Radiation-Induced Changes in Gene Expression Involve Recruitment of Existing Messenger RNAs to and away from Polysomes

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Abstract

Although ionizing radiation has been shown to influence gene transcription, little is known about the effects of radiation on gene translational efficiency. To obtain a genome-wide perspective of the effects of radiation on gene translation, microarray analysis was done on polysome-bound RNA isolated from irradiated human brain tumor cells; to allow for a comparison with the effects of radiation on transcription, microarray analysis was also done using total RNA. The number of genes whose translational activity was modified by radiation was ~10-fold greater than those whose transcription was affected. The radiation-induced change in a gene's translational activity was shown to involve the recruitment of existing mRNAs to and away from polysomes. Moreover, the change in a gene's translational activity after irradiation correlated with changes in the level of its corresponding protein. These data suggest that radiation modifies gene expression primarily at the level of translation. In contrast to transcriptional changes, there was considerable overlap in the genes affected at the translational level among brain tumor cell lines and normal astrocytes. Thus, the radiation-induced translational control of a subset of mRNAs seems to be a fundamental component of cellular radioresponse. (Cancer Res 2006; 66(2): 1052-61)

Introduction

Cellular radioresponse is a complex biological process regulated by such fundamental processes as cell cycle arrest, DNA repair, apoptosis, senescence, and likely others that have yet to be identified. The best-defined mechanisms for the initiation of these processes after irradiation involve constitutively expressed proteins. However, because radiation induces the transcription of a wide variety of genes, the modulation of gene expression has also been assumed to contribute to cellular radioresponse. That is, analogous to prokaryotic cells, radiation-induced gene expression in eukaryotic cells may comprise an adaptive or protective response against radiation-induced injury or death. Thus, defining the inducible and putatively protective genes may not only provide insight into the fundamental mechanisms regulating radioresponse but may also lead to the identification of targets for modifying radiosensitivity.

Radiation-induced gene expression profiles generated from microarray analysis of total cellular RNA have been reported for a number of normal cells and tissue as well as for a variety of tumor cells grown *in vitro* and *in vivo* (1–6). Comparison of these profiles reveals few commonly affected genes among the cell types evaluated and even among tumor cell lines originating from the same histology. Moreover, whereas these microarray analyses accurately reflect changes in transcription, there has been an overall lack of data correlating radiation-induced changes in mRNAs with their corresponding proteins. Although there are exceptions involving individual genes (1), the vast majority of mRNA changes detected after irradiation have not been extended to the protein level. Given that protein is the operational end product of gene expression, the lack of correlation between mRNA and protein changes combined with the heterogeneity among cell lines has made it difficult to assign a functional consequence to radiation-induced gene expression. Along these lines, Birrell et al. showed that after irradiation of yeast, there was little or no relationship between radiation-induced transcriptional changes and survival (7).

These DNA microarray studies were based on the assumption that radiation-induced gene expression occurs primarily through changes in transcription. However, gene expression is dependent not only on transcriptional activity but on a variety of post-transcriptional events, including the initiation of mRNA translation. In contrast to prokaryotic cells, eukaryotic transcription and translation are not directly coupled with each event confined to separate cell compartments (nucleus versus cytoplasm). Indeed, after stress, in eukaryotic cells, there is often a poor correlation between mRNA levels and protein production (8, 9). Accounting for the disengagement of the transcriptome and proteome is translational control (10–12), which has been shown to play a significant role in regulating gene expression during such fundamental processes as T-cell activation (13), growth factor signaling (14), and tumorigenesis (15). Given that translational control can provide a critical regulatory point for gene expression, we hypothesized that radiation modulates the translation of a subset of mRNAs. The initiation of translation involves recruitment of mRNAs to polysomes (polyribosomes): the association of an mRNA with polysomes can then be used as an indicator of translational activity (11). Therefore, to obtain a genome-wide perspective of the effects of radiation on translation control, we have done microarray analysis on polysomal-bound RNA; to allow for a comparison with the effects of radiation on transcription, microarray analysis was also done using total RNA. Because radiation remains a primary treatment modality for brain tumors, these gene expression analyses were done on human brain tumor cell lines and normal astrocytes. The data presented indicate that gene translation is considerably more susceptible to radiation-induced modifications than is transcription,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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and that the changes in translational activity involve the recruitment of existing mRNAs to and away from polysomes. Moreover, there was a correlation between the genes whose expression was modified at the translational level and the expression of their corresponding proteins. These results suggest that radiation primarily affects gene expression at the level of translation.

Materials and Methods

Tissue culture and irradiation. The human brain tumor cell lines U87, SF126, and SF539 used in this study were kindly provided by Dr. Dennis Deen (Brain Tumor Research Center, University of California, San Francisco, CA). U87 cells were grown in DMEM containing glutamate (5 mmol/L) and 10% fetal bovine serum (FBS); SF126 and SF539 were grown in RPMI 1640 (Life Technologies, Rockville, MD) containing glutamate (5 mmol/L) and 5% FBS. Primary normal human astrocytes were purchased from Cambrex BioScience (Walkersville, MD) and grown in Astrocyte Growth Media (Cambrex BioScience, Walkersville, MD) for no more than 3 weeks with a change of media every 2 days until cells were 80% confluent. All cultures were maintained at 37°C in an atmosphere of 5% CO_2 and 95% room air. Monolayer cultures were irradiated using a Pantak (Solon, OH) X-ray source at a dose rate of 1.55 Gy/min.

RNA sample preparation, probe labeling, and microarray procedure. Cells were scraped from tissue culture flasks, and total RNA was extracted from each sample using TRIZOL reagent (Invitrogen, Carlsbad, CA) passed through an RNeasy spin column (Qiagen, Valencia, CA) and then amplified using RiboAmp RNA kits (Arcturus, Mountain View, CA) according to manufacturer's protocol. Amplified RNA (1.5-3.0 μg) was labeled with Cy3-dUTP (experimental RNA) or Cy5-dUTP (Stratagene Universal Reference, La Jolla, CA) using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Each cDNA microarray chip contained 7680 human cDNA clones (National Cancer Institute ROSP 8K Human Array), and methods for microarray hybridization and washing were described previously (16). Hybridized arrays were scanned with 10-μm resolution on a GenePix 4000A scanner (Axon Instruments, Inc., Foster City, CA) at wavelengths 635 and 532 nm for Cy5- and Cy3-labeled probes, respectively. The resulting TIFF images were analyzed by GenePix Pro 4.0 software (Axon Instruments). The ratios of the sample intensity to the reference [green (Cy3)/red (Cy5)] intensity for all targets were determined, and ratio normalization was done to normalize the center of ratio distribution to 1.0. Tumor cell lines had a biological replicate, and each replicate was run on duplicate slides. The biological replicates (i.e., independent experiments) had correlation coefficients of ≥0.78 for total RNA and ≥0.83 for polysome RNA, indicative of high reproducibility (17). Microarray analysis was done on primary normal human astrocytes using duplicate slides.

Polysome preparation and analysis. Polysomes were isolated using sucrose-gradient fractionation basically as described by Galban et al. (18). Cells were grown to ~80% confluence in 150-mm^2 tissue culture dishes and incubated in 100 μg of cycloheximide/mL for 15 minutes before collection. Cytoplasmic RNA was obtained by lysing cells in 1 mL of polysome buffer [10 mmol/L Tris-HCl (pH 8), 140 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.5% NP40, 10 mmol/L DTT, 100 μg/mL cycloheximide, 500 μg/mL heparin, 1 mmol/L phenylmethanesulfonyl fluoride, and 500 units/mL RNasin (Promega, Madison, WI)]. After 10 minutes on ice, lysates were centrifuged (10,000 × *g* for 10 minutes), and the resulting cytosolic supernatant was layered onto a 10% to 50% sucrose gradient. Gradients were then centrifuged at 35,000 × *g* for 3 hours at 4°C and 1-mL fractions collected using an ISCO Density Gradient Fractionation System (ISCO, Lincoln, NE) with continuous monitoring based on A_{254}. The RNA in each fraction was extracted using TRIZOL and used for Northern analysis, or fractions 5 to 11 (corresponding to polysome-bound RNA) were pooled and subjected to microarray analysis as described above.

Microarray data analysis. Raw intensity profiles were analyzed using the mAdb tools (National Center for Biotechnology Information, NIH) to perform microarray normalization and statistical analysis. All nonflagged raw fluorescent intensities were subjected to a spot quality filter with signal: background ratios of >2, a minimum background corrected signal of 250 counts, and 60% of pixels in the spots with an intensity greater than a SD plus background. Scatter plots were created and correlation coefficients calculated using the mAdb software.[3] As a supervised approach for analyzing the function of genes whose levels were modified by ≥2-fold, GOstat was done.[4] This program automatically obtains the Gene Ontology annotations from a database and generates a statistical analysis of the functional annotations that are overrepresented in the inputted list of genes (19).

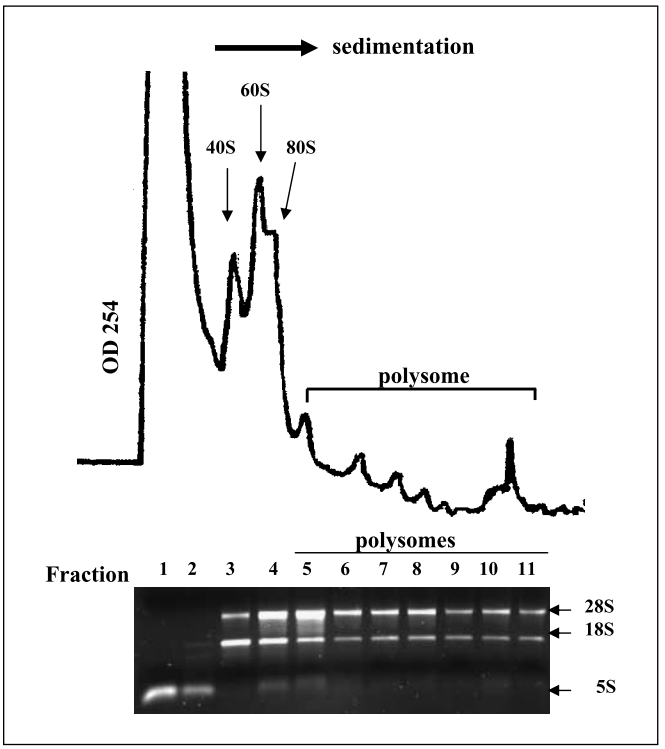

Figure 1. Sucrose-gradient fractionation of RNA from U87 cells. *Top,* representative profile of U87 cytoplasmic cell extracts subjected to sucrose-gradient centrifugation followed by fractionation with continuous monitoring of the absorbance at 254 nm. Sedimentation of the 40 S, 60 S, and 80 S ribosome peaks (*arrows*). *Bottom,* RNA distribution within the gradient. RNA was extracted from each of the 11 fractions and subjected to agarose gel electrophoresis. Fraction numbers are indicated above the corresponding lanes, and fractions 5 to 11, which contain polysomes, were pooled and used for microarray analysis.

Ingenuity Pathway Analysis (IPA)[5] was used as an additional method for evaluating functional significance of the radiation-induced gene profiles. IPA uses a curated database to construct functional regulatory networks from a list of individual genes. A data set containing gene identifiers and their corresponding expression values was uploaded as an Excel spreadsheet using the template provided in the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. A log 2–transformed cutoff of 1.0 was set to identify genes whose expression was significantly differentially regulated. These genes (referred to as focus genes) were then used as the starting point for generating biological networks. To build networks, the program uses its knowledge base to identify interactions between focus genes as well as other genes. IPA then determines a statistical score for each network

[3] http://nciarray.nci.nih.gov/.
[4] http://gostat.wehi.edu.au.
[5] https://analysis.ingenuity.com.

according to the fit of the network to the set of focus genes. The score is the negative log of *P* and denotes the likelihood of the focus genes in the network being found together due to chance. Biological functions were assigned to each gene network by using the findings that have been extracted from the scientific literature and stored in the Ingenuity Pathways Knowledge Base. The biological functions assigned to each network are ranked according to the significance of that biological function to the network. A Fisher's exact test is used to calculate *P*, determining the probability that the biological function assigned to that network is explained by chance alone.

Northern analysis. Northern blot analysis was done using RNA isolated from whole cells or from the individual fractions generated from sucrose gradients, which used equal volumes from each fraction. Denatured RNA was separated using 1.5% agarose gels containing GelStar for RNA visualization (Nucleic Acid Gel Stain, Cambrex BioScience, Rockland, ME) and transferred to positively charged Zeta-Probe blotting Membranes (Bio-Rad, Hercules, CA). The mRNAs encoding GADPH, enolase 1 (ENO1), superoxide dismutase (SOD2), glutathione synthetase (GSS), and β-actin were detected with specific oligonucleotides (Sigma-Genosys, Woodlands, TX): TTATTGATGGTACATGACAAGGTGCGGCTC, GGAGATGACACGGCTCACATGAGTGTAG, TGCTATGATTGATATGACCACCACCATTGA, CAATTCTGTAGACTGTACTGACGAGGCATG, and GTCAAGAAAGGGTGTAACGCAACTAAGTCA, respectively, that were end labeled with digoxin (DIG Oligonucleotide 3′-End Labeling kit, Roche Applied Science, Indianapolis, IN). Oligonucleotide and anti-DIG-alkaline phosphatase hybridization were done by using DIG OMNI System for Oligonucleotide Probes kit (Roche Applied Science) according to manufacturer's instructions. Visualization of the chemiluminescent signal was done with a Typhoon scanner (Molecular Dynamics, Sunnyvale, CA). The distribution of mRNA among the sucrose-gradient fractions was determined by densitometry.

Immunoblots. Cells were rinsed with ice-cold PBS and scrapped into lysis buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, supplemented with Roche protease inhibitor cocktail and Sigma phosphatase inhibitors I and II (St. Louis, MO). After centrifugation at 14,000 rpm to separate insoluble material, proteins were subjected to SDS-PAGE using NuPage 4% to 12% gels and NuPage MES or MOPS buffers according to the manufacturer's instructions (Invitrogen, San Diego, CA). After electrophoresis, gels were electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The nonspecific sites on the membranes were blocked at room temperature for 30 minutes with 5% nonfat milk in TBS supplemented with 0.2% Tween 20 (TBS-T). Membranes were probed in blocking solution overnight at 4°C with the following antibodies: ADAM9, carbonic anhydrase 1 (Abcam, Cambridge, MA); FMRP, aquaporin 3, β-actin (Chemicon, Temecula, CA); cyclin C, enolase 2, mitogen-activated protein kinase 6 (MAPK6), Ataxin 2 (BD Biosciences, San Jose, CA); Elk-3 (Net), TNFRSF6 (Fas), glyceraldehyde 3 phosphate dehydrogenase (GAPDH), p53R2, eIF4G, glutathione synthetase (Santa Cruz Biotechnology, Santa Cruz, CA); SOD2 (Upstate, Waltham, MA); and enolase 1 (GenWay, San Diego, CA). Membranes were then washed thrice in TBS-T and incubated with the appropriate horseradish peroxidase–conjugated secondary antibody at a 1:2,000 dilution in blocking solution for 1 hour at room temperature. Membranes were again washed thrice in TBS-T, developed with enhanced chemiluminescence Western blotting detection reagents (Amersham, Buckinghamshire, United Kingdom), and visualized with a Typhoon scanner (Molecular Dynamics).

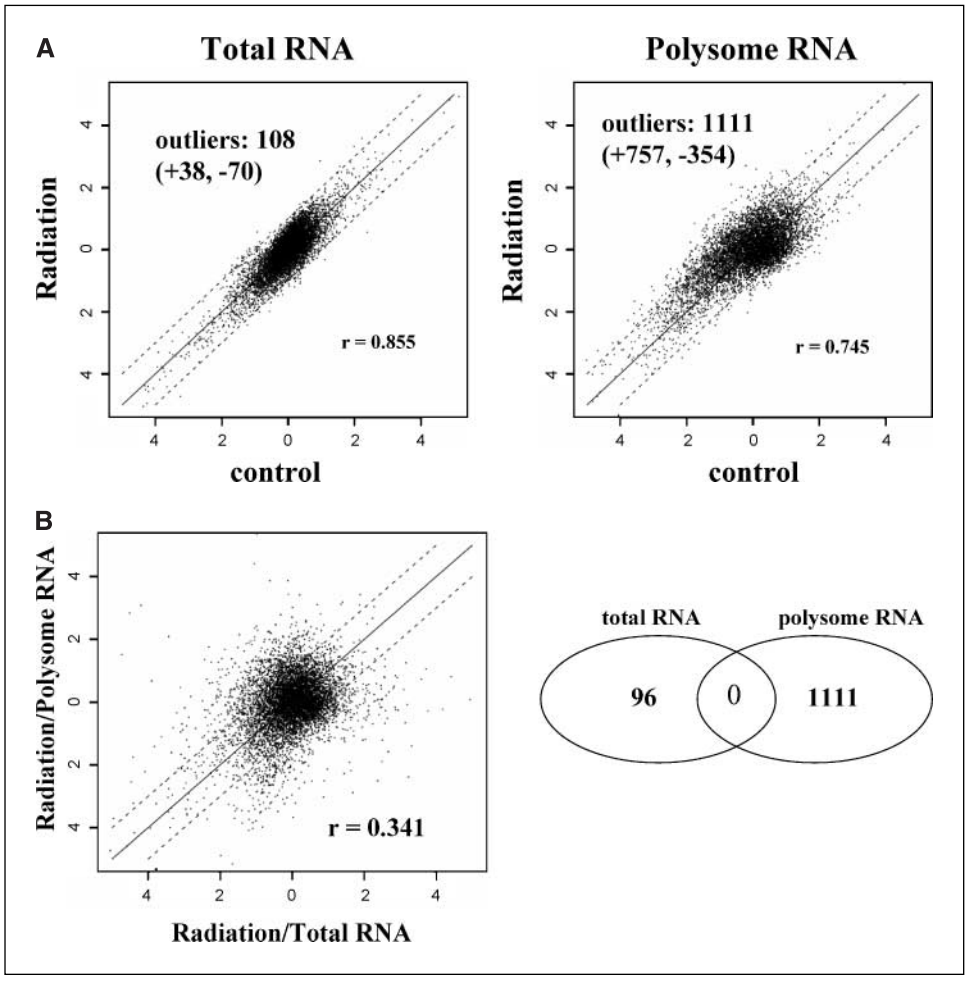

Figure 2. Comparison of radiation-induced gene expression profiles generated from microarray analysis of total and polysome RNA isolated from U87 cells. Cells were irradiated (7 Gy) and collected 6 hours later for isolation of total cellular RNA or polysome-bound RNA. *A,* gene expression was directly compared between irradiated and unirradiated control cells for total RNA (*left*) and polysome RNA (*right*) using scatter plot analysis. Correlation coefficients (*r*) were calculated for each comparison and are listed on each scatter plot. *Points on solid line,* genes with similar expression levels; *points outside dotted line,* genes with expression levels that differ by >2-fold between irradiated and unirradiated control cells. The total number of outliers (i.e., those that differed by ≥2-fold) are listed on each scatter plot. *B,* the radiation-induced gene expression profiles obtained from total and polysome-bound RNA were then directly compared by scatter plot analysis (*left*). The number of genes altered by radiation in total RNA, polysome-bound RNA, and in both is shown in the Venn diagram (*right*).

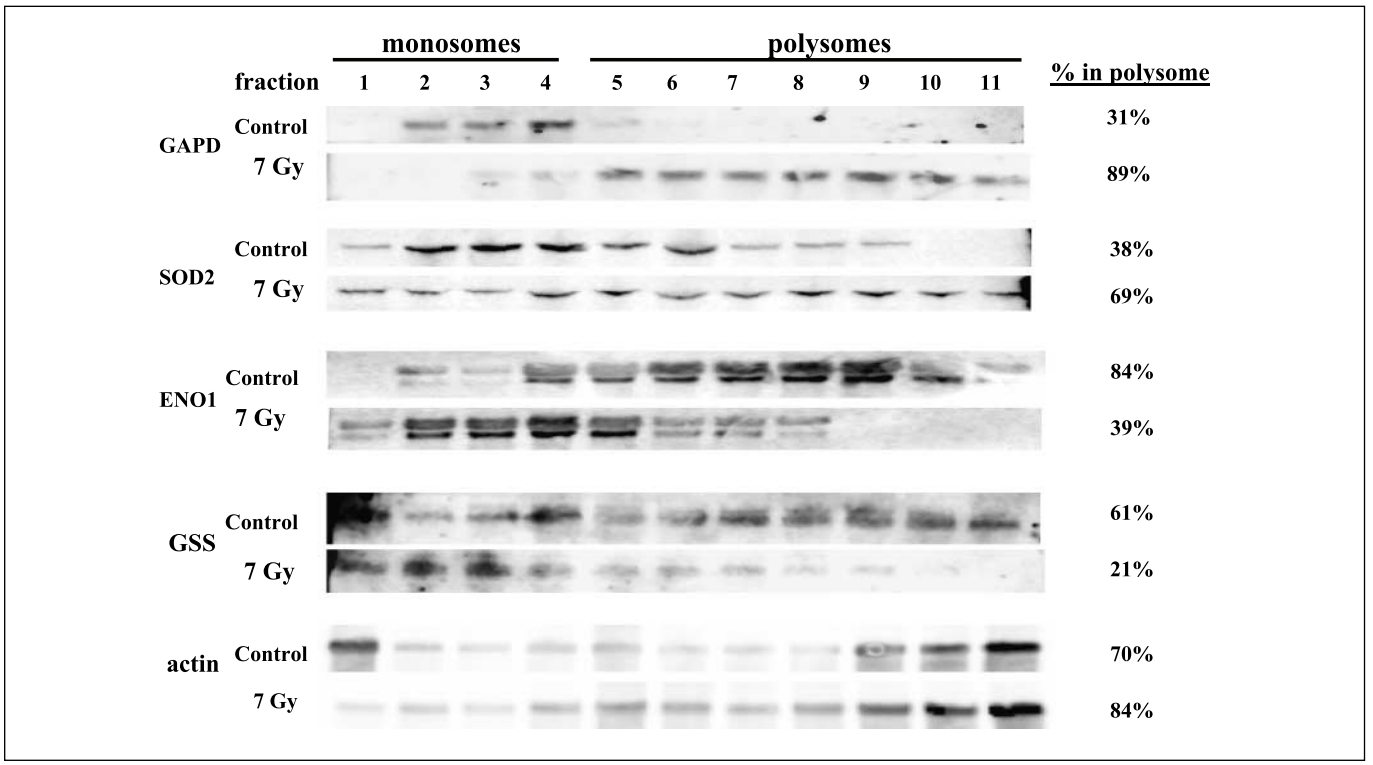

Figure 3. Northern blot analyses of the radiation-induced shift of mRNA to and away from polysomes. U87 cells were irradiated (7 Gy) and collected 6 hours later as cytoplasmic extracts for polysome isolation. Northern blots were generated from RNA extracted from each of the fractions collected from sucrose gradients as illustrated in Fig. 1. Fraction number is indicated on top with polysomes corresponding to fractions 5 to 11. *Top,* isolated from control untreated cells (for each mRNA); *bottom,* isolated from irradiated cells (for each mRNA). The percentage of mRNA found in the polysome fractions for control and irradiated samples was determined by densitometry (*right column*).

Results

Comparison of radiation-induced changes in transcription and translation. Initial studies were done using the human brain tumor cell line U87 exposed to 7 Gy, which results in ~1 log of cell killing, and collected 6 hours later. The radiation treatment protocol was chosen to correspond to previous studies indicating that the maximum number of genes induced at the transcriptional level (i.e., using total RNA) is typically around 6 hours after a dose of ~4 to 10 Gy (2, 6). In addition, the 6-hour time point has been typically used to evaluate the effects of radiation on the expression of individual transcripts (20). To identify genes under translational control after cellular exposure to radiation, cytoplasmic lysates from irradiated and control U87 cells were centrifuged through sucrose gradients and then collected in 11 continuous 1-mL fractions from lightest, which are devoid of ribosomes, to the heaviest, which contain the largest polysome complexes (18). A representative absorption profile (A_{254}) from a sucrose gradient generated from U87 cells along with the RNA content of each 1-mL fraction is shown in Fig. 1. Radiation had no detectable effect on the polysome profile or the RNA content of each fraction (data not shown). The polysome containing fractions 5 to 11 were pooled; RNA was extracted and used for microarray analysis. In the same experiment, total RNA was isolated from whole-cell lysates from duplicate irradiated and untreated cultures and subjected to microarray analysis. Using both polysome-bound RNA and total RNA in these studies allowed for the identification of genes whose expression was regulated by radiation at the translational and/or transcriptional levels, respectively.

Analysis of radiation-induced changes in gene expression using total RNA and polysome-bound RNA provided dramatically different results (Fig. 2). The number of genes affected by radiation in the two groups was determined using scatter plots of control versus irradiated U87 cells (Fig. 2*A*). Points appearing on or outside the dotted lines corresponded to genes whose expression was changed by ≥2-fold after irradiation, which were then classified as outliers. In total RNA, radiation resulted in 108 outliers (38 increased and 70 decreased), which is similar to the number of genes previously reported to be affected after irradiation of U87 cells (2). However, when microarray gene expression analysis was done using polysome RNA 1111 (757 increased and 354 decreased) outliers were detected. The list of genes whose expression was affected by ≥2-fold after irradiation in polysome and total RNA is presented in Supplementary Table 1. These data suggest that at least in U87 cells, although radiation affects gene transcription (total RNA) and translation (polysomal RNA), translation control is considerably more susceptible to radiation. A direct comparison of the radiation-induced gene expression profiles obtained from total RNA versus polysome RNA appears in Fig. 2*B*. As indicated by the correlation coefficient generated by the scatter plot, the radiation-induced expression profiles obtained from these two sources of mRNA were significantly different. Importantly, as shown in the accompanying Venn diagram, there were no outlier genes commonly affected by radiation in total RNA and polysome-bound RNA.

Radiation-induced recruitment of mRNAs to and away from polysomes. To verify that the radiation-induced changes

detected in the microarray analysis of polysomal RNA reflected modifications in the polysomal association of an mRNA, Northern blots were generated for the RNA fractions collected from the sucrose gradients (see Fig. 1). Fractions 1 to 4 correspond to unbound RNA or RNA associated with monosomes (poorly or not translated) and fractions 5 to 11 to RNA bound to polysomes (actively translated). In this study, four representative genes were evaluated whose expression levels were modified by radiation in the microarray analysis of polysomal RNA but not in the analysis of total RNA (Supplementary Table 1): *GAPDH* and *SOD2*, which were increased, and *ENO1* and *GSS*, which were decreased in polysomal RNA. These genes were expressed at relatively high levels in U87 cells, which allowed for detection across the 11 fractions. The effects of radiation on the monosome/polysome distribution of these mRNAs are presented in Fig. 3. In control untreated U87 cells, *GAPDH* mRNA was found primarily in the monosome fractions; however, after irradiation, there was a definite shift to the polysome fraction indicative of an increase in translational activity. A similar radiation-induced shift from monosomal to polysomal fractions was found for *SOD2*. In contrast, *ENO1* and *GSS* mRNAs underwent a shift from polysomal to monosomal fractions after irradiation, indicative of a decrease in translation. Radiation had no effect on the monosome/polysome distribution of actin whose expression was not effected at the transcription or translation levels (Supplementary Table 1). The lack of an effect of radiation on the steady-state levels of these mRNAs as indicated in the total RNA microarray analysis was verified by Northern blotting of whole-cell lysates (data not shown). Thus, these data confirm that radiation selectively regulates the association of existing mRNAs with polysomes, consistent with a modification of translational activity.

Correlation between radiation-induced changes in polysome-bound mRNA and protein. To determine whether the radiation-induced change in a gene's translational activity (as indicated by polysome binding) corresponds with a change in its protein product, immunoblots were generated for irradiated U87 cells. Based on the list of genes generated from the microarray analysis of polysomal RNA (Supplementary Table 1), 16 proteins were screened, for which there were commercially available antibodies; the immunoblots shown are representative of at least two independent experiments. Figure 4*A* shows the immunoblots for 11 proteins that were predicted from the polysome microarray analysis to be increased after irradiation. ADAM9, aquaporin 3 (AQP3), cyclin C (CCNC), GAPDH, FMRP (*FMR1*), p53-dependent ribonucleotide reductase (RRM2B), and SOD2 were each increased at 6 and 16 hours after irradiation. The increases in TNFRSF6 (CD95/FAS) and MAPK6 (extracellular signal-regulated kinase-3) were transient over this time course, increasing at 6 hours and returning to unirradiated levels by 16 hours. TNFRSF6 was previously reported to be increased in U87 cells after irradiation (21). In contrast to these nine proteins, no reproducible changes were detected in irradiated cells for levels of carbonic anhydrase 1 (CA1) or ELK-3, which were predicted to increase by the polysome microarray analysis. Immunoblots for proteins corresponding to mRNAs that were decreased in the polysome fraction after

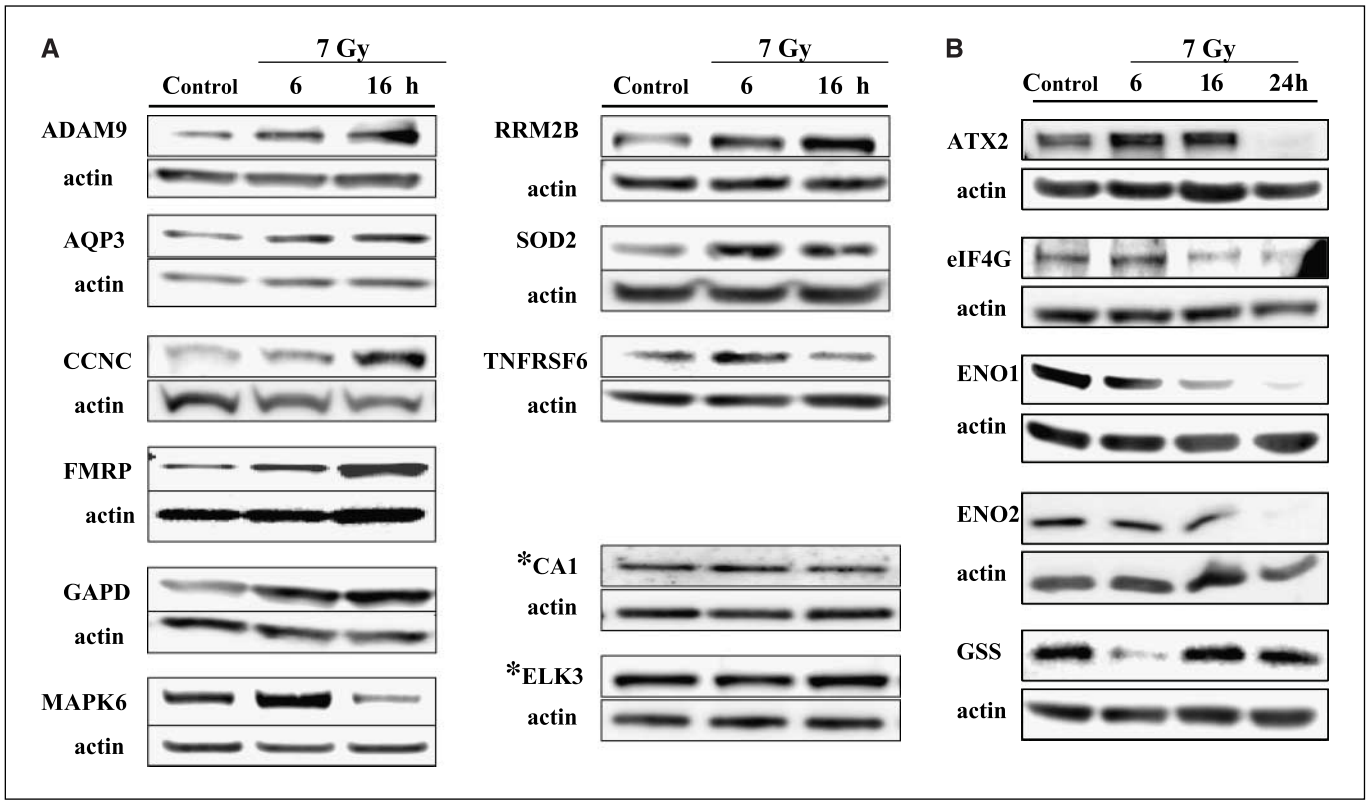

Figure 4. Immunoblot analyses of proteins predicted by the polysome-bound RNA gene expression profile to be modified by radiation. U87 cells were irradiated (7 Gy), and total protein was collected 6 to 24 hours later and subjected to immunoblot analysis for the specified proteins. Each blot used actin as a loading control, and the blots shown are representative of at least two independent experiments. *A,* proteins predicted to increase after irradiation. *, reproducible increase was not detected. *B,* proteins predicted to decrease after irradiation.

Table 1. Selected processes overrepresented by the polysome-bound mRNAs affected by radiation

Overrepresented categories	U87		SF126		SF539		Astrocyte	
	GOstat score	No. genes	GOstat score	No. genes	GOstat score	No. genes	GOstat score	No. genes
Genes with increased activity								
Cell cycle	7.00e−03	51	1.32e−09	60	6.50e−11	47	1.64e−04	67
Regulation of cell cycle	3.00e−03	34	2.23e−08	39	1.27e−09	31	5.86e−07	49
Transcription regulator activity								
Transcription factor binding	6.92e−04	20	8.87e−02	17	1.00e−04	18	3.64e−06	34
Transcription cofactor activity	1.82e−04	19	—	—	1.28e−03	15	3.30e−06	31
RNA polymerase II transcription factor	1.39e−03	16	7.12e−02	14	1.05e−02	13	7.03e−04	24
Nucleotide metabolism	4.32e−05	164	4.07e−03	181	2.65e−03	127	1.36e−05	281
Transcription from RNA polymerase II	3.74e−09	36	5.99e−06	35	4.99e−05	25	4.99e−08	51
RNA metabolism								
RNA processing	9.96e−03	22	7.10e−03	25	1.30e−03	20	2.84e−06	42
mRNA metabolism	5.34e−04	18	4.38e−06	23	1.76e−06	18	4.54e−08	33
mRNA processing	1.10e−03	16	4.83e−06	21	3.81e−04	16	1.86e−06	28
Genes with decreased activity								
Monosaccharide catabolism	1.16e−03	9	6.51e−02	9	9.99e−04	13	4.80e−02	12
Glycolysis	2.01e−03	9	—	—	2.05e−03	11	3.26e−02	11

NOTE: The polysome-bound mRNAs whose levels were modified by radiation by ≥2-fold were subjected to GOstat analysis. The categories were selected from the Gene Ontology pathways under biological process that were statistically overrepresented. The GOstat score refers to statistical significance (*P*).

irradiation and thus predicted to decrease are shown in Fig. 4*B*. Of these five proteins, the levels of ataxin 2 (ATX2), eukaryotic initiation factor 4G (eIF4G), ENO1, and ENO2 were reduced by 24 hours after irradiation. However, the reduction in GSS was transient with levels decreased at 6 hours and returning to control levels by 16 hours after exposure to 7 Gy. Thus, for 14 of the 16 mRNAs whose polysome association was modified after irradiation (Supplementary Table 1; Fig. 3), analogous changes were detected in the corresponding protein using standard immunoblot analyses.

Pathway analysis of genes affected at translational level after irradiation. To gain insight into the potential functional consequences of the radiation-induced changes in U87 cell gene translation the pathway analysis tool GOstat was applied (19). GOstat distributes genes into biological processes corresponding to Gene Ontology pathways (22), which are then organized in hierarchical clusters with the most general function at the primary node and more specific functions at each subsequent node. The number of genes expected to occur randomly in each pathway is compared with the actual distribution of genes in the sample set, which results in a list of biological pathways that are statistically overrepresented in a given list of genes. As shown in Table 1, for the genes increased in the polysome fraction after irradiation the predominant overrepresented Gene Ontology pathways were those pertaining to cell cycle, transcription, and DNA/RNA metabolism. Interestingly, the only pathway that was overrepresented by genes whose translation was decreased after irradiation was monosaccharide catabolism, specifically glycolysis. Whereas GAPDH is a glycolytic enzyme and yet was increased in the polysome fraction after irradiation, recent reports indicate that it has additional activities, including RNA binding (23).

As an additional tool for investigating the biological pathways represented by the genes whose translational activity was affected by radiation the IPA was used (Table 2). IPA distributes genes into networks defined by known interactions culled from the literature and then associates these networks with biological pathways (see Materials and Methods). Compared with GOstat, IPA uses a different database and a different statistical method. IPA identified 22 statistically significant networks with each containing at least six genes affected after irradiation; the first 10 are shown in Table 2. Of note, in the first four networks, the genes affected by radiation comprised the maximum number of genes (n = 35) allowed by the program. This analysis suggests that there is significant interaction between the protein products of genes whose translational activity was modified by radiation, which in turn suggests that radiation influences the activity of a variety of biochemical pathways at multiple points. Moreover, these IPA-generated networks were associated with cell cycle, gene expression, and DNA replication and repair (Table 2; Fig. 5*B*), functional processes consistent with those identified by the GOstat analysis.

Comparison of glioma cell lines and normal astrocytes. Microarray analysis of radiation-induced gene transcription (i.e., using total RNA) has identified relatively few commonly effected genes among cell lines, even those of the same tumor histology (2, 6). To determine whether a similar cell type specificity applies to the analysis of radiation-induced changes in translational activity, microarray analysis of polysomal RNA was used to compare the genes whose expression was affected by radiation in the human brain tumor cell lines U87, SF126, and SF539 (Fig. 5*A*). There were no commonly affected genes among the three cell lines when comparing genes whose expression was affected by radiation at the transcriptional level (total RNA), consistent with previous results. However, although there was some cell type specificity with respect to the radiation-induced changes in translation, there was also a significant number of genes (n = 296)

Table 2. Top 10 networks generated from IPA for polysome-bound RNAs affected by radiation

Network ID	Genes in network	Score	No. focus genes	Top categories
1	*ALAS2, BATF, BGLAP,* **CASR, CCL2, CXCR4, DKK1,** *FOSL1, FY,* **HMGCS2, JUN, MAP2K4, MAP3K8, MMP9, NPTX2,** *NR1H2*, **NR1H4, NR2F1, NR2F2, PIAS2,** *PIN1*, **RARB, RCN2, RQCD1,** *RXRA, SOCS3*, **STAT1, STAT4, TAF1B, TFPI,** *THBS1*, **TIEG, TOP2A, TOP2B, VDR**	37	35	Gene expression, cellular growth and proliferation, skeletal and muscular system development and function
2	**AR,** *BIRC5, CAMK1, CAMK2B*, **CASP7, CASP8, CASP8AP2, CAST (CALPASTATIN), CCNA2, CDC2,** *CDC34*, **CDKN1B,** *EEF2*, **GADD45A, GADD45G, HMGA2, JAG1, KLF4,** *M6PRBP1*, **MARCKS, MDM2, MSH2, NUP50, PCNA,** *PLEC1*, **PRKR,** *RAD9A*, **RFC3,** *SYN1*, **TAX1BP1, TNFRSF6,** *TNFSF13*, **TP73L, UBE2A, ZNF148**	37	35	Cell cycle, cancer, cell death
3	**CBL, CCNT1, CCNT2,** *CCRK*, **CD2AP,** *CDK9*, **EFEMP1, EGF,** *ERBB2*, **ERBB3,** *GRN, IL11RA*, **IL6ST,** *IRF7*, **KLF2,** *LAMR1, LAT, LIF, MYBL2*, **PMAIP1,** *POLR2C, POLR2H*, **PRDX2, PSG2, PTPRC, RNGTT, SMAD4, SMAD5, SPRY2, SPTBN1,** *SUPT5H*, **SYK,** *TCF7L2, TEGT*, **VAV1**	37	35	Cellular development, gene expression, viral function
4	*ACTL6B*, **ACTR2, ACTR3,** *ALDOA*, **ARID4A,** *ARPC4*, **CBFA2T1, CDH11, CPA3, CSNK1G2, DAG1, FABP5,** *FIBP*, **FMR1,** *GNAI2*, **HMGB2,** *IGFBP6*, **MAPK6, NFYB, OGDH, PIK3CB, PIK3R3, PSMC3,** *PSMC6, PSMD2*, **PSMD10,** *PSMD13*, **RFX5, SAP30, SGCD, SGCG, SMARCA2, SNCA, STAU,** *TH*	37	35	Cardiovascular disease, organismal injury and abnormalities, small molecule biochemistry
5	**ASK**, *CDC7, CDC10, CEP2, CLK2*, **CPNE3, CR1, DCAMKL1, G3BP, HNRPA2B1, IL2RB,** *ITSN2*, *JAK3, LCK,* **LSM8**, *MBP, MCM2, MCM3, MCM4, MCM5*, *MCM6, MCM7*, **MMP9**, *NEDD5, NFKBIA, NRBP, NRG1, PGD, PNUTL1*, *POU3F1,* **PTPRC**, *SEPT6, STX4A, VCP*, **VRK1**	13	21	Cell cycle, DNA replication, recombination, and repair, cell death
6	*ACTB*, **ATP5I, CCT3**, *CCT4,* **CETN3, CLK1, COX17**, *COX5B, DCTN1*, *DCTN2, DNCH1, DNCI1, DST,* **H3F3A**, *JRK,* **KIF11**, *MAGOH, MAPRE1, MAPRE3, NUDC, NXF1*, *PAFAH1B1, PPIH,* **PRPF3**, *PRPF4,* **PRPF18, PRPF4B, RPL36A, RSN**, *SFRS1, SFRS6, SFRS12, SRPK1,* **SRPK2**, *U2AF1*	10	18	RNA post-transcriptional modification, protein folding, protein synthesis
7	**ACVR2, ACVR1B,** *ALDOC*, *BLOC1S1, BLOC1S2, BLOC1S3,* **BTEB1, CPSF1, DEK, ELF1, HAT1,** *HMGA1*, *INHA, INHBA, MYCN, MYST3, MYST4, NME1,* **PAH, PCAF, PLDN, RNF6,** *RPL29, RPS15*, **RPS23**, *SRRM1, STAF42, STAF65* (gamma), *SUPT3H,* **TADA3L, TAF5**, *TAF12,* **TAF5L**, *TAF6L, TCF1*	10	18	Post-translational modification, protein synthesis, DNA replication, recombination, and repair
8	*AKAP8, ANAPC1, ANAPC4,* **ANAPC10, APOBEC1, AQP2, AQP3,** *ARVCF*, *BAG4, C3orf15, CDC27,* **CDH1**, *CFTR, DNAJA1, DNAJB11, EGFR,* **EIF2S2,** *EIF5A*, **FKBP4**, *GPR37, HSPA8, HSPA1B,* **MYCBP,** *MYO7A*, **PDZK1**, *PRKACA, PRKACB, PRKACG, PRKAG1,* **PRKAR1A, PRKAR2B**, *PRKDC,* **SDCBP,** *STUB1*, **XRCC4**	10	18	Protein folding, protein synthesis, cancer
9	**ACTN1**, *ANXA2,* **BRD2**, *CASP3, CDKN1A,* **DDX21**, *E2F2, E2F4,* **ENO1**, *FOS,* **GBP2, GEM**, *HIF1A,* **LAMP2**, *MYC, MYCN,* **PEG3, PGK1, PLS3, PRDX3**, *ROCK1, ROCK2,* **RPL23, RPS13, SUCLA2, TMSB4X**, *TP53*	9	15	Cell cycle, cellular development, cellular growth and proliferation
10	**ANGPT1, ASE-1, BMX**, *CHUK,* **ELF2, GRB14,** *HSPA1L, HSPCB*, *IKBKB, IKBKE,* **IKBKG**, *IL1R1, IRAK1, MAP3K14, NFKBIB, STIP1,* **TANK**, *TBK1, TEBP,* **TEK, TERF1**, *TINF2, TLR2, TLR4,* **TLR5**, *TNFAIP3, TNFRSF8, TNFRSF1B,* **TNKS2, TOLLIP, TRAF3**, *TRAF5, TRAF6,* **TTRAP, ZA20D2**	9	17	Endocrine system development and function, organismal survival, cell death

NOTE: The polysome-bound mRNAs whose levels were modified by radiation by ≥2-fold were subjected to IPA; the top 10 networks and their top 3 network function categories are shown. The score is the negative log of *P* and indicates the likelihood of the focus genes in a network being found together due to chance. No. focus genes refers to the genes affected by radiation within an IPA defined network; the maximum number of genes allowed by the IPA in a network is 35. Bold: genes increased by radiation; italicized bold: genes decreased by radiation; standard text: genes in network not affected by radiation.

that were commonly affected among the three tumor cell lines (Supplementary Table 2). Analysis of the radiation translational control profiles using GOstat (Table 1) and IPA (Fig. 5*B*) indicated that although there are differences in specific categories, genes modified after irradiation of SF126 and SF539 cells distributed to similar functional pathways as for those in U87 cells.

The studies above focused on brain tumor cell lines. To determine whether radiation has similar effects on normal cells of the central nervous system (CNS), microarray gene expression analysis was done on total and polysomal RNA isolated from normal human astrocytes grown in monolayer culture. As for the tumor cell lines, polysome-bound RNA was substantially more susceptible to radiation-induced changes than total RNA (Fig. 6*A*). The number of genes affected at the translational level (n = 1,399) was significantly greater than the number of genes (n = 39) modified by radiation at the transcriptional levels (total RNA). Ten genes were commonly affected in both total and polysomal RNA. The genes whose expression was modified in polysomal

RNA in astrocytes were then compared with those identified as common outliers in the tumor cell lines (Fig. 6*B*). Although there were no genes commonly affected among the four cell types with respect to transcription changes (total RNA), there were 184 genes commonly affected at the level of translation (polysome RNA). As shown by GOstat analysis (Table 1) and the IPA in Fig. 5*B*, the genes affected after irradiation of normal astrocytes distributed to similar functional categories as those affected in the brain tumor cell lines. These results suggest that although there is cell type specificity, a fundamental cellular response to radiation involves modifying the translational control of a subset of genes.

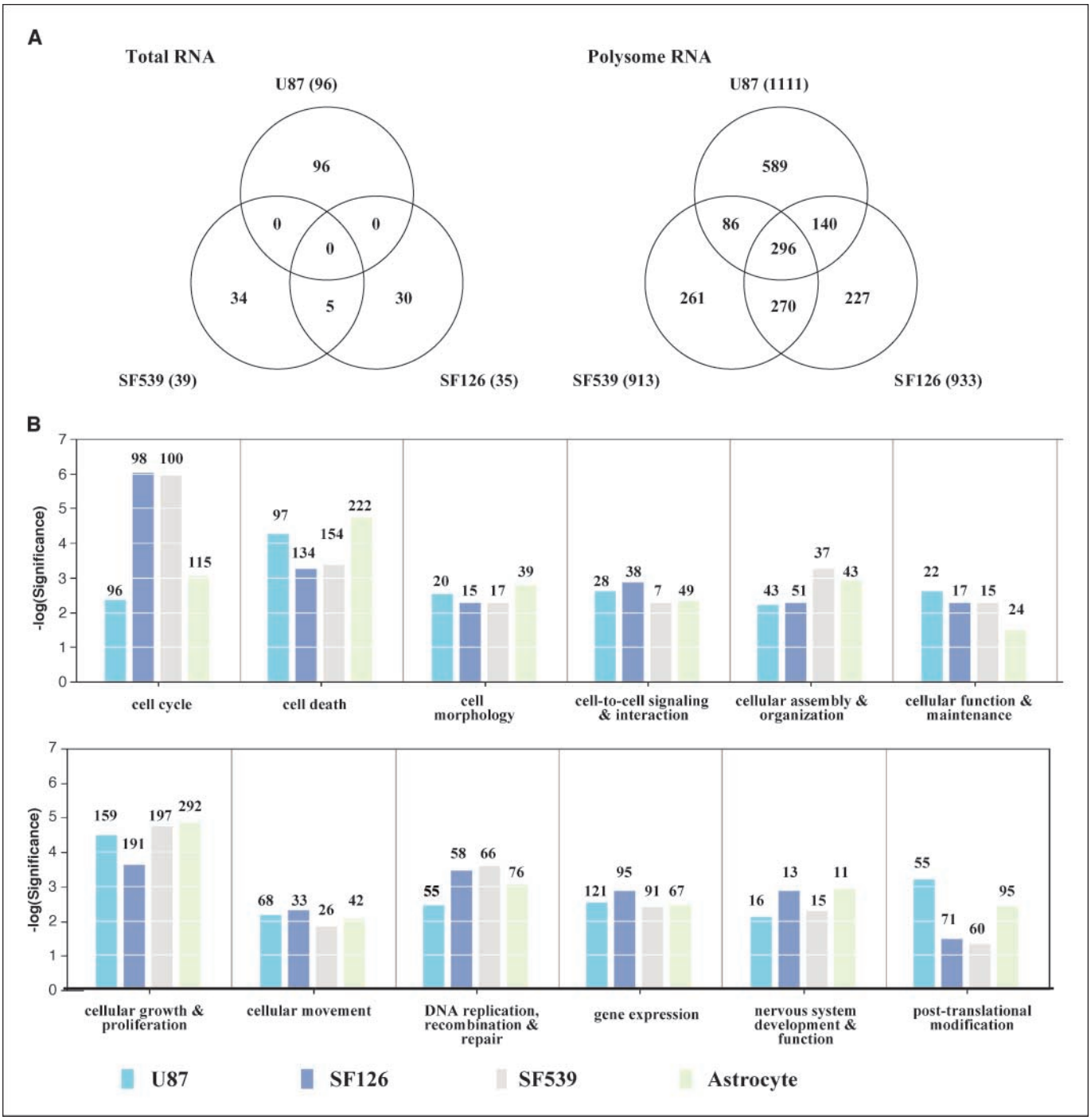

Figure 5. Comparison of radiation-induced gene expression profiles generated from total and polysome-bound RNA for three brain tumor cell lines. Each cell line was irradiated (7 Gy) and collected 6 hours later for isolation of total cellular RNA or polysome-bound RNA. The number of outlier genes (≥2 fold) was determined by scatter blot analysis comparing control with irradiated samples for each cell line. *A,* Venn diagrams comparing the number of outlier genes obtained from each cell after microarray analysis of total RNA (*left*) and polysome-bound RNA (*right*). *B,* selected IPA assigned global functional categories for the radiation-induced outlier genes identified in each cell line. Increasing value of −log (significance) indicates increasing confidence for each category. The number of genes is indicated above each bar. This graph also includes data from normal astrocytes as described in Fig. 6.

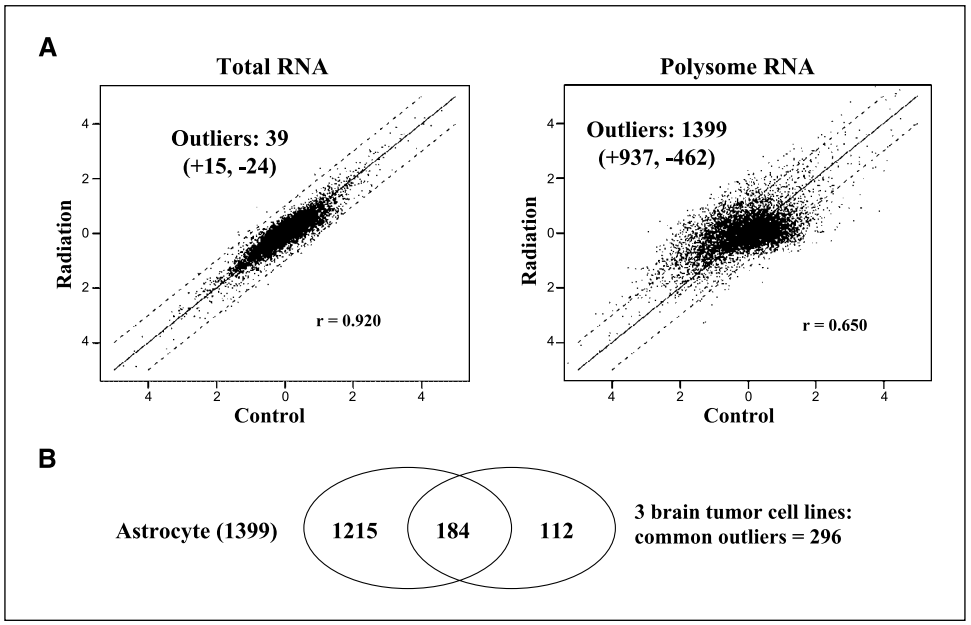

Figure 6. Comparison of radiation-induced gene expression profiles generated from microarray analysis of total and polysome-bound RNA isolated from normal human astrocytes. Astrocyte cultures were irradiated (7 Gy) and collected 6 hours later for isolation of total cellular RNA or polysome-bound RNA. *A,* Gene expression was directly compared between irradiated and unirradiated control cells for total RNA (*left*) and polysome RNA (*right*) using scatter plot analysis. Correlation coefficients (*r*) were calculated for each comparison and are listed on each scatter plot. *Points on solid line,* genes with similar expression levels; *points outside dotted line,* genes with expression levels that differ by >2-fold between irradiated and unirradiated control cells. The total number of outliers (i.e., those that differed by ≥2-fold) are listed on each scatter plot. *B,* Venn diagram comparing the number of radiation-affected genes detected in the analysis of polysome-bound RNA of astrocytes and the 296 genes that were commonly affected in the three brain tumor cell lines.

Discussion

Radiotherapy continues to be a primary treatment modality for brain tumors as well as for many other solid tumors. Recent approaches aimed at improving the efficacy of radiation involve the development and application of molecularly targeted agents, a strategy that requires a thorough understanding of the fundamental processes comprising cellular radioresponse. Towards this end, radiation-induced transcriptional and post-translational modifications, such as phosphorylation, have been the subjects of extensive research. However, although translation is an essential step in gene expression, the global effects of radiation on mRNA translational activity remained undefined. The findings presented here comparing microarray analyses of total and polysome-bound RNA indicate that whereas radiation does modify gene transcription, it also modifies the translational activity of a subset of mRNAs. Surprisingly, compared with transcription, radiation was found to affect substantially more genes at the level of translation. Furthermore, there were few, if any genes affected at both the transcriptional and translational levels. These results suggest that the radiation-induced changes in transcription and translation are not coordinated with each proceeding through different mechanisms. The independence of these two events is further supported by data indicating that the radiation-induced changes in translation as detected in the microarray analysis of polysomal RNA occur through the recruitment of existing mRNAs to and away from polysomes.

The mechanism through which radiation regulates the association of specific mRNAs with polysomes remains to be investigated. At a relatively high dose of 20 Gy radiation has been reported to decrease overall translation through inhibiting the disassociation of 4E-BP1 and eIF4E (24). However, at a clinically relevant dose of 2 Gy, radiation was shown to enhance the activities of S6 kinases via ErbB-dependent pathways, which suggested an increase in translational activity (25). Whereas these reports have associated radiation with changes in translation, the mechanism accounting for the recruitment to and away from polysomes of a specific subset of mRNAs as observed herein is unclear. To account for this specificity and because the mRNAs targeted by radiation were found to be components of functional pathways and interactive networks the post-transcriptional operon model put forth by Keene and Tenenbaum (26), which hypothesizes that RNA-binding proteins regulate the translation of functionally related mRNAs, may be applicable. Along these lines, Murmu et al. have reported that after whole body irradiation the RNA-binding protein CUGBP2 is induced in the mouse intestine, which was proposed to regulate cyclooxygenase-2 translation (27). In addition, epidermal growth factor receptor stimulation, which can occur after irradiation (25), was shown to result in the phosphorylation of CUGBP1 and increase its activity in mammary epithelial cells (28). Consistent with a potential role for RNA-binding proteins in the effects of radiation on translation control (Fig. 4*A*), irradiation of U87 cells resulted in an increase in the levels of FMRP, an RNA-binding protein involved in Fragile X syndrome (29) and GAPDH, a glycolytic enzyme recently shown to have RNA binding activity and to play a role in post-transcriptional regulation (23). Clearly, although the data presented here indicate that radiation regulates the translational activity of a subset of mRNAs, defining the mechanisms mediating this effect will require further investigations.

The disconnect between changes in transcription and translation after irradiation is consistent with previous reports involving eukaryotic cells undergoing other forms of stress (8, 9) and would seem to account for the general inability to correlate radiation-induced changes in gene transcription as detected by microarray analyses with changes in corresponding protein. In contrast, as shown here, the radiation-induced change in a gene's translational activity as detected by microarray analysis of polysome-bound RNA correlated for the most part (14 of 16) with a change in its protein product. Clearly, there are a number of processes distal to translation initiation, including protein stability and half-life that could account for the lack of a correlation between a change in the polysome association of an mRNA and its protein. However, given that polysome recruitment is a critical regulatory event through which environmental signals/stress can affect gene expression (7–14), the data presented are consistent with translational control serving as the primary mechanism mediating radiation-induced

gene expression. Moreover, this study suggests that microarray analysis of polysome-bound RNA may provide an initial high-throughput screening strategy for identifying proteins that play a role in regulating cellular radioresponse.

The mRNAs whose polysome association was modified after irradiation were not simply a random collection but were components of a number of functional pathways. Several of the general pathways represented, such as cell cycle, cell death, and DNA replication, recombination and repair would be predicted to have a role in determining radiosensitivity. However, the affected genes were also preferentially distributed in other pathways that may be indicative of more novel aspects of cellular radioresponse. For example, IPA identified a statistically significant number of the genes that participate in cellular movement and cell morphology. Further investigation of these genes and their associated networks and pathways may provide insight into the previous observation that radiation increases glioma cell migration and invasiveness after irradiation (30). In addition, there were a number of genes increased in polysomes after irradiation involved in transcription regulation (GOstat) and gene expression (IPA). This may suggest that the effects of radiation on translational control may actually serve as regulators of transcription. More detailed time course analyses of radiation-induced translational and transcriptional changes will address this possibility. Whereas distributed to specific functional categories, the analyses presented indicate that the genes affected by radiation at the translational level were also components of interacting networks. While serving to illustrate the complexity of events and processes involved in cellular radioresponse, distribution of genes to interacting networks also suggest that it may be possible to target a single protein and affect the activities of multiple pathways operative in an irradiated cell.

Comparison of the three brain tumor cell lines regarding the genes whose transcription was modified by radiation revealed no commonly affected genes, which is consistent with previous reports. Such results have been interpreted as indicating that radiation-induced gene transcription is highly dependent on cellular genotype (6). In contrast, at the translational level radiation affected a significant number of the same genes (n = 296) in each of the brain tumor cell lines. Moreover, of the 296 genes commonly affected in the brain tumor cell lines, 184 (62%) were also modified by radiation in primary cultures of normal human astrocytes. These findings suggest that radiation-induced translational control is a fundamental component of cellular radioresponse, at least for cells of CNS origin. Whether there are similarities with genes modified at the translational level after irradiation of other tumor and normal cells remains to be determined. However, these initial results suggest that understanding translational control should generate insights into the mechanisms regulating the cellular radiosensitivity.

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Radiation-Induced Changes in Gene Expression Involve Recruitment of Existing Messenger RNAs to and away from Polysomes

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