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Quantification of Ribonucleotide Reductase Expression in Wild-Type and Hydroxyurea-Resistant Cell Lines Employing *in Situ* Reverse Transcriptase Polymerase Chain Reaction and a Computerized Image Analysis System[1]

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Ribonucleotide reductase (RR) is the enzyme responsible for converting nucleoside diphosphates to deoxynucleoside diphosphates, ensuring a balanced supply of deoxyribonucleotides for DNA synthesis. Expression of RR is tightly regulated, but it is affected by exogenous agents, such as hydroxyurea (HU), which inactivates the tyrosyl free radical on the small subunit of RR, R2. We have previously employed *in situ* reverse transcriptase (RT)-PCR to estimate expression of R2 in wild-type and HU-resistant human colon carcinoma cell lines and to correlate altered expression of R2 with changes in cell size and morphology. The current studies were undertaken to render this methodology more quantitative. Both wild-type and resistant cells were grown on partitioned glass slides and analyzed with *in situ* RT-PCR. Because both wild-type and resistant cells were analyzed under a single cover slip, protease digestion, reverse transcription, PCR, and color development were all performed under identical conditions. Images were analyzed with NIH Image 1.59 software. There was a highly significant correlation between expression of R2 and cell size for both sensitive and resistant cells ($P = 0.0001$, for both). When cell size was compared either with expression of R2 or cell shape, however, these correlated only in wild-type cells ($P = 0.001$ and 0.0001, respectively). These data demonstrate that normal cell growth in the unperturbed wild-type cell line was closely linked to expression of R2, whereas in the resistant variants which overexpress R2, these correlations were absent, suggesting that HU resistance is related to loss of linkage between R2 expression and cell growth and confirming previous data relating overexpression of R2 with multiple other changes in the cell growth repertoire. Thus, we have demonstrated for the first time a quantitative application of *in situ* RT-PCR.

Key Words: **drug resistance; ribonucleotide reductase; hydroxyurea; *in situ* RT-PCR; gene expression.**

Currently, there is great interest in methods that allow analysis and quantification of gene expression in single cells, such as a recent approach which combined fluorescent *in situ* hybridization (FISH) analysis with digital imaging microscopy (1). We now describe a method that combines *in situ* reverse transcriptase (RT)[3]-PCR hybridization techniques and a computerized image analysis system to study drug effects in cultured human colon carcinoma cells *in vitro.* This technique allows both quantification of single gene expression in individual cells and simultaneous analysis of the cell morphology and volume, which can give important information about the cytokinetic state of the cell.

In situ RT-PCR has been employed extensively (2–6), but not to our knowledge in a quantitative fashion. Thus, this is the first system to attempt to quantify the findings from such an assay. A previous report (7) focused on the development of a micropreparative technique for cultured cells, with the goal of ensuring identical fixation, protease digestion, PCR, and hybridization under a single coverslip for the various cell populations to be studied. While this system guaranteed uniformity of RT-PCR assay conditions, differ-

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[3] Abbreviations used: RT, reverse transcriptase; HU, hydroxyurea; RR, ribonucleotide reductase; FISH, fluorescent *in situ* hybridization.

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ences in gene expression and morphology remained qualitative. In the current studies, we have now added a computer-driven image analysis system to quantify the observed changes in cell size and morphology with differences in gene expression, which improves the utility of such a method, specifically for correlating level of gene expression with cell morphology and size.

The problem that was studied was resistance to the drug hydroxyurea (HU) in human colon carcinoma cell lines, but this technique has applicability to a wide range of other questions. The cellular target for HU is ribonucleotide reductase (RR), the enzyme responsible for a balanced supply of nucleotide precursors for DNA synthesis (8). In mammalian cells, this enzyme exists as an $\alpha_2\beta_2$ heterodimer, with a 170-kDa subunit, which binds nucleotide substrates and allosteric effectors (R1), and an 88-kDa subunit, which contains a nonheme iron tyrosyl free radical, which facilitates the reduction reaction (R2) (9). Expression of RR is closely linked to cellular events leading to DNA synthesis (10); however, regulation of the expression of each subunit differs. R1 is expressed constitutively and in excess in cycling cells, but not in cells in G0, independent of cell cycle phase and of DNA replication (11, 12). In contrast, regulation of R2 is closely tied to the cell cycle and DNA synthesis.

Regulation of RR is complex and may be specific to particular cell types and tissues (10, 13, 14). In a heterogeneous population, levels of R2 mRNA vary among different cells. Thus, in a population of unsynchronized cells *in vitro*, estimation of expression of R2 using conventional analytic methods such as Northern analysis or quantitative RT-PCR represents an average of a kinetically heterogenous population. Enrichment or depletion of specific cell subpopulations may result in under- or overrepresentation of levels of R2 mRNA. Furthermore, comparisons of different cell populations, either different cell lines, variants of the same cell line, or a single cell line treated with exogenous agents, may be compromised by these considerations. Therefore, the methodology we have described has potential utility in such a complex, cell-type-specific system.

METHODS

Cell lines. SW480 human colon carcinoma cells and $SW480_{R1000}$, a hydroxyurea-resistant variant, were grown in RPMI 1640 with 10% fetal bovine serum and 1% penicillin–streptomycin–neomycin (GIBCO, Grand Island, NY) with or without continuous exposure to hydroxyurea (1000 μM), kindly provided by Terry Dugan (Bristol-Myers-Squibb, Princeton, NJ).

Primers/probe. Primers were synthesized at the Albert Einstein Oligonucleotide Synthesis Facility. PCR primers were selected and optimized using PC/Gene software (Intelligenetics Corp, Mountain View, CA). The upstream primer, P49, was TGAGAGAAAACCCCCGCC-

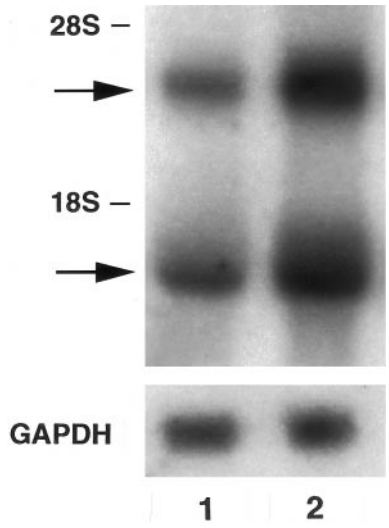


FIG. 1. Northern blot analysis of R2 RNA. (Top) Total RNA from wild-type cells (lane 1) and the resistant variant (lanes 2) was analyzed. Twenty micrograms of RNA was loaded in each lane. Two species of mRNA are identified as described (16) (arrows). (Bottom) GAPDH was employed as an internal control. Figure is representative of three replicates.

GCTTT and the downstream primer, M18, was GTGAGGCCAGGCATCAGTCCTCGT, corresponding to locations 412–434 and 938–961, respectively, on R2 (15). The R2 probe encompassed a 550-bp region from 412 to 961, which optimally combined properties of specificity and ability to diffuse into the cell nucleus following cell fixation.

Northern analysis. Total RNA was extracted from SW480 cells and variants with Trizol (GIBCO, Grand Island, NY). Twenty micrograms of total RNA was electrophoresed on a 0.8% agarose gel and transferred to a Nytran membrane overnight. The R2 probe was labeled as above, and hybridization of the blot was performed as previously described (16). The blots were rehybridized with a [2-^{32}P]dCTP-labeled probe for GAPDH. Bands were compared by densitometry (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA).

In situ RT-PCR. SW480 cells and the variants were grown on glass slides exactly as described (7). Fixation of the slides, protease digestion, DNase digestion, and reverse transcription were performed as described. PCR was then performed using the P49 and M18 primers. Detailed studies of the kinetics of amplification of R2 target cDNA were employed to determine the plateau phase. Nineteen cycles were employed, which was on the linear portion of the curve and at least 10 cycles below the plateau. The PCR products were detected using the digoxigenin-labeled (Boehringer-Mannheim, Indianapolis, IN) R2 probe. Slides were developed as previously described. For all slides, negative controls (with DNase treatment and without reverse transcrip-

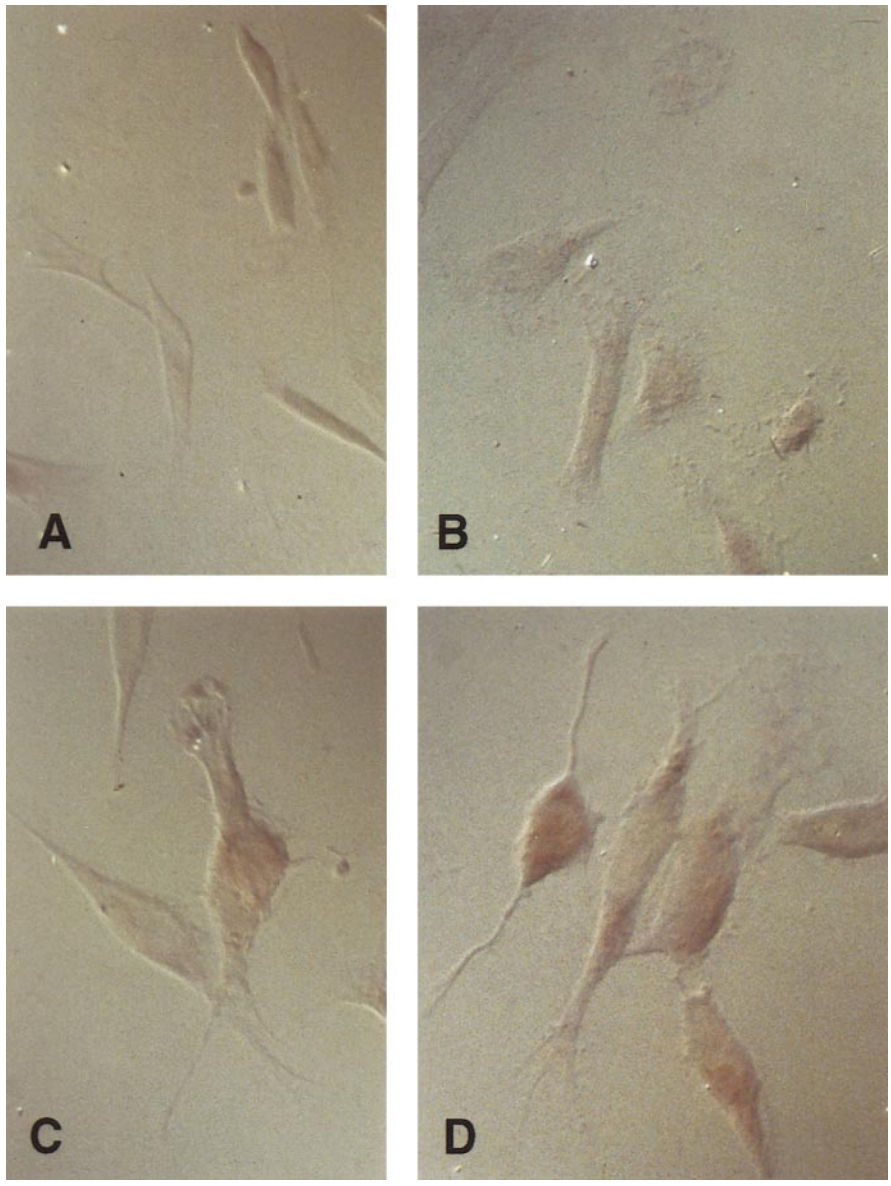


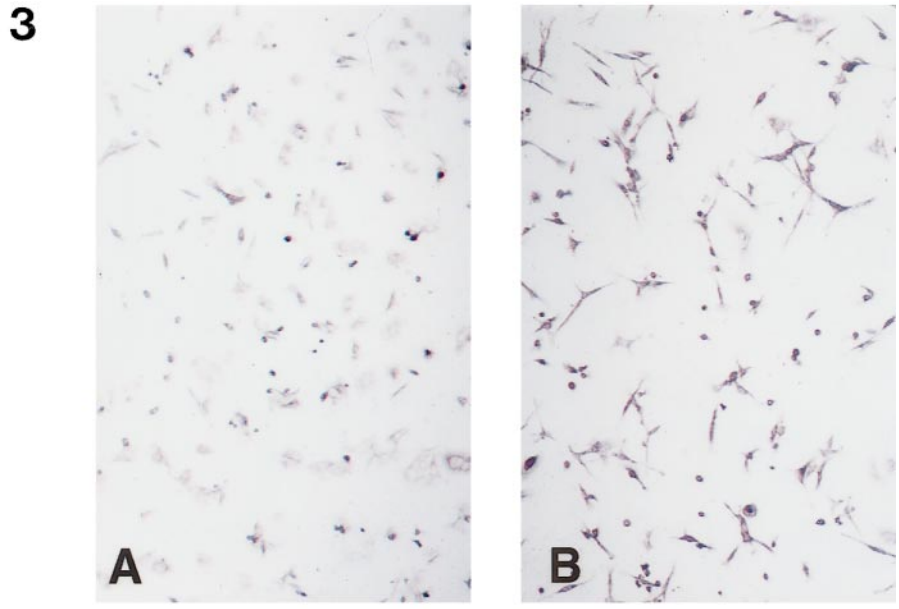
FIG. 2. Imaging of wild-type and resistant cells. Detailed structural characteristics of representative wild-type (A, B) or HU-resistant (C, D) cells were photographed using Nomarski optics (630× for all frames). There was considerable overlap in morphology between the two cell populations; however, the wild-type cells were generally flatter, smaller, and blander than the resistant cells, which often exhibited both broad, flat, and extended processes or thinner processes.

tase) and positive controls (no DNase treatment, with reverse transcriptase) were included exactly as described. Additional controls were employed periodically as appropriate, including samples undergoing PCR in the absence of *taq* polymerase, samples developed in the absence of probe, and samples analyzed without colorimetric development.

Image analysis. Images were acquired and analyzed at the Albert Einstein Analytical Imaging Facility. Images were collected with a Photometrics PXL cooled CCD camera on an Olympus IX70 microscope with a HG-100R power supply (Chiu Technical Corporation) and an infinity-corrected 40× NA 0.75 objective. Images were analyzed with NIH Image 1.59 software (Bethesda, MD) for intensity, roundness, and area. Area was determined by pixel counting. Roundness was determined as the ratio, p^2/A, where p is perimeter and A is area. Intensity was defined as

$$\frac{\Sigma S/X}{A},$$

where $\Sigma S/X$ is the gray-scale reading per pixel summed over the area of the cell body and A is the



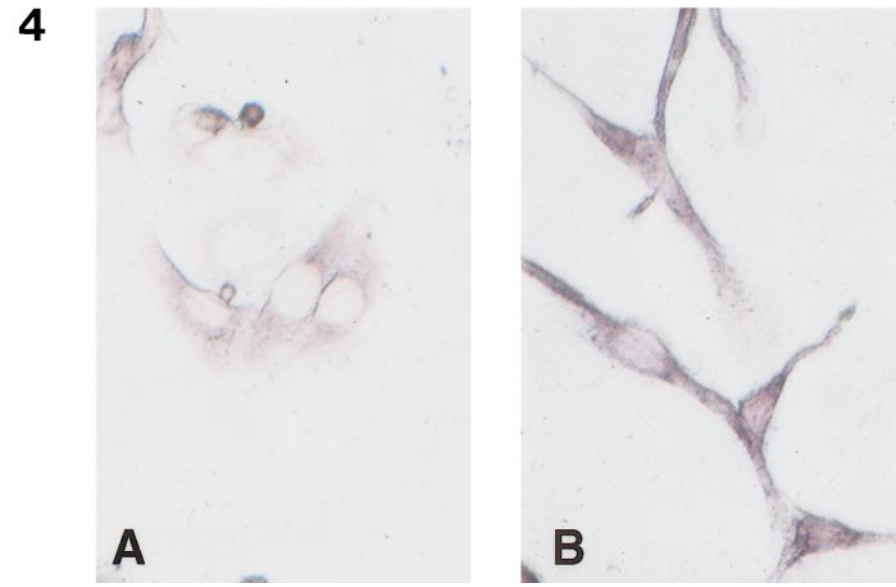


FIG. 3. Wild-type (A) and resistant (B) cells following *in situ* RT-PCR (100×). Both populations were morphologically distinct. The resistant cells almost uniformly overexpressed R2 relative to the wild-type cells. The small, very dark, round bodies are dead cells.
FIG. 4. Wild-type (A) and resistant (B) cells following *in situ* RT-PCR (1000×). Cells were examined under oil immersion. The HU-resistant cells stained much more intensely than the wild-type cells. Staining occurred primarily in the cytoplasm with some diffusion into the nucleus, confirming the specificity of the method.

area of the cell body. The intensity was normalized to extracellular background by choosing two areas (three pixels each) adjacent to the cell body, averaging their intensity, and subtracting that from the intensity of the cell. Scoring for intensity was on a scale of 0 to 255, where the lower number represented black and the higher number, white. For structural imaging studies, images were acquired with a Zeiss Axioplan microscope (Thornwood, NY) at 630× using Nomarski optics.

Statistical analysis. Each *in situ* RT-PCR assay was performed three times using different cell populations with each experiment employing two or three slides. This resulted in analysis of eight slides. Because the results were reproducible among separate experiments, the eight slides were considered as a single group for the analysis. Mean differences in intensities, areas and roundness between sensitive and HU-resistant cells were tested by Student's *t* test. In order to analyze the correlations between

TABLE 1

Expression of R2, Cell Size, and Cell Shape in Wild-Type and HU Resistant SW480 Cells

Parameter[a]	Wild type (n = 177)	HU-resistant (n = 126)	P value
Area	1322.11 ± 806.70	1548.63 ± 705.79	<0.05
Roundness	0.19 ± 0.031	0.15 ± 0.026	<0.0001
Intensity	218.43 ± 21.05	180.09 ± 29.85	<0.0001

[a] All values are means ± SD. Intensity is ranked on scale of 0 (black) to 255 (white). Area is expressed in pixels; all other values are in arbitrary units.

intensity and area, intensity and roundness, and area and roundness, Pearson correlation coefficients were calculated and tested. All analyses were performed on SAS (17).

RESULTS

Analysis of R2 DNA and mRNA in wild-type and variant cells. $SW480_{R1000}$ cells were selected for low-level resistance to HU and had been stably grown in HU-containing medium for about 1 year. As shown in Fig. 1, levels of R2 mRNA were 3.5 ± 1.5 (mean ± SEM)-fold higher in the resistant variants relative to the wild-type cells.

In situ RT-PCR and image analysis of wild-type and resistant cells. Wild-type and variant cells grown on glass slides were photographed using Nomarksi optics. Structural characteristics of the cells are shown in Fig. 2. The wild-type cells were smaller, blander, and rounder, whereas the HU-resistant variants were generally larger with elongated processes that increased the area of the cells and decreased their overall roundness. There was substantial heterogeneity among the two cell populations with the wild-type cells being, in general, more uniform.

Figure 3 shows wild-type and resistant SW480 cells following *in situ* RT-PCR and treatment with 5-bromo-4-chloro-3-indoyl phosphate. Expression of R2 was uniform in both populations, with the resistant cells demonstrating more intense staining. Under higher magnification (Fig. 4) the colorimetric reaction occurred mainly in the cytoplasm, as expected, with a small amount of stain diffusing into the nucleus. The HU-resistant variants had higher levels of R2 expression.

In order to quantify these results, images of both sensitive and resistant cells were acquired electronically and then analyzed for intensity of staining, area, and roundness using parameters defined above (Table 1). The intensity of staining for the resistant cells was 21% higher than that for sensitive cells (180 vs 218; $P < 0.0001$), confirming the visual impression (Fig. 4) that the variants overexpressed R2. When cells were analyzed for morphologic features, the resistant cells were 17% larger (1549 vs 1322 pixels; $P < 0.05$) and 27% less round (0.15 vs 0.19, arbitrary units; $P < 0.0001$) than the sensitive cells, also consistent with the morphologic appearance.

As shown in Table 2, there was a highly significant correlation between expression of R2 and cell size for both sensitive and resistant cells ($P = 0.0001$, for each), demonstrating that expression of R2 increased with cell growth. In contrast, when expression of R2 was compared with cell shape, the level of gene expression correlated with the absence of spindle-shape (or presence of cell roundness) only in the wild-type cells ($P = 0.001$). Cell size and shape were also compared; there was a linear correlation only for the wild-type cells ($P = 0.0001$), demonstrating that as these cells grew in size, they became less spindle-shaped, whereas the resistant cells consistently maintained a less rounded morphology.

DISCUSSION

Northern analysis of R2 expression in SW480 cells and their hydroxyurea-resistant variants correlated well with both the visual impression of the results of *in situ* RT-PCR and a formal image analysis of these characteristics. This supports similar previous observations in mouse L cells grown in HU (18) and in human KB oropharyngeal cells transfected with R2 (19).

Among sensitive cells, expression of R2 varied as a function of cell growth. This likely reflected, in part, increased expression of the enzyme as cells approached the G1/S border; however, the correlation coefficient was only −0.40, possibly reflecting nonlinear, and specifically cell cycle-related, changes in expression of the enzyme. Among resistant cells, expression of R2 also correlated with cell size, a surprising observation given that these cells constitutively overexpress R2. This suggests that despite higher amounts of enzyme required by resistant cells in order to function in HU-containing medium, levels of enzyme are still at least partially regulated in a cell cycle-dependent fashion.

The correlation between shape and size and between shape and expression of R2 in the sensitive cells was

TABLE 2

Correlations between Expression of R2, Cell Size, and Cell Shape

	Wild type		HU resistant	
Correlation of	r	P	r	P
Intensity and area	−0.60416	0.0001	−0.47465	0.0001
Intensity and roundness	−0.24614	0.0010	−0.05448	0.5446
Area and roundness	0.36876	0.0001	0.036532	0.6847

not surprising. In contrast, the absence of a correlation between shape and either size or expression of R2 in the resistant cells was of interest. While the variants grew more slowly than the wild-type cells (data not shown), it was apparent that growth was more disordered, resulting in a more aberrant morphology compared with the wild-type cells. Clearly, this was not solely a function of altered cytokinesis. In studies of KB cells transfected with R2 (19), the transfectants also grew more slowly, but with a normal morphology.

The relationship between growth, as estimated by cell size, and expression of R2 is plausible, because of the intimate relationship between expression of R2 and transition through late G1 and early S. Our studies take this observation further and suggest that overexpression of R2 may lead to recruitment of other cell growth regulatory genes, which determine not only the rate of cell growth, but also cell morphology and size as well. This is plausible given the seamless relationship between regulation of cell growth and regulation of cell shape. Furthermore, this confirms emerging data that overexpression of R2 is associated with increased expression of other E2F transition state enzymes (20, 21). The absence of such an effect in KB cells (19) may either be cell-type specific or related to the level of expression of R2.

The current studies extend these observations in a quantitative fashion, rather than employing a purely descriptive approach. While labor intensive and requiring optimization of fixation, protease digestion, and PCR technique for specific cell lines, this approach potentially lends itself to a more quantitative description of the relationship of cell growth to expression of various growth-related genes. Furthermore, while this approach may be useful in dealing with the heterogeneous but relatively predictable variations in cell growth in culture, the major utility of this method is potentially in the analysis of tumor samples, which are often complex mixtures of cancer cells, normal tissue, and multiple stromal elements.

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