs changed when the SC2 mutant cell line was grown in the presence or absence of hydroxyurea, a more quantitative dot blot analysis was carried out (Figure 4B). The results indicated that the levels of both the M1 and the M2 transcripts

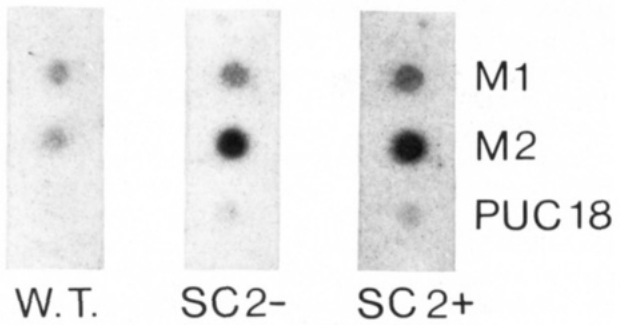

FIGURE 5: Nuclear run-off transcription analysis of M1 and M2 genes in wild-type, SC2–, and SC2+ cells. Cells were harvested and isolated; nuclei were incubated in the presence of [^{32}P]UTP. Following isolation of RNA from nuclei, equivalent amounts of ^{32}P-labeled transcripts were hybridized to filter-immobilized plasmid D 65 (M1 protein), C 10 (M2 protein), and pUC 18 as described under Experimental Procedures. The autoradiograms were exposed for 4 days at –70 °C with intensifying screens.

were essentially unchanged when the mutant cell line was grown under the two different conditions. Densitometric scanning indicated approximately a 2–3-fold increase in M1 message levels and a 35–40-fold elevation in M2 message levels in the SC2 cell line, regardless of whether it was grown in the presence or absence of hydroxyurea, compared to the wild-type cell line.

Transcription of M1 and M2 Genes in Isolated Nuclei. To determine if the increases in the steady-state levels of M1 and M2 mRNA in the SC2 mutant cell line were a result of an increase in the rate of transcription, or in posttranscriptional events, we measured the relative rate of transcription of the two genes by using the nuclear run-off transcription assay in isolated nuclei. In this assay, preinitiated nascent transcripts are elongated in vitro in the presence of [α-^{32}P]UTP and are subsequently hybridized to filter-immobilized cDNAs under conditions of DNA excess. Since new transcripts are not initiated in vitro, this technique provides a relatively accurate measure of transcriptional activity of different genes at the time of cell lysis (McKnight & Palmiter, 1979; Greenberg & Ziff, 1984).

Nuclei isolated from SC2– and SC2+ cells showed a small but reproducible increase in M1 gene transcription when compared to nuclei isolated from wild-type cells (Figure 5). The rate of transcription of the M2 gene is dramatically increased in nuclei isolated from either SC2– or SC2+ cells when compared to wild-type nuclei. The extremely low levels of M1 and M2 gene transcription in wild-type nuclei make it difficult to accurately quantitate the magnitude of the changes in transcription rate between wild-type and mutant cell lines. However, it is clear that M1 and M2 gene transcription is increased in the SC2 cell line. The specificity of the assay is demonstrated by the fact that little or no hybridization occurred when ^{32}P-labeled transcripts were incubated with pUC 18.

M1 and M2 Genes in Genomic DNA from Wild-Type and Hydroxyurea-Resistant Cell Lines. It has now been firmly established that gene amplification commonly underlies overexpression of mRNA in drug-resistant cell lines (Stark & Wahl, 1984; Schimke, 1984). A Southern blot analysis of wild-type, SC2–, and SC2+ genomic DNA, digested with *Eco*RI, *Bam*HI, and *Hin*dIII restriction endonucleases, probed with the M1 cDNA is shown in Figure 6A. Both the wild-type and the mutant cell line showed a similar restriction banding pattern with no obvious changes in band intensity, suggesting that no M1 gene amplification has occurred. An identical Southern blot probed with the M2 cDNA showed

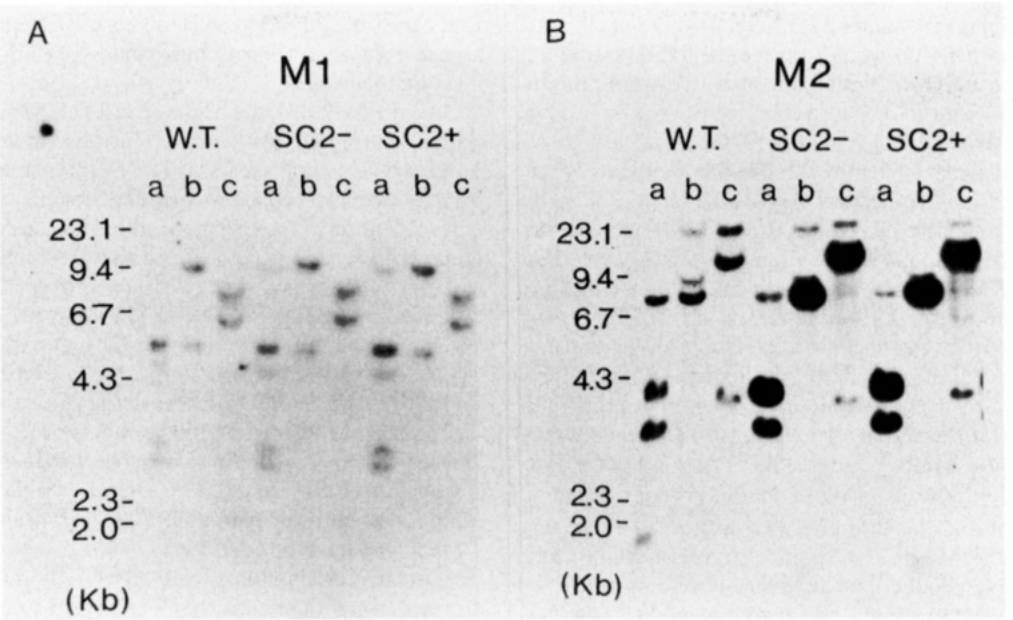

FIGURE 6: Southern blot analysis of M1 and M2 genes in genomic DNA from wild-type, SC2–, and SC2+ cells. High molecular weight DNA (20 μg) was digested with the indicated endonucleases, electrophoresed through a 0.75% agarose gel, and then transferred to nitrocellulose. Blots were hybridized with a ^{32}P-labeled *Nco*I fragment of the M1 cDNA or a ^{32}P-labeled *Pst*I fragment of the M2 cDNA. Restriction endonucleases used for M1 were (a) *Hin*dIII, (b) *Bam*HI, and (c) *Eco*RI and for M2 (a) *Eco*RI, (b) *Bam*HI, and (c) *Hin*dIII. Autoradiograms were exposed for 48 h at −70 °C with intensifying screens.

a complex banding pattern with clear amplification of several but not all bands in the SC2 hydroxyurea-resistant cell line (Figure 6B). There was no evidence of M2 gene rearrangement in the mutant cell line. Results from probings with different segments of the M2 cDNA (5′ end, coding sequence, 3′ end) indicated that there was no selective amplification of a certain region but rather the entire gene had been amplified (data not shown). The presence of unamplified bands in the Southern blot probed with an M2 cDNA suggests that the mouse genome may contain more than one M2-related gene. In fact, recent chromosome mapping results indicate that the mouse genome does indeed contain M2 pseudogenes (Yang-Feng et al., 1987). Densitometric scans of the most intense bands hybridizing with the M2 cDNA in both wild-type and the SC2 mutant cell line gave an estimate of 6-fold gene amplification. As expected, there was no obvious difference in gene copy number for either M1 or M2 genes when DNA from SC2 mutant cells grown in the presence of hydroxyurea was compared to DNA from cells grown in the absence of drug.

Discussion

The current results demonstrate that the SC2 hydroxyurea-resistant cell line contains approximately 50-fold more M2 than its wild-type counterpart. This conclusion was reached on the basis of results from EPR spectroscopy, which measures the M2-specific tyrosyl free radical (Akerblom et al., 1981; Graslund et al., 1982), and Western blot analysis using specific monoclonal antibody against the M2 component of ribonucleotide reductase. In a previous study, M2 activity titration experiments indicated that further overproduction of protein M2 activity occurred when mutant cells were grown in the presence of hydroxyurea. This earlier observation has now been confirmed by EPR spectroscopy (Table I) and Western blot analysis (Figure 2), both of which showed an increase in protein M2 levels when SC2 cells were grown in the presence of hydroxyurea.

Results from Western blot analysis using anti-M1 monoclonal antibody clearly demonstrated that protein M1 was overproduced 2–3-fold in SC2– cells compared to wild-type cells (Figure 3). When SC2 cells were cultured in the presence of hydroxyurea, protein M1 levels were further elevated. This is the first reported example of a cell line that can regulate protein M1 levels in response to hydroxyurea. The 5-fold increase in ribonucleotide reductase activity previously observed in these mutant cells compared to wild-type cells (McClarty et al., 1986a) is most likely a result of the large overproduction of the M2 subunit. Originally, we suggested that the further, 4-fold increase in enzyme activity that occurred when SC2 cells were cultured in the presence of hydroxyurea, as compared to the absence of drug, was a result of a further increase in protein M2 activity levels (McClarty et al., 1986a). However, considering the huge excess of protein M2 already present in SC2 cells, we now suggest that the rapid increase in ribonucleotide reductase activity seen when SC2 cells are cultured in hydroxyurea-containing medium is a result of the drug-induced, reversible, increase in protein M1. The increase in subunit M1 concentration in response to hydroxyurea is surprising since the site of action of the drug is known to be the M2 component (Akerblom et al., 1981; Kjoller-Larsen, 1982). In wild-type cells, protein M2 is normally the limiting subunit, and, therefore, it regulates ribonucleotide reductase activity during the cell cycle (Eriksson et al., 1984; Engstrom et al., 1985). When the SC2 cell line is grown in the absence of hydroxyurea, there is a large overproduction of radical containing protein M2 (Figure 1) that can combine with protein M1 to form an active complex capable of producing the deoxyribonucleotides required for DNA synthesis. However, when the same cells are cultured in the presence of hydroxyurea, they contain a large pool of radical-lacking, and therefore inacive, protein M2 (Figure 1, Table I). In the case of the homologous *Escherichia coli* ribonucleotide reductase subunits B1 and B2, it has been shown that radical-lacking, inactive protein B2 can form a complex with protein B1 and, therefore, compete with radical containing active B2 (Brown et al., 1969; Larsson & Sjoberg, 1986). In anology, the inactive protein M2, present in SC2+ cells, may combine with subunit M1 to form a ribonucleotide reductase complex in-

capable of reducing ribonucleotides. Under these particular circumstances, protein M1 would become the limiting subunit. Presumably, the cells are triggered to produce more protein M1 to combine with the few active M2 components present in hydroxyurea-treated cells. Although the exact nature of this hypothetical signal is unknown, a possible candidate is the deoxyribonucleoside triphosphate pools which are known regulators of ribonucleotide reductase activity (Thelander & Reichard, 1979; Wright, 1983). Such a regulatory role has been postulated for polyamine pools in controlling ornithine decarboxylase expression (McConglogue et al., 1986). Other reported examples of enzymes that are increased in concentration in response to the presence of the appropriate inhibitory agents include dihydrofolate reductase (Domin et al., 1983; Cowan et al., 1986), ornithine decarboxylase (McConglogue et al., 1986), and 3-hydroxy-3-methylglutaryl-CoA reductase (Luskey et al., 1983).

Northern blot and dot blot analyses indicate that the increases in M1 and M2 protein levels observed in SC2- cells are a result of overproduction of the corresponding mRNAs (Figure 4A,B). Interestingly, the increases in M1 and M2 protein levels observed when SC2 cells were cultured in the presence of hydroxyurea were not reflected in similar increases in the levels of M1 and M2 mRNAs (Figure 4B). The discrepancy between the magnitude of change in protein M1 and M2 levels and their corresponding mRNA levels could be attributed to a variety of posttranscriptional processes such as protein turnover rates or translational efficiencies. The ability to increase the efficiency of protein synthesis has been shown to be an important mechanism leading to overproduction of dihydrofolate reductase (Bastow et al., 1984; Cowan et al., 1986) and ornithine decarboxylase (McConglogue et al., 1986). Immunoprecipitation experiments are currently being carried out in order to measure the protein half-life of M1 and M2, and as well to determine if translational efficiency of M1 and M2 mRNA is enhanced when SC2 cells are grown in the presence of hydroxyurea.

Johnston et al. (1986) have recently reported that the level of dihydrofolate reductase mRNA increases in methotrexate-resistant cells in response to hydroxyurea treatment. Unlike the SC2 cell line, hydyroxyurea blocks these methotrexate-resistant cells at the G1–S phase border of the cell cycle, and they suggest that this block could result in preferential accumulation of proteins expressed from cell cycle regulated genes, which would, thereby, be activated for an artificially prolonged period.

Results from nuclear run-off experiments indicate that there is a small but reproducible increase in the rate of transcription of the M1 gene and a substantial increase in M2 gene transcription in the hydroxyurea-resistant cell line (Figure 5). Although difficult to quantitate, the observed increases in transcription rates are probably sufficient to account for the elevated M1 and M2 mRNA levels in the SC2- mutant cell line. However, we cannot exclude the possibility that there may also be increases in mRNA half-life. The transcription rates of the two genes do not change appreciably when SC2 cells are grown in the presence of hydroxyurea, a result consistent with the observation that the level of mRNA for the two subunits does not change significantly under the two growth conditions.

Southern gel analysis of restriction endonuclease digested genomic DNA demonstrated that SC2 cells have a 6-fold amplification in the number of copies of the gene for protein M2 but no apparent increase in the number of copies of the gene for protein M1 (Figure 6A,B). Although the number of copies of the M2 gene is increased in the SC2 cell line, the gene does not appear to have undergone any gross structural reorganization.

In the SC2 cell line, the M2 mRNA levels are elevated by much more than the observed increase in gene copy number (35–40-fold compared to 6-fold). Interestingly, M1 mRNA levels are enhanced approximately 2-fold in the absence of gene amplification. Furthermore, the M1 and M2 protein levels are enhanced 2–3-fold and about 50-fold, respectively, in mutant cells grown in the absence of hydroxyurea. A similar nonlinear increase in M1 and M2 mRNA levels compared to gene copy number was reported for hydroxyurea-resistant cell lines by Thelander and Berg (1986) and by Wright et al. (1987). The ability to increase the size of a mRNA pool in the absence of gene amplification has been shown to occur in other cell lines including those overproducing dihydrofolate reductase (Bastow et al., 1984), (hydroxymethyl)glutaryl-CoA reductase (Luskey et al., 1983), and ornithine decarboxylase (McConglogue et al., 1986).

The overproduction of enzyme activity through the process of gene amplification is a well-established mechanism of drug resistance in cultured cells (Stark & Wahl, 1984; Schimke, 1984). The ability of drug-resistant cell lines to respond to drug challenge through qualitative changes in gene expression is clearly a mechanism that can play an important role in drug resistance. The ability of the SC2 cell line to elevate both the M1 and M2 subunits of ribonucleotide reductase in response to hydroxyurea provides us with an excellent system, not only to continue our studies on drug resistance, but also to investigate how the expression of the two genes is coordinated.

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