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DIFFERENTIAL REGULATION OF POLYSOME mRNA LEVELS IN MOUSE HEPA-1C1C7 CELLS EXPOSED TO DIOXIN

Jessica A. Thornley^{1,6}, Heidi W. Trask¹, Christian J. A. Ridley^{1,6}, Murray Korc^{1,3,5}, Jiang Gui^{1,2}, Carol S. Ringelberg⁴, Sinny Wang¹, and Craig R. Tomlinson^{1,3,5,*}

¹ Norris Cotton Cancer Center, Dartmouth Hitchcock Medical Center, Lebanon, New Hampshire 03756, USA

² Department of Community and Family Medicine, Dartmouth Hitchcock Medical Center, Lebanon, New Hampshire 03756, USA

³ Department of Medicine, Dartmouth Hitchcock Medical Center, Lebanon, New Hampshire 03756, USA

⁴ Department of Genetics, Dartmouth Hitchcock Medical Center, Lebanon, New Hampshire 03756, USA

⁵ Department of Pharmacology and Toxicology, Dartmouth Hitchcock Medical Center, Lebanon, New Hampshire 03756, USA

⁶ Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY, United Kingdom

Abstract

The environmental agent 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) causes a multitude of human illnesses. In order to more fully understand the underlying biology of TCDD toxicity, we tested the hypothesis that new candidate genes could be identified using polysome RNA from TCDD-treated mouse Hepa-1c1c7 cells. We found that (i) differentially expressed whole cell and cytoplasm RNA levels are both poor predictors of polysome RNA levels; (ii) for a majority of RNAs, differential RNA levels are regulated independently in the nucleus, cytoplasm, and polysomes; (iii) for the remaining polysome RNAs, levels are regulated via several different mechanisms, including a “tagging” of mRNAs in the nucleus for immediate polysome entry; and (iv) most importantly, a gene list derived from differentially expressed polysome RNA generated new genes and cell pathways potentially related to TCDD biology.

Keywords

Polysomes; gene expression; gene regulation; TCDD; microarrays; Hepa-1c1c7

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*Address correspondence to: Craig R. Tomlinson, Department of Medicine, Dartmouth Hitchcock Medical Center, Norris Cotton Medical Center, One Medical Center Drive, Lebanon, NH 03756, Tel.: 603-653-6088, Fax: 603-653-9952, Craig.R.Tomlinson@Dartmouth.edu.

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INTRODUCTION

The pervasive environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) is a halogenated polycyclic aromatic hydrocarbon ubiquitous in the atmosphere, soil, water, and food (Zedeck, 1980). The major source of TCDD arises from combustion of fossil fuels (Kimbrough, 1987). TCDD is one of the most potent tumor promoters known (Graham et al., 1988; Pitot et al., 1980), and in humans, TCDD causes chloracne, a scarring skin disease (Suskind, 1985; Zuger, 1990), endometriosis (Yoshida et al., 2000), immunotoxicity and liver damage (Pohjanvirta and Tuomisto, 1994), cardiovascular disease and diabetes (Flesch-Janys et al., 1995; Steenland et al., 1999), and cancer (Bertazzi, 1991; Bertazzi et al., 1993; Fingerhut et al., 1991; Manz et al., 1991). *In utero* exposure of mice and other rodents to TCDD causes teratogenic abnormalities (Birnbaum, 1995) including hydronephrosis and cleft palate (Abbott, 1995; Couture et al., 1990).

Most of the effects of TCDD exposure are mediated via the aryl hydrocarbon receptor (AHR) signaling pathway (Fernandez-Salguero et al., 1995; Lucier et al., 1993). There are at least several hundred identified compounds that serve as agonists to activate the AHR, and TCDD is one of the most potent AHR activators known. Upon ligand binding, the AHR translocates to the nucleus where it complexes with the AHR nuclear translocator (ARNT) (Hoffman et al., 1991). The AHR/ARNT heterodimer regulates the transcription of genes in the cytochrome P450 *Cyp1* family, several Phase II detoxification genes (Gonzalez et al., 1984; Hankinson, 1995), as well as hundreds if not thousands of other genes (Guo et al., 2004; Karyala et al., 2004) by binding to response elements known as AHR-, dioxin-, or xenobiotic-response elements (Whitlock, 1993). Thus, the AHR/ARNT heterodimer activates the transcription of a battery of genes encoding proteins capable of catabolizing most AHR ligands, thereby completing an elegant feedback loop directed to eliminate such toxicants. TCDD is an exception in that the halogen side groups on TCDD render it highly resistant to degradation by the induced Phase I and II enzymes presumably leading to chronic activation of the AHR and the subsequent harmful biological effects (Uno et al., 2004).

One of the goals in our laboratory is to understand the underlying biology of TCDD toxicity. In pursuit of that goal, we had profiled the transcriptomes of several cell types following TCDD exposure using traditional microarray approaches to determine differential steady-state mRNA levels (Guo et al., 2004; Karyala et al., 2004). That is, we followed the methods of every known published microarray study by isolating whole cell RNA to generate labeled target for the microarray probes. However, in later work, we showed that the use of whole cell RNA is not an accurate measure of steady-state mRNA levels because the inclusion of nuclear RNA introduces a large number of missed and false positive genes (Schwanekamp et al., 2006; Trask et al., 2009). Thus, steady-state mRNA levels are best represented by using purified cytoplasm RNA; however, the determination of steady-state mRNA levels does not provide insight into many of the other events that contribute to gene expression regulation. For instance, there are many examples in which changes in mRNA levels do not correlate with changes in protein levels (Chen et al., 2002; Gygi et al., 1999; Haynes et al., 1998; Tew et al., 1996), and much of the discrepancy between the whole cell transcriptome and proteome is likely via regulation at the polysome level (Harding et al., 2000; Lu et al., 2006; Pradet-Balade et al., 2001; Wilkie et al., 2003).

In the study reported here, we tested the hypothesis that novel TCDD-regulated genes and pathways could be identified using polysome RNA. The premise for the hypothesis is that (i) except for relatively few examples, cell structure and functions are carried out by proteins, thus, the proteome provides a more accurate view of a cell's disease and functional states; (ii) because polysome RNA are those RNAs in the process of translation into proteins,

polysome RNA is representative of a cell's proteome, and (iii) therefore, polysome RNA profiles are an accurate reflection of the proteome and may uncover new key genes involved in the underlying disease biology. The premise is supported by a study of irradiated U87 cells that compared polysome RNA and whole cell RNA levels to protein levels (Lu et al., 2006). Using Western immunoblotting, 14 of 16 (87.5%) proteins showed analogous changes to polysome RNA levels while whole cell RNA levels showed little correlation.

Thus, to better understand the effects of TCDD on gene expression, we have carried out microarray analysis of polysome-bound RNA to generate profiles of mRNAs undergoing translation. That is, we used an RNA-based approach to profile newly synthesized proteins. We present results showing that differentially expressed cytoplasm RNA and whole cell RNA levels are poor predictors of polysome RNA levels; that polysome RNA levels are regulated via multiple mechanisms; and that polysome RNA profiling may be a powerful means to identify new genes and pathways involved in cell toxicity and disease states.

METHODS AND MATERIALS

Cell cultures

Mouse hepatoma Hepa-1c1c7 cells (Hankinson et al., 1991) were grown in α -minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum, 100 μ g/ml penicillin, and 100 μ g/ml streptomycin. Vehicle- (dimethyl sulfoxide) and TCDD-treated (10 nM TCDD for 2 hr) Hepa-1c1c7 cells were collected at 80% confluence. Cells were maintained in monolayer cultures at 37°C in humidified air with 5% CO₂, and cell numbers were determined by counting the cells with a hemocytometer. For some of the polysome preparations (6 and 20 hr), after the 2-hr vehicle- and TCDD exposure, the original medium was replaced with unadulterated fresh medium.

Nucleus and cytoplasm fractionation

The nucleus and cytoplasm fractions were isolated as described (Trask et al., 2009; Yasutome et al., 2005). The Hepa-1c1c7 cells were incubated for 30 min at 4°C in lysis buffer (20 mM HEPES, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, 25 mM NaF, 25 mM β -glycerophosphate, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin). Following centrifugation at 1700 rpm for 5 min, supernatants were used for the cytoplasm fraction. The pelleted nuclei were washed with 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH of 7.4) and incubated for 10 min at 4°C in nuclear extraction buffer (hypotonic buffer containing 500 mM NaCl) and cleared by centrifugation.

Polysome isolation

The Hepa-1c1c7 cells were harvested at 4°C, washed with 1X PBS containing 100 μ g/ml cycloheximide, and lysed in 1 ml of polysome buffer (10 mM Tris-HCl, pH 8, 140 mM NaCl, 1.5 mM MgCl₂, 0.5% NP-40, 10 mM dithiothreitol, 100 μ g/ml cycloheximide, 500 mg/ml heparin, 1 mM PMSF, and 500 units/ml RNasin [Promega, Madison, WI]). The lysates were incubated for 10 min on ice, centrifuged at 10,000 x g at 4°C for 10 min, and 400–500 μ l of the resulting supernatant layered onto a 10%–50% sucrose gradient (10% and 50% sucrose in 10 mM Tris, pH 7.5, 1.5 mM MgCl₂, 140 mM NaCl, 1 mM DTT, 100 μ g/ml cycloheximide, 0.5 mg/ml heparin [Sigma, St. Louis, MO]). The gradients were centrifuged at 164,000 x g at 4°C for 3.5 hr in a Superspeed 630 rotor using a Sorvall Discovery 90SE centrifuge (Thermo Scientific, Waltham, MA). Fractions (0.5 ml) were collected using a Gilson gradient fraction collector system (Middleton, WI) with continuous monitoring at

A₂₅₄ with a Bio-Rad UV monitor (Hercules, CA) and Windaq software (Akron, OH). The fractions corresponding to polysome RNA (Fig. 1A) were pooled and prepared for RNA purification.

RNA purification

Cells and cell fractions were homogenized in TRIzol Reagent (Invitrogen Corp., Carlsbad, CA), and the RNA was further purified using RNeasy Kits (Qiagen, Valencia, CA). RNA purity, quantity, and quality were determined using a NanoDrop spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA) and Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) (Wang et al., 2006).

Microarrays

The RNA gene expression microarray experiments were carried out by the Dartmouth Genomics & Microarray Laboratory (DGML). The MouseRef-8 v2.0 Expression BeadChip array (Illumina, San Diego, CA) with approximately 25,600 annotated RefSeq transcripts covering 19,100 unique mouse genes were used for the RNA profiling. Fluorescent images were obtained with an Illumina 500GX scanner in the DGML and processed with the BeadScan software (Illumina, San Diego, CA). Following the accompanying protocol, reverse transcription was carried out using an oligo(dT) primer bearing a T7 promoter and the high yield ArrayScriptTM reverse transcriptase to make cDNA. The cDNA was made double-stranded with DNA polymerase, and the dsDNA was purified to use as a template for *in vitro* transcription with T7 RNA polymerase and the included biotin-NTP mix. The labeled cRNA was purified and 1.5 µg used for hybridization to the beadarrays for 16 hr at 55°C. Following hybridization, the beadarrays were washed and stained with streptavidin-Cy3 (GE Healthcare, Piscataway, NJ). Fluorescent images were obtained with an Illumina 500GX scanner in the DGML and processed with the BeadScan software (Illumina, San Diego, CA).

Microarray data analysis

Four biological replicates per experimental condition were carried out. The data analysis was carried out by performing Quantile normalization (Smyth and Speed, 2003) without background correction to pre-process the image files from the Illumina software and an intensity-based, modified t-test (Sartor et al., 2006) to characterize the significance level of each feature using limma R packages from Bioconductor (Smyth, 2005). The data were also analyzed using Pathway Studio (Ariadne, Rockville, MD) to generate gene lists with designated *p*-values and false discovery rates (FDR) (Benjamini and Hochberg, 1995; Reiner et al., 2003). Statistically significant, differentially expressed genes were annotated with functional assignments to help determine category enrichment using the biological process (BP-FAT) branch of the Gene Ontology (GO) database (Ashburner et al., 2000) via the DAVID program of the National Institute of Allergy and Infectious Diseases (Huang et al., 2008; Huang et al., 2009). Venn diagrams of the statistical results were constructed using the GeneVenn software package (Pirooznia et al., 2007).

RESULTS

We followed the scheme shown in Fig. 1A to determine differentially expressed RNA levels in polysomes between vehicle-treated and TCDD-treated Hepa-1c1c7 cells. Cell lysates from cultures grown to 80% confluence were placed on 10–50% sucrose gradients, and the polysomes were resolved by ultracentrifugation. Fractions representing the heavier two-thirds of the polysomes (those polysomes under the bracket in Fig. 1A) were pooled, purified, and prepared for microarray analysis. In order to follow mature, fully processed mRNA from nucleus to polysomes and eliminate possible confounding results that may have

arisen from labeling unprocessed RNAs, we used oligo (dT) primers rather than random primers in the synthesis of the cDNA for labeling. Representative polysome profiles are shown in Fig. 1B and C. Intact polysomes were isolated because treatment with 100 μ M EDTA, which chelates the Mg^{++} necessary for polysome integrity (Pestka and Hintikka, 1971), disassociated the polysomes to form exclusively the 40S and 60S ribosomal subunits (Fig. 1D). The microarray results for all differentially expressed genes from whole cell, nucleus, cytoplasm, and polysomes are listed in Table S1; and all unique and shared genes among nucleus, cytoplasm, and polysomes are listed in Table S2.

Most polysome RNAs reach steady-state by 4 hr following TCDD exposure

There are several known mechanisms which regulate the translation of mRNA in polysomes, including cap-dependent translation, internal ribosome entry site translation under stress conditions, and the mostly inhibitory actions of miRNA (Djuranovic et al., 2011; Spriggs et al., 2008). Thus, the time lag between RNAs expressed in the nucleus and cytoplasm to those that have entered the polysomes can vary considerably for different mRNAs. In order to suitably compare RNA levels between the nucleus/cytoplasm/whole cell and the polysomes, we selected a single TCDD exposure time (2 hr) to act as the reference for the nucleus/cytoplasm/whole cell RNA level measurements and three post-TCDD exposure times (0, 4, and 18 hr) for the polysome RNA measurements following the 2-hr TCDD exposure (Fig. 1E). Time matched controls were used for all but the 6- and 20-hr time points for the TCDD-exposed polysome samples, which were compared to the 2-hr vehicle-exposed polysome samples. The distribution of microarrays and different comparisons of the microarray data sets are shown in Fig. 1F.

The polysome results were analyzed by two methods to compare polysome RNA levels. First, a time series/ANOVA test was used to show significant changes over the 2-, 6-, and 20-hr time points relative to the 2-hr time point. An FDR cutoff of ≤ 0.5 generated 1412 genes (Table S3). A heat map and dendrogram (Fig. 2) show that at the 2-hr time point there were relatively few statistically significant, differentially expressed genes and that most of the genes that were differentially expressed are observed by 6 hr (4-hr post-TCDD exposure). Most of the differentially expressed genes at 6 hr were little changed by 20 hr indicating that most TCDD-induced polysome RNA levels had attained steady-state by 6 hr. The second analysis method used multiple t-tests to compare the three polysome time points, in which a p -value cutoff of ≤ 0.05 generated a total of 4549 genes over the three time points (Table S4). Again, most of the polysome differentially expressed genes peaked by 4-hr post-TCDD exposure with a slight overall drop at 20 hr (Table 1). Fig. 3 and Table 1 summarize a comparison of the polysome RNA levels at the three time points. Most of the differentially expressed polysome RNA levels at 6 hr showed an increase in RNA levels (63.2%) as a result of TCDD exposure rather than decrease (20.8%) or remain unchanged (16.0%) relative to the 2- and 20-hr time points.

Cytoplasm RNA levels and whole cell RNA levels are poor predictors of polysome RNA levels. (Yasutome et al., 2005)

Because whole cell RNA levels poorly correlate with protein levels (Chen et al., 2002; Gygi et al., 1999; Haynes et al., 1998; Tew et al., 1996), we reasoned, in turn, that if polysome RNA is representative of a cell's protein synthesis, which can be determined by integration over a period of time in concert with protein degradation, then a view of the proteome can be revealed. Thus, polysome RNA profiles are a more accurate reflection of newly synthesized proteins and may uncover new key genes involved in the underlying disease biology. Furthermore, because there is little association between cytoplasm mRNA levels and whole cell RNA levels (Trask et al., 2009), we thought it possible that cytoplasm mRNA profiles may be representative of polysome mRNA profiles.

However, of the 4549 differentially expressed polysome RNAs (p -value ≤ 0.05), only 1511 (19.0%) and 1579 (19.2%) differentially expressed genes were shared between polysomes and cytoplasm and between polysomes and whole cell, respectively (Fig. 4). Of those shared genes with polysomes, a mere 930 (11.7%) and 928 (11.3%) genes were commonly differentially expressed in the cytoplasm and whole cell (Table 2). Thus, although there is little correlation between whole cell and cytoplasm RNA levels (Trask et al. 2009), there was also little correlation between polysome RNA and cytoplasm RNA levels and the differential RNA levels of the cytoplasm were no different in predicting polysome RNA levels than were whole cell RNA levels. In fact, the differential RNA levels of the whole cell and cytoplasmic compartments were less predictive than were the differential RNA levels of the nucleus (Fig. 4 and Table 2). These findings are similar to the results we found (unpub. results) comparing *SMAD4*^{-/-} and *SMAD4*⁺ human pancreatic cancer BxPC3 cell lines (Farivar et al., 2003; Yasutome et al., 2005).

RNA levels are highly differentially regulated at multiple cellular junctures

Recent work from our laboratory demonstrated that differentially expressed RNA levels between the whole cell, nucleus, and cytoplasm of a cell are dissimilar (Schwanekamp et al., 2006; Trask et al., 2009), and as described above, cytoplasm and polysome RNA levels are also quite dissimilar. We therefore wanted to further compare gene expression profiles of these cell compartments. Differential gene expression profiles from the nucleus, cytoplasm, and polysomes of TCDD-treated vs. vehicle-treated Hepa-1c1c7 cells were compared (Table S5). We found that the number of differentially expressed genes in the nucleus (5594) surpassed those of the cytoplasm and polysomes (3390 and 4549, respectively), and of the 3390 differentially expressed genes of the cytoplasm, 2193 of the genes were also differentially expressed in the nucleus (64.7%), whereas, only 1511 genes were shared with the differentially expressed genes of the polysomes (44.6%) (Fig. 5).

Table 3 summarizes how the gene expression profiles of the nucleus and cytoplasm compared to that of the polysomes. Contrary to the common expectation that a sizable proportion of the differential polysome RNA levels would have followed an unaltered continuum of differential expression from nucleus to cytoplasm to polysomes, only 617 of 4549 polysome RNAs (13.6%) were regulated similarly. The largest group of genes differentially expressed in the polysomes showed an increase or decrease in polysome RNA levels with no change in the RNA levels of the nucleus and cytoplasm (1671 genes, 36.7%). The surprise was the larger number and percentage of genes shared between the nucleus and polysomes (647 genes, 14.2%) relative to that of the polysomes and cytoplasm (343 genes, 7.5%). From these results, we conclude that (i) there is extensive and independent gene regulation in the nucleus, cytoplasm, and polysomes and (ii) a goodly proportion of differentially expressed RNAs are “tagged” in the nucleus to directly enter the polysomes once transported to the cytoplasm. Again, these findings are similar to the results we found (unpub. results) comparing *SMAD4*^{-/-} and *SMAD4*⁺ human pancreatic cancer BxPC3 cells.

Different genes, common pathways

The next question asked was whether these shared genes encoded proteins that comprised any common cell pathways. Pathway analyses of the significantly changed genes from the different cell compartments were performed based on the Gene Ontology (GO) databases (Ashburner et al., 2000) to determine which gene categories and cellular pathways were enriched. Although there were relatively few shared differentially expressed genes among polysomes, nucleus, and cytoplasm; there were a higher proportion of shared biological pathways (Fig. 6 and Table S6). For example, there were 2,393 shared differentially expressed genes between the nucleus and polysomes, which represented 42.8% and 52.6% of the total number of differentially expressed genes in the two cellular compartments,

respectively; however, there were 24 shared GO biological pathways ($FDR \leq 0.05$), which was 45.3% and 64.9% of the GO biological pathways in the nucleus and polysome compartments, respectively. Thus, although a large proportion of differentially expressed genes in the nucleus, cytoplasm, and polysomes are unshared, the encoded proteins nevertheless share a greater proportion of common biological pathways. The results suggest that while there is independent regulation of gene expression at the different cellular locations, the genes share functional roles, as if the regulation of RNA levels for the genes in a given biological pathway is distributed among the nucleus, cytoplasm, and polysomes.

New TCDD-regulated genes

Almost all published studies using high-throughput microarray approaches rely on RNA from whole cells in the belief that whole cell RNA is a valid measure of steady-state mRNA levels. In a study to determine whether whole cell RNA is indeed representative of steady-state mRNA levels, we showed that nuclear RNA greatly distorts mRNA profiles and that precise mRNA profiling is possible only by the preparation of unadulterated cytoplasm RNA (Trask et al., 2009). A comparison of relative RNA levels for the 50 genes with the greatest fold change in differential expression between polysomes and the conventionally used whole cells, as well as from nucleus and cytoplasm (Table 4) illustrates how disparate the results can be for different RNA sources from the same cells. Two points can be made. One, Table 5 lists the relatively few shared genes between polysomes and whole cell, nucleus, and cytoplasm, in which 48 of the top 50 listed genes in polysomes are unique to the polysomes (only polysome genes Cxcl1 and Akap12 were shared with whole cell and nucleus, respectively). Two, the differential changes are much less pronounced in the polysomes relative to whole cell, nucleus, and cytoplasm (Table S7 and Table 4). On the latter point, the opposite result was found in BxPC3 cells (unpub. results), in that the differential RNA levels in polysomes were considerably greater than that found in whole cell, nucleus, and cytoplasm. Thus, depending on the RNA source, whether cell type or cell compartment, very different results may be obtained.

A portion of the studies reported here was a replication of earlier work. In the earlier study, we attempted to determine whether whole cell RNA was an accurate representation of cytoplasm steady-state mRNA levels (Trask et al., 2009). Using the Agilent microarray platform (Agilent Technologies, Santa Clara, CA), we showed that whole cell RNA did not accurately measure steady-state mRNA levels because the nuclear RNA contribution strongly biased the results, and that the cytoplasm RNA fraction produced a more precise mRNA steady-state profile. For the current study, the Illumina bead array system (Illumina, San Diego, CA) was used with the same nucleus, cytoplasm, and whole cell RNA preparations (Fig. 7A and B). Although the numbers and proportions of differentially expressed genes for the two microarray platforms are markedly different for some of the cell fractions, e.g., the nucleus (probably due to the increased sensitivity of the Illumina platform), it is reassuring that the same conclusions can be drawn from both studies. That is, the RNA profiles for the whole cell and cytoplasm fractions are very different, and the nuclear RNA has a large impact on the whole cell RNA profile.

DISCUSSION

One goal of our work was to identify novel genes and pathways that may be involved in TCDD toxicity. Based on the premise that the proteome is a better measure of a cell's biological state than the transcriptome, we sought to determine the differential expression of a subset of cytoplasm mRNAs that are in the active process of being translated, i.e., the polysomes. In the attempt to identify more genes encoding products that dictate functional states, polysome profiling using microarrays was carried out to reveal new genes and pathways that may contribute to the ill effects following TCDD exposure. There were a total

of 403 genes uniquely differentially expressed in polysomes, i.e., the genes were not differentially expressed in whole cells, nucleus, or cytoplasm (Table S8). For example, the gene with the greatest RNA level fold change unique to polysomes was *Exosc2* (*Rrp4*), which encodes a component of the exosome involved in rRNA and cytoplasmic RNA processing (de la Cruz et al., 1998; van Dijk et al., 2007). Some of the most affected biological pathways involved associated with the differential polysome RNA levels involved RNA processing (Table S6), cellular events that the exosome plays major roles. Thus, a considerable number of differentially expressed genes with relative closer ties to the cell's functional state would have been missed following conventional approaches to microarrays. In the future, selected unique polysome genes will be examined to determine their role in TCDD toxicity.

Table S8 lists differentially expressed genes unique to the polysomes. Presented are just two examples of what would have been missed genes that may play important roles in TCDD toxicity and tumor promotion. For example, *Atf4* was expressed nearly 5-fold greater in TCDD-treated cells to that of vehicle-treated cells. ATF4 functions as a transcriptional activator or repressor. ATF4 is protective and involved in regulating the adaptation of cells to stress factors, e.g., ER and oxidative stress; and also plays roles in skeletal and eye development and haematopoiesis (Ameri and Harris, 2008). Another gene uniquely differentially expressed in polysomes was *Psmb5*, a proteasome subunit, was expressed nearly 4-fold greater in TCDD-treated cells to that of vehicle-treated cells. Mutations in the *Psmb5* gene are often the underlying cause of drug resistance in treatments for multiple myeloma (Oerlemans et al., 2008), and in the dysregulation of different stress responses (Ri et al., 2010).

Our studies also showed that there was independent regulation of gene expression at the level of the nucleus, cytoplasm, and polysomes (Fig. 5, Table 3). In regard to the latter, four classes of differentially regulated polysome mRNAs were apparent: (i) RNAs regulated similarly between nucleus and polysomes, (ii) mRNAs regulated independently at the polysome level, (iii) RNAs regulated similarly among nucleus, cytoplasm, and polysomes, and (iv) mRNAs regulated similarly between cytoplasm and polysome. The four polysome mRNA classes are illustrated by the four models presented in Fig. 8, in which a given mRNA species for each polysome mRNA class is depicted. For example, in each of the models in Fig. 8A–D, of the 2083 differentially expressed polysome mRNAs, only one of the 2083 mRNA species is shown. Thus, if the given mRNA was expressed four-fold greater in the TCDD-treated vs. vehicle-treated Hepa-1c1c7 cells, then with 400 mRNAs in the polysomes of the TCDD-treated cells there would be 100 mRNAs in the vehicle-treated polysomes for that given polysome mRNA transcript.

In Model 1 (Fig. 8A), which represents those RNA levels regulated similarly between nucleus and polysomes, the polysome mRNAs are somehow “tagged” in the nucleus for immediate polysome entry. The apparent nuclear tagging of mRNAs directing them to the polysomes could be based on the status of the 3' terminus poly (A) tract (Munroe and Jacobson, 1990); miRNA targeting (Kren et al., 2009); or more likely as a result from an exposure to TCDD, via specific sequence motifs, such as internal ribosome entry sites that are utilized in stress responses (Fitzgerald and Semler, 2009), possibilities we are currently investigating. This is the second largest class of polysome mRNAs (1321/4549 = 29.0%) and were differentially expressed in nucleus and polysomes but not cytoplasm. Model 2 (Fig. 6B) represents those mRNAs regulated independently at the polysome level. The largest class of polysome mRNAs (1717/4549 = 37.7%) were differentially expressed only in the polysomes but not nucleus and cytoplasm. Model 3 (Fig. 6C) represents those polysome mRNAs (1072/4549 = 23.6%) that were similarly differentially expressed in nucleus, cytoplasm and polysomes. This is typically what is thought to occur for most

mRNAs but is obviously not the case, at least in Hepa-1c1c7 cells. Model 4 (Fig. 6D) is surprisingly the smallest group of polysome mRNAs (439/4549 = 6.5%) and were mRNAs similarly differentially expressed in cytoplasm and polysomes but not in the nucleus.

Little is known how polysome mRNA levels are regulated. Cell location plays a large role in the regulation of gene expression (Corral-Debrinski, 2007). In a study of liver regeneration in rats, at least 85 miRNAs were associated with three different polysome types (free, cytoskeleton-bound, and membrane-bound), and differential protein expression of c-myc and p53 among the different polysome populations appeared to be due, at least in part, to regulation by miRNAs (Kren et al., 2009). Other studies using genome-wide approaches to examine mRNA and protein levels in parallel showed that miRNA can repress the expression of many proteins by directly repressing translation without affecting mRNA levels, although the same studies found that for the majority of mRNAs, the major effect of miRNAs on mRNA expression is by promoting mRNA turnover (Baek et al., 2008; Hendrickson et al., 2009; Selbach et al., 2008). Our tentative hypothesis is that regulation of the differential mRNA levels in the polysomes and cytoplasm rests heavily on the actions and distribution of miRNAs at the different cell locations, a hypothesis we are testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Differentially expressed whole cell and cytoplasm RNA levels are both poor predictors of polysome mRNA levels.
- mRNA levels are regulated independently in the nucleus, cytoplasm, and polysomes.
- Polysome mRNA is regulated via several mechanisms including a “tagging” of nuclear RNA for immediate polysome entry.
- Differentially expressed polysome mRNA generated new genes and biological pathways potentially related to TCDD toxicity.

Figure 1.

Experimental design (A) and representative polysome profiles at the 2-hr time point of vehicle (dimethyl sulfoxide)-treated (B), TCDD-treated (10 nM, 2 hr) (C), and EDTA-treated (100 μ M, 10 min) polysomes (D) from Hepa-1c1c7 cells. Timeline showing the duration of vehicle and TCDD exposures and harvest times of control and experimental Hepa-1c1c7 cells (E). Distribution of the microarrays and comparisons of the different microarray data sets (F). Four biological replicates per experimental condition were used for the microarray experiments. WC, whole cell; Cyto, cytoplasm; Nuc, nucleus; Poly, polysomes; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Cont., control.

Figure 2.

A heat map and dendrogram comparing the differentially expressed genes from polysomes collected at 2 hr, 6 hr, and 20 hr from Hepa-1c1c7 cells exposed to 10 nM TCDD for 2 hr. Red color indicates an increase, green color a decrease, and black no change in statistically significant, differentially expressed genes among the three groups (p -value ≤ 0.05).

Figure 3.

Venn diagram depicting the numbers of differentially expressed genes between, among, and exclusively of polysomes from vehicle-treated vs. TCDD-treated (10 nM, 2 hr) Hepa-1c1c7 cells collected at 2, 6, and 20 hr (p -value ≤ 0.05).

Figure 4.

Venn diagrams depicting the numbers of differentially expressed genes between, among, and exclusively of the cytoplasm, polysomes, and whole cells from TCDD-treated (10 nM, 2 hr) vs. vehicle-treated Hepa-1c1c7 cells.

Figure 5.

The numbers of differentially expressed genes (DEGs) between, among, and exclusively of the nucleus, cytoplasm, and polysomes from TCDD-treated (10 nM, 2 hr) vs. vehicle-treated Hepa-1c1c7 cells shown in a Venn diagram (A) and in the different cell compartments (B). The percentages in parentheses are the percent of shared DEGs of the total DEGs for the two corresponding RNA populations being compared.

Figure 6.

The numbers of differentially expressed genes (DEGs) and corresponding Gene Ontology biological pathways (BPs) in the different cell compartments between and exclusively of the nucleus, cytoplasm, and polysomes in TCDD-treated vs. vehicle-treated Hepa-1c1c7 cells. The percentages in parentheses are the percent of shared DEGs and shared BPs of the total DEGs and total BPs, respectively, for the two corresponding RNA populations being compared.

Figure 7. Venn diagrams depicting the numbers of differentially expressed genes between, among, and exclusively of the cytoplasm, polysomes, and whole cells from TCDD-treated vs. vehicle-treated Hepa-1c1c7 cells using the Illumina bead array platform (A) and the Agilent microarray platform (B).

Figure 8.

Models showing how differentially expressed polysome RNA populations may be distributed in the cell. The polysome population under consideration is identified by a circle in the corresponding Venn diagram. Only one of the 4549 polysome RNA species is depicted in each model to be representative of a given polysome RNA population. Model 1 (A) represents those RNAs regulated similarly between nucleus and polysomes but not cytoplasm (1321/4549=29.0%). Model 2 (B) represents those RNAs regulated independently at the polysome level and are differentially expressed only in the polysomes but not nucleus and cytoplasm (1072/4549=23.6%). Model 3 (C) is of polysome RNAs that were similarly differentially expressed in nucleus, cytoplasm and polysomes RNAs (439/4549=9.7%). Model 4 (D) is of polysome RNAs similarly differentially expressed in cytoplasm and polysomes but not in the nucleus (1717/4549=37.7%).

Table 1

Number of Genes in Mouse Hepa 1c1c7 Cells with Differential RNA Expression Levels in Polysomes at the 2- and 20-hr Time Points Relative to the 6-hr Time Point after a 2-hr Exposure to 10nM TCDD.

Polysome RNA levels at 2-hr and 20- hr TCDD exposure	Polysome RNA levels at 6hr TCDD exposure		
	Polysomes↑ (%)	Polysomes↓ (%)	Polysomes↔ (%)
2 hr↑, 20 hr↑	96 (3.3)	0 (0)	32 (4.4)
2 hr↑, 20 hr↓	0 (0)	9 (1.0)	3 (0.4)
2 hr↑, 20 hr↔	52 (1.8)	3 (3.2)	14 (1.9)
2 hr↓, 20 hr↑	27 (0.9)	1 (0.1)	140 (19.3)
2 h↓, 20 hr↓	5 (0.2)	71 (7.5)	32 (4.4)
2 h↓, 20 hr↔	39 (1.4)	45 (4.8)	114 (15.7)
2 h↔, 20 hr↑	1138 (39.6)	7 (0.7)	350 (48.2)
2 h↔, 20 hr↓	28 (1.0)	726 (76.7)	41 (5.6)
2 h↔, 20 hr↔	1491 (51.8)	85 (9.0)	NA
Total number of genes	2876 (63.2)	947 (20.8)	726 (16.0)

↑ = increase in relative RNA levels

↓ = decrease in relative RNA levels

↔ = no change in relative RNA levels

Table 2

Differential RNA Levels from the Whole Cell, Cytoplasm, and Nucleus Compartments Are All Poor Predictors of Differential Polysome RNA Levels.

Relative RNA Level Measurement	Cell Compartment			
	Cytoplasm	Whole Cell	Nucleus	Polysomes
Number of differentially expressed genes	3390	3694	5594	4549
Number of differentially expressed genes shared with polysomes (% of RNA levels shared between polysomes and cell compartment)	1511 (19.0)	1579 (19.2)	2393 (23.6)	NA
Number of shared genes with polysomes with same fold change direction (% of shared RNA levels with similar fold changes between polysomes and cell compartment)	930 (11.7)	928 (11.3)	1264 (12.5)	NA
Number of shared genes with polysomes with different fold change direction (% of shared RNA levels with opposite fold between polysomes and cell compartment)	581 (7.3)	651 (7.9)	1129 (11.1)	NA

NA = Not Applicable

Table 3

Number of Genes in the Nucleus and Cytoplasm with Similar and Dissimilar Differential RNA Expression Levels to that of Polysomes.

Nucleus and Cytoplasm Compartments	Polysomes ↑ (%)	Polysomes ↓ (%)	Polysomes ↔
Nuc↑, Cyto↑	528 (11.6)	77 (1.7)	496
Nuc↑, Cyto↓	39 (0.9)	4 (0.1)	37
Nuc↑, Cyto↔	486 (10.7)	127 (2.8)	929
Nuc↓, Cyto↑	44 (1.0)	3 (0.1)	57
Nuc↓, Cyto↓	287 (6.3)	89 (2.0)	531
Nuc↓, Cyto↔	589 (12.9)	119 (2.6)	1151
Nuc↔, Cyto↑	219 (4.8)	58 (1.3)	407
Nuc↔, Cyto↓	133 (2.9)	76 (1.7)	350
Nuc↔, Cyto↔	972 (21.4)	699 (15.4)	NA
Total	3297 (72.5)	1252 (27.5)	3958

↑ = increase in relative RNA levels

↓ = decrease in relative RNA levels

↔ = no change in relative RNA levels

NA = Not Applicable

Table 4

The 50 Most Differentially Expressed RNAs in Whole Cell, Nucleus, Cytoplasm, and Polysomes (ave.) of TCDD-Treated (10 nM, 2 hr) vs. Vehicle-Treated Mouse Hepa-1c1c7 Cells ($p \leq$ value 0.05).

Gene Symbol	Whole Cell	Gene Symbol	Nucleus	Gene Symbol	Cytoplasm	Gene Symbol	Polysomes
PIK3R3	34.94	TNFAIP2	34.29	TPCN2	34.56	ALDH3A1	12.38
GADD45B	30.02	CYP2S1	24.65	CYP2S1	33.88	HIST1H2AF	9.05
VASN	27.87	BST2	19.46	TNFAIP2	29.43	HIST1H2AN	8.45
NR1H4	25.28	OSBPL2	18.08	NHEDC2	28.85	SPRR2E	7.24
IRF1	24.77	SMPDL3B	17.26	GMPR	28.54	SPRR1B	6.65
TNFAIP2	24.13	CYP1B1	17.16	IFNAR2	25.43	PTPRE	6.63
SNAI1	21.24	LMAN2L	16.75	NUCB1	24.88	Dbi	6.53
CYP2S1	18.97	VASN	16.54	SLC22A5	20.78	Tpi1	6.44
PTPN14	18.27	GPC1	16.32	CLN6	20.75	SHFM1	6.41
MED24	17.74	IRF1	16.27	LOC100048721	19.82	MYBBP1A	6.25
MPP2	16.54	IGFBP6	14.94	TCIRG1	19.57	ALDOA	6.22
Fiz1	16.13	MAFF	14.93	FLRT3	18.63	NACA	6.11
ATOH8	15.95	USP18	14.04	TNXB	18.48	LOC100047935	6.05
CYP1B1	15.76	ARRDC3	14.02	SLC30A6	18.44	LMNB1	5.61
NR1D1	14.62	PTPN14	13.87	ARRDC3	18.13	Exosc2	5.60
ARHGDIB	14.60	FBLN1	13.74	LMAN2L	17.93	CHI3L1	5.57
LRIG1	14.59	H6PD	13.42	PTGES	17.20	Lgals1	5.56
BCL2L1	14.38	TLR7	13.38	SEC61A2	16.60	YWHAB	5.47
SLC39A4	14.15	GMPR	13.01	GFSN2	16.43	ARPC1B	5.46
ST5	13.54	LOC100044322	13.00	PIK3R3	16.31	LOC100048622	5.36
SNX30	13.46	TXNDC5	12.60	OLEML3	16.16	KRT7	5.29
TPST2	13.36	PRKCSH	12.49	PCDH1	16.02	LOC100044204	5.27
MYOZ2	13.23	ENTPD5	12.03	DERL2	15.78	SUMO2	5.10
0610037D15RIK	13.05	ABCB6	11.86	SLC1A4	15.51	Mdh2	5.10
LOC100047427	13.03	HIST2H4	11.80	SEC61A1	15.45	NT5C	5.09
SOX9	-9.97	ZMIZ1	-8.44	C430003P19RIK	-13.54	Myo1a	-2.99
LOC100044190	-10.02	ZEB1	-8.77	RRAGC	-13.62	Tnfrsf14	-3.00

Gene Symbol	Whole Cell	Gene Symbol	Nucleus	Gene Symbol	Cytoplasm	Gene Symbol	Polysomes
120003C05RIK	-10.03	D4BWG0951E	-8.89	HHEX	-13.77	LRRC42	-3.07
RNF6	-10.06	CMPK	-8.97	SNN	-13.86	Nnat	-3.07
ANKRD12	-10.53	ZFP219	-8.98	5730410E15RIK	-14.21	Junb	-3.10
FZD5	-10.63	PDGFA	-9.04	NFAT5	-14.28	Vwde	-3.12
ARPC5	-10.73	PRKRA	-9.05	UBTD2	-14.29	ATP1B1	-3.13
1200016B10RIK	-10.97	MTMR12	-9.23	FBXO9	-14.42	Serp1b13	-3.14
GADD45G	-11.25	GARNL4	-9.29	ARHGAP24	-14.61	Timd2	-3.14
ZFHX3	-11.50	FBXL14	-9.54	TOLLIP	-14.62	Tspan6	-3.16
CXCL1	-11.57	LGALS8	-9.60	LOC100041725	-14.81	RGS16	-3.18
ZFP318	-11.69	ZFP52	-9.82	HDAC2	-15.12	Clec1a	-3.20
PCYT1A	-11.80	SERPINB1A	-9.95	8430410K20RIK	-15.27	BC004728	-3.20
ASAH1	-12.27	EGR1	-10.71	SMAD1	-16.08	Gpr171	-3.24
9130422G05RIK	-12.56	BC057627	-10.84	FEM1B	-16.75	Upk1b	-3.26
PDCD10	-12.56	SOX9	-10.86	PAPSS1	-16.77	Prokr1	-3.29
TWISTNB	-12.87	ZFHX3	-10.90	D030056L22RIK	-16.78	Zfp36	-3.36
ANKRD1	-13.27	STAU2	-11.05	8430427H17RIK	-16.96	Tceal	-3.41
ZFP292	-13.72	FOXA2	-11.12	MCC	-17.63	Dynlrb2	-3.42
HHEX	-14.26	RREB1	-12.13	ARHGAP12	-17.71	AKAP12	-3.57
NUAK2	-14.78	CAMK2N1	-13.40	VPS24	-17.97	4930519N16RIK	-3.69
ZDHHC21	-17.93	AKAP12	-14.13	SCHIP1	-18.14	Lyz1	-3.71
D0H4S114	-18.23	ENC1	-15.60	DAP	-20.24	EDN1	-3.74
BMPR2	-22.26	HHEX	-15.83	SOC5	-25.22	Lrrc3b	-4.41
Lrig3	-23.99	5730410E15RIK	-18.35	2810003C17RIK	-26.56	CXCL1	-4.42

Shared Genes Among the 50 Most Differentially Expressed RNAs in Whole Cell, Nucleus, Cytoplasm, and Polysomes (ave.) of TCDD-Treated (10 nM, 2 hr) vs. Vehicle- Treated Mouse Hepa-1c1c7 Cells ($p \leq$ value 0.05).

Table 5

Cell Compartment Comparison					
Whole Cell to Nucleus	Whole Cell to Cytoplasm	Whole Cell to Polysomes	Nucleus to Cytoplasm	Nucleus to Polysomes	Cytoplasm to Polysomes
CYP1B1	CYP2S1	CXCL1	5730410E15RIK	AKAP12	None
CYP2S1	HHEX		ARRDC3		
HHEX	PIK3R3		CYP2S1		
IRF1	TNFAIP2		GMPR		
PTPN14			HHEX		
SOX9			LMAN2L		
TNFAIP2			TNFAIP2		
VASN					
ZFHX3					

Genes in bold are shared among multiple cell compartments.