Identification of Actively Translated mRNA Transcripts in a Rat Model of Early-Stage Colon Carcinogenesis

Laurie A. Davidson,1,2 Naisiyin Wang,3 Ivan Ivanov,4 Jennifer Goldsby,2 Joanne R. Lupton1,2 and Robert S. Chapkin1,2

Abstract With respect to functional mapping of gene expression signatures, the steady-state mRNA expression level does not always accurately reflect the status of critical signaling proteins. In these cases, control is exerted at the epigenetic level of recruitment of mRNAs to polysomes, the factories of ribosomes that mediate efficient translation of many cellular messages. However, to date, a genome-wide perspective of the effect of carcinogen and chemoprotective bioactive diets on actively translated (polysomal) mRNA populations has not been done. Therefore, we used an established colon cancer model, i.e., the azoxymethane (AOM)-treated rat, in combination with a chemoprotective diet extensively studied in our laboratory, i.e., n-3 polyunsaturated fatty acids, to characterize the molecular processes underlying the transformation of normal colonic epithelium. The number of genes affected by AOM treatment 10 weeks after carcinogen injection was significantly greater in the polysome RNA fraction compared with the total RNA fraction as determined using a high-density microarray platform. In particular, polysomal loading patterns of mRNAs associated with the Wnt-β-catenin, phospholipase A2-eicosanoid and the mitogen-activated protein kinase signaling axes were significantly upregulated at a very early period of tumor development in the colon. These data indicate that translational alterations are far more extensive relative to transcriptional alterations in mediating malignant transformation. In contrast, transcriptional alterations were found to be more extensive relative to translational alterations in mediating the effects of diet. Therefore, during early stage colonic neoplasia, diet and carcinogen seem to predominantly regulate gene expression at multiple levels via unique mechanisms.

Total steady-state mRNA levels have utility in the prediction of the expression levels of an array of proteins. However, in many cases, the steady-state mRNA expression level does not accurately reflect the expression rate of proteins. This is especially relevant in the case of Ras-transformed cells (1–4). Because prolonged activation of p21 Ras drives colonic tumor development (5), it is likely that translational regulation plays an important role in the control of colonic gene expression during malignant transformation.

It has been recently reported that several tumor suppressors and proto-oncogenes can influence the formation of the mature ribosome or regulate the activity of translation factors (6, 7). Traditionally, the differential mobilization of mRNAs onto polyribosomes has been used to identify genes whose transcripts are translationally controlled (8). Translational control has been verified by demonstrating the redistribution of mRNAs on polysome gradients at various stages of colon tumor cell line transformation (3). Recently, Provenzani and colleagues (9), using SW480 colon carcinoma cells and their relative, lymph node metastasis SW620 cells, showed that the colorectal cancer genome acquires changes impacting by far the translational more than the transcriptional control of gene expression. These changes would have been completely missed in a conventional transcriptosome analysis detecting cellular mRNAs irrespective of their degree of polysomal loading.

Although the analysis of cancer cells in tissue culture is informative, it is possible that this model system may not accurately represent the state of cancer cells in vivo. Therefore, we have applied a systems biology approach to the characterization of gene expression changes occurring during carcinogen-induced colon tumorigenesis. Specifically, to take full advantage of the polysomal isolation method, we have combined ribosome-free and polysome-bound mRNAs with an open, high throughput quantitative mRNA analysis detection platform that simultaneously measures and identifies existing mRNA species to provide a panoramic overview of gene expression at both the total...
transcript and posttranscriptional levels. To our knowledge, the effect of a colon-specific carcinogen on the pattern of existing mRNAs that are recruited to polysomes has never been attempted in vivo.

With respect to environmental risk factors, there is growing evidence that long-chain n-3 polyunsaturated fatty acids (PUFA), e.g., eicosapentaenoic acid and docosahexaenoic acid, suppress colon cancer risk in humans (10, 11). Recently, we have shown that chemoprotective n-3 PUFA reprogram genetic signatures during colon cancer initiation and progression and reduce colon tumor formation (12). Therefore, we further investigated whether n-3 PUFA modulate the levels of actively translated mRNAs in colonic mucosa following carcinogen exposure.

Materials and Methods

Animals

Sixty-four 8-wk-old male Sprague-Dawley rats (Harlan) were acclimated for 2 wk in a temperature and humidity controlled facility on a 12-h light/dark cycle. The animal use protocol was approved by the University Animal Care Committee of Texas A&M University. The study was a 2 × 2 × 2 factorial design with two types of dietary fat (n-6 PUFA or n-3 PUFA), two types of dietary fiber (cellulose or pectin), and two treatments [injection with the colon carcinogen, azoxymethane (AOM), or with saline]. Animals (n = 6-9 per group) were stratified by body weight after the acclimation period so that mean initial body weights were not different between groups. Body weight was monitored throughout the study.

Carcinogen treatment

After a 1-wk acclimation on standard pelleted diet, rats were assigned to one of four diet groups that differed in the type of fat and fiber as previously described (13). Diets contained (grams/100 grams diet) the following: dextrose, 51.00; casein, 22.40; D,L-methionine, 0.34; AIN-76 salt mix, 3.91; AIN-76 vitamin mix, 1.12; choline chloride, 0.13; and pectin or cellulose, 6.00. The total fat content of each diet was 15% by weight with the n-6 PUFA diet containing 15.0 grams of corn oil/100 grams diet, and the n-3 PUFA diet containing 11.5 grams of fish oil/100 grams diet plus 3.5 grams of corn oil/100 grams diet as previously described (12).

Polysome and total RNA isolation

Upon termination, each colon was cut open longitudinally, was flushed clean with PBS, 1-cm from the distal colon was collected for fixation and embedding for immunohistochemical assays, an adjacent centimeter was taken for total RNA isolation, and the remainder of the colon was used for polysome RNA isolation. For total RNA isolation, epithelial cells were scraped from the underlying muscle layer with a glass microscope slide, were homogenized on ice in lysis buffer (mirVana miRNA Isolation kit, Ambion), and were frozen at −80°C until RNA was isolated. Using the mirVana kit, total RNA enriched with
microRNA was isolated followed by DNase treatment. For polysome RNA isolation, colons were incubated for 5 min at room temperature in PBS containing 100 μg/mL cycloheximide, an inhibitor of translation that locks the mRNA/ribosome complex, facilitating its isolation and preventing RNA degradation. Following incubation, epithelial cells were scraped with a microscope slide on a chilled glass plate and immediately processed according to the procedure of Ju et al. (14). Briefly, epithelial cells were allowed to swell in LSB [20 mmol/L Tris (pH 7.5), 10 mmol/L NaCl, and 3 mmol/L MgCl₂] containing 1 mmol/L DTT and 50 units RNase inhibitor for 2 min followed by lysis in LSB containing 0.2 mol/L sucrose and 1.2% Triton X-100. After removal of nuclei by centrifugation, the supernatant was layered over a 15% to 50% linear sucrose gradient (in LSB) and centrifuged at 247,000 × g for 2 h at 4°C in a swinging bucket rotor. Gradients were fractionated using a pipette, an aliquot was taken for absorbance at 254 nm and 3-volume denaturation solution

**Fig. 2.** A, number of genes significantly affected by treatment with AOM compared with saline (control) in polysome and total RNA fractions. Polysomes were isolated from colonic mucosa by sucrose gradient centrifugation followed by RNA isolation from the polysome fraction. Total RNA was also isolated from the same animals (n = 6-9). Following microarray analysis, genes expressed differentially (P < 0.05) between the AOM- and saline-treated rats were compared in the polysome and total RNA fractions. B, effect of carcinogen on various biological process gene categories. Data are expressed as the percent of AOM affected genes (P < 0.05) in a biological process category for the polysome fraction divided by the percent of genes for the total RNA fraction.
Fig. 3. Regulated genes in the Wnt signaling pathway following carcinogen treatment at 10 wk. See legend of Fig. 2 for details. Gene expression in AOM- and saline-treated rats was compared using GenMAPP pathway analysis. Colors indicate significantly ($P < 0.05$) up- or down-regulated genes. Numbers show the fold changes. A, polysome RNA fraction; B, total RNA fraction.
Table 1. Carcinogen-induced changes in actively translated mRNA transcripts

A. Translationally upregulated genes significantly affected by carcinogen exposure*

<table>
<thead>
<tr>
<th>NCBI accession #</th>
<th>Polysome RNA AOM/saline</th>
<th>Total RNA AOM/saline</th>
<th>Translatability index</th>
<th>Polysome RNA P</th>
<th>Total RNA P</th>
<th>Gene description</th>
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<td>Cell division cycle 5-like (S pombe) (Cdc5)</td>
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</table>

(Continued on the following page)
Global Alterations in Colonic Polysomal mRNA

Table 1. Carcinogen-induced changes in actively translated mRNA transcripts (Cont’d)

A. Translationally upregulated genes significantly affected by carcinogen exposure*

<table>
<thead>
<tr>
<th>NCBI accession #</th>
<th>Polysome RNA AOM/saline</th>
<th>Total RNA AOM/saline</th>
<th>Translatability index</th>
<th>Polysome RNA P</th>
<th>Total RNA P</th>
<th>Gene description</th>
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<td>RNase H1 (Rnaseh1)</td>
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B. Translationally downregulated genes significantly affected by carcinogen exposure†

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Abbreviation: NCBI, National Center for Biotechnology Information.

* Abundance of mRNAs in polysome and total RNA fractions in AOM vs saline treatment was quantified by microarray analysis. Translatability index was calculated as the fold change in polysome RNA ratio (AOM/saline) over fold change in total RNA ratio. Genes shown have a translatability index of 1.3 or greater and a false discovery rate of <0.05.

† Abundance of mRNAs in polysome and total RNA fractions in AOM vs saline treatment were quantified by microarray analysis. Translatability index was calculated as the fold change in polysome RNA ratio (AOM/saline) over fold change in total RNA ratio. Genes shown have a translatability index of 0.7 or less and a false discovery rate of <0.05.

(Ambion Totally RNA kit) was immediately added to the remainder of each fraction. Samples were frozen at −80°C until RNA was isolated using the Totally RNA kit as per manufacturer’s instructions followed by DNase treatment. Both total RNA and polysome RNA were analyzed on an Agilent Bioanalyzer to assess RNA integrity. In select experiments, 10 mmol/L EDTA was added to the post–nuclear supernatant to examine the shift in elution due to disruption of polyribosome formation by EDTA.

**Microarray**

CodeLink rat whole genome bioarrays (Applied Microarray) were used to assess gene expression. For total RNA arrays, the Ambion MessageAmp II-Biotin Enhanced aRNA amplification kit was used to prepare labeled cRNA. For polysome arrays, the Ambion MessageAmp II aRNA amplification kit followed by the MessageAmp II-Biotin Enhanced aRNA amplification kit were used with two rounds of in vitro transcription to generate labeled cRNA. For both total RNA and polysome RNA, 10 μg of biotin-labeled cRNA was hybridized to the CodeLink genome array following manufacturer’s instructions (12). Slides were scanned with an Axon GenePix 4000B scanner.

**Immunohistochemistry**

At necropsy, a 1-cm section of distal colon was removed, fixed in 4% paraformaldehyde for 4 h, and paraffin embedded. Following antigen retrieval in 1× TE containing 0.05% Tween 20 brought to a boil and maintained at sub-boiling temperature for 10 min in a microwave, followed by cooling on the bench for 30 min, sections were treated with 3% hydrogen peroxide in methanol for 15 min to quench endogenous peroxidase activity. Sections were then processed using rabbit eukaryotic translation initiation factor 4E (eIF4E; Cell Signaling Technology) at 1:100 or rabbit phospho-eIF4E (Cell Signaling) at 1:7 to quantify in situ eIF4E and phosphorylated eIF4E staining as previously described (13). Images were quantified using NIS Elements.
software (Nikon) by collecting staining intensity at the base, middle, and top of at least 20 crypts per animal, eight animals per treatment group (AOM or saline).

Statistics and pathway analysis
Polysome and total RNA expression data were analyzed using GenMapp\textsuperscript{5} and GO\textsuperscript{6} and the approaches were described below using the R computation platform.\textsuperscript{7} Raw data were first normalized through a robust procedure that eliminates the intensity-based chip-by-chip bias and reduces the between-chip variation and the potential outlier effects. Robust local median estimation was applied to all observations from chips of the same treatment and potential outlying observations identified (15). A within-treatment quantile normalization was then applied to all nonoutlying observations (16). An alternative of replacing the within-treatment target distribution in quantile normalization by the cross-all-chip method was also investigated, testing outcomes remained unchanged between the two alternatives. Mixed-effect ANOVA was applied to normalized logarithm based 2 transformed data to obtain \( P \) values, \( q \) values, and positive false discovery rate (17). Significant genes for the AOM versus saline as well as diet comparisons were identified. Genes that met the criteria of \( q \) value of <0.05 were submitted to GenMapp and GO for pathway profiling.

Results

Whole genome polysomal profiles
Colonic mucosa polysome isolation was monitored by examining sucrose gradient fractions on an Agilent Bioanalyzer. As shown in Fig. 1, the top of the gradient contained small ribonuclear proteins (region A). Through the remainder of the gradient, the progression to heavier particles was seen first with the elution of the 40S (B) then 60S (C) ribosomal subunits, followed by 80S ribosomes (D) and finally polysomes (E), which eluted as a broad area due to mRNA transcripts loaded with varying numbers of ribosomes. The entire broad region in the second half of the gradient, excluding the final fraction containing RNA granules (18), was collected as the polysome fraction. Addition of EDTA to the sample before gradient fractionation resulted in the expected shift away from the bottom of the gradient due to the disruption of polysome and 80S ribosome formation (Supplementary Fig. S1).

Carcinogen-induced changes in gene expression differentially affect recruitment of mRNAs to polysomes
Polysome analysis and microarray technology was used to assess the impact of a colon-specific carcinogen (AOM) on global mRNA polysome recruitment. Total and polysomal mRNA from scraped colonic mucosa were isolated, processed, and hybridized using the Codelink (Applied Microarray) microarray platform. The number of genes affected by AOM treatment 10 wk after carcinogen treatment was significantly \( (P < 0.05) \) greater in the polysome RNA fraction compared with the total RNA fraction. As shown in Fig. 2A, 3,239 genes were significantly altered \( (P < 0.05) \) by AOM versus saline treatment in the polysome RNA fraction only. In contrast, the total RNA fraction resulted in only 69 unique genes affected by the AOM treatment. There were 233 genes significantly \( (P < 0.05) \) affected by the carcinogen treatment identified in both the polysome and total RNA fractions. These data indicate that translational alterations were more extensive (11.5-fold higher) relative to transcriptional alterations in mediating malignant transformation.

Analysis of translationally activated genes in colonic mucosa
Because an increase in polysomal RNA can reflect changes in mRNA abundance and/or translatability, the relative translatability of each differentially (AOM versus saline) expressed mRNA was calculated. Specifically, data were normalized to determine the change in abundance in polysomal RNA relative to the change in abundance in total RNA for each mRNA (polysome/total; ref. 19). Table 1A shows that 35 genes were translationally enhanced following carcinogen exposure, i.e., polysome-associated mRNAs were enriched in AOM versus saline-treated animals. We have defined translationally enhanced mRNAs as those that are significantly increased in AOM polysomes by 30% or greater (poly/total ratio, >1.3; \( P < 0.05 \)). These mRNAs are present on polysomes to a greater extent than can be explained by increases in total mRNA abundance alone. Notable genes maintained on polysomes included CD47, involved in regulating neutrophil transepithelial migration (20); phosphatidylinositol-glycan biosynthesis class S protein (PIGS), essential for the transfer of GPI to proteins (21); cyclin G1, associated with G2-M phase arrest in response to DNA damage (22); and SMAD4, a tumor suppressor gene implicated in intestinal cell cycle regulation (23). Our analysis also identified eight genes (Table 1B) that were reduced by 30% or greater (poly/total ratio, <0.7; \( P < 0.05 \)) in AOM versus saline-injected rats. Of interest is the observation that several of these genes are associated with immune response. Among these were C-C motif chemokine 17 (CCL17; ref. 24), Best5 (25), and CX5 chemokine ligand (CXCL9; ref. 26).

Microarray chips from a total of six rats per treatment were processed and data mining was done as described in the Materials and Methods. Functional analysis of differentially expressed polysome fraction genes was done by differential pathway analysis (GSEA; ref. 27). The systematic profiling approach shown in Fig. 2B revealed that carcinogen affected gene sets classified into 26 well-defined biological process categories. Interestingly, the top biological processes affected by AOM exposure were related to electron transport and coenzyme and prosthetic group metabolism.

The canonical Wnt cascade has emerged as a critical regulator of stem cells (28). There is also compelling evidence that oncogenic K-ras stimulates Wnt signaling in colon cancer and that Myc is required for the majority of Wnt target gene activation (29, 30). Therefore, we examined the effect of carcinogen (AOM) on gene activation of the Wnt pathway. Colonic mucosal polysome and total RNA were isolated from rats terminated at 10 weeks postinjection (saline or AOM). Only significantly \( (P < 0.05) \) altered genes are shown \( (n = 6-9 \) rats per group; Fig. 3). The data clearly show the impact of carcinogen on the mobilization of mRNAs onto polyribosomes for genes associated with the Wnt pathway (Fig. 3A). Interestingly, at this “early” promotional stage (tumors typically do not appear until 30 weeks after carcinogen injection), almost no changes were observed in the total mRNA transcriptome.

\textsuperscript{5} http://www.genmapp.org
\textsuperscript{6} http://www.geneontology.org
\textsuperscript{7} http://www.R-project.org
(Fig. 3B), indicating minimal perturbation at the transcriptional level. Furthermore, because cyclooxygenase-2 (COX-2)–derived prostaglandin E₂ can promote colonic tumor initiation and progression by enhancing cell proliferation, angiogenesis, cell migration, and invasion, while inhibiting apoptosis (31), we also investigated the impact of carcinogen exposure on the pattern of eicosanoid-related mRNAs that are recruited to polysomes. Overall, eight enzymes were significantly (P < 0.05)
Microarray analysis also revealed that a number of genes associated with Ras and mitogen-activated protein kinase (MAPK) cascades were significantly \((P < 0.05)\) upregulated \((\text{expressed as fold increase — AOM versus saline; e.g., Map2k1/MAP/ERK kinase 1-2.0})\) predominantly in polysomes \((\text{Supplementary Figs. S2 and S3})\). This is noteworthy, because blockade of the MAPK pathway suppresses growth of tumors in vivo \((5, 32)\).

**Diet preferentially influences global transcriptional profiles**

The number of genes affected by diet \((n-3 \text{ versus } n-6 \text{ PUFA})\) 10 weeks following placebo \((\text{saline})\) treatment was much greater in the total RNA fraction compared with the polysome RNA fraction. As shown in Supplementary Fig. S4, only 63 genes were significantly altered \((P < 0.05)\) by PUFA treatment in the polysome RNA fraction. In contrast, the total RNA fraction contained 328 unique genes affected by diet. In addition, there were two genes significantly \((P < 0.05)\) affected by diet treatment identified in both the polysome and total RNA fractions. Interestingly, a similar trend was observed in n-3– versus n-6 PUFA-fed animals injected with carcinogen \((\text{Supplementary Fig. S5})\). These data indicate that transcriptional alterations were more extensive relative to translational alterations in mediating the effects of diet.

In previous experiments, we have shown that diets containing both a fermentable fiber source, e.g., pectin and n-3 PUFA as the lipid component, e.g., fish oil, are maximally protective against experimentally induced colon cancer compared with cellulose and corn oil \((33, 34)\). Therefore, we examined the effect of dietary combination chemotherapy on the mucosal translatability index. As shown in Supplementary Table S1, 13 genes were modulated translationally following carcinogen exposure and corn oil + cellulose relative to fish oil + pectin feeding. Notably, the tumor suppressor retinoblastoma-associated protein \((\text{RB1})\) gene was translationally suppressed in corn oil + cellulose relative to fish oil + pectin–fed animals \((\text{translatability index, 0.57})\). A similar trend was also noted with respect to epoxide hydrolase 2, a xenobiotic metabolizing phase I enzyme \((\text{translatability index, 0.15})\).

**Effect of carcinogen on expression levels of colonocyte eIF4E and phospho-eIF4E**

Because elevated eIF4E function can induce malignancy by selectively enhancing translation of key malignancy-related transcripts \((35)\), eIF4E expression in tissue sections was quantified. Expression of eIF4E was higher \((P < 0.01)\) in the carcinogen \((\text{AOM})\)-treated compared with saline-control animals \((\text{Fig. 5})\). Tissue polysomal mRNA and protein eIF4E levels \((\text{AOM/saline ratio})\) were similar, 1.22 and 1.19, respectively. Interestingly, eIF4E was higher \((P < 0.01)\) at the base of the crypt \((\text{proliferative zone})\) and progressively decreased toward the luminal surface \((\text{Supplementary Fig. S6})\). We also evaluated whether the phosphorylation status of eIF4E, which typically correlates with the translation rate and growth status of the cell, was affected by carcinogen exposure. Similar to eIF4E, phospho-eIF4E levels were significantly \((P < 0.01)\) elevated in carcinogen treated animals \((\text{Fig. 5})\).

**Discussion**

In this study, we examined ribosome-free and polysome-bound mRNAs using a high throughput microarray platform to provide a panoramic overview of gene expression at both the total transcript and posttranscriptional levels during the
early stages of colon carcinogenesis. To our knowledge, this has never been attempted in the context of a highly relevant in vivo colon cancer model. The AOM-induced rat colon tumor model was selected because it provides a clear distinction between tumor initiation and promotion (36). Similar to humans, tumors from AOM-injected animals exhibit the presence of mutated/nuclear β-catenin (37) and the overexpression of COX-2 (31, 32), consistent with the dramatic upregulation of the Wnt signaling pathway (32). In addition, rat colonic tumors closely parallel human colonic neoplasia in pathologic features (38). Therefore, in the absence of comprehensive human data, the AOM chemical carcinogenesis model serves as a highly relevant means of assessing human colon cancer risk.

The examination of global alterations in gene expression in colonic mucosa at a very early stage (10 weeks post-AOM injection) of colorectal cancer development revealed that the colon acquires changes predominantly impacting the translational control of gene expression. This finding is supported by the elevation in eIF4E and phospho-eIF4E in colonic sections, in addition to a previous study in which colon carcinoma cell lines of cancer progression to metastasis was examined (9). Collectively, these data support a model whereby oncogenic signaling leads to cellular transformation by altering the transcriptome and producing a radical shift in the composition of mRNAs associated with actively translating polysomes (2). We argue, therefore, that studies using polysome purification and microarray analysis are needed to fully elucidate the mechanisms of translational deregulation associated with colon tumor development. Indeed, major advancements in genome-wide techniques for systematically monitoring protein translation in response to environmental factors have recently been made (39).

By determining mRNA relative translatability (polysome/total mRNA ratio), we catalogued which mRNAs are translated efficiently under malignant transformation conditions and are thus refractory to translational repression. Our data indicated a profound alteration in translational control following AOM treatment, suggesting that signaling pathways can cooperate to differentially translate existing pools of mRNA. The shift of existing mRNAs into and out of the polysome fraction seems to be more rapid and extensive than the change in the total mRNA pool. Because conditions such as cell stress and malignant transformation often result in a poor correlation between mRNA levels and protein production (9, 40, 41), it is evident that complementary experiments investigating the causal relationship between translational control and oncogenesis are needed. In addition, having determined the level of concordance between steady-state and translated mRNA populations at a very early stage of malignant transformation, future analyses will need to focus on how the expression of translated mRNA is altered in (late stage) tumor samples compared with uninvolved mucosa. This will involve running separate analyses on uninvolved mucosa and tumors, because there is growing evidence that epigenetic perturbations in cancer are not confined to tumor cells but also involve adjacent cells (7).

It is well known that the APC gene is mutated, truncated, or deleted in the majority of human colon tumors. This is significant because mutant forms of the APC protein cause activation of Wnt-β catenin-dependent signaling (42), COX-2 expression/prostaglandin/leukotriene synthesis (43), MAPK signaling (32), and crypt stem cell survival (44). Similarly, colon tumors induced in rodents by chemical alkylating carcinogens, e.g., AOM, exhibit deregulation of the Wnt and MAPK signaling pathways (45). However, from the perspective of the development of a lethal cancer, it is not clear precisely at which point in the evolution of a colonic tumor does Wnt, COX-2, or MAPK-dependent signaling become chronically perturbed. Our data show that polysomal loading patterns of mRNAs associated with the Wnt-β catenin, phospholipase A₂-eicosanoid, and the MAPK signaling axes are significantly upregulated at a very early period of tumor development in the colon. Interestingly, previous reports indicate that MAPKs are highly activated during late progression of colorectal carcinoma. Indeed, in colorectal cancer, at least 21 pathways have been identified to be enriched in mutations from different pathway databases (46). We have applied a systems biology approach involving simultaneous microarray analysis of rat colonic total (steady-state) mRNA and actively translated (polysomal) mRNA populations to show that two pathways (Wnt and eicosanoid), previously implicated in colorectal tumorigenesis, are affected at the level of translation at a very early stage of tumor development. It seems that transcriptional differences seen with AOM-induced signaling alterations are generated secondarily to translational effects on mRNAs. It is likely, therefore, that differential recruitment into polysomes of a given pool of mRNA may be sufficient to promote tumor formation. This interpretation is consistent with previous reports indicating that as cancer cells become more transformed, they acquire resistance to translation repression (47, 48). To the best of our knowledge, this is the first report demonstrating a profound alteration of translational control in early colorectal progression in vivo. These data complement previous studies using sequencing and transcriptome profiling approaches to examine early stage tumor evolution (49, 50).

Our data also offer insight into the effects of chemoprotective n-3 PUFA on colonic polysomal mRNA and total mRNA profiles. In striking contrast to the effects of AOM, transcriptional alterations were found to be more extensive relative to translational alterations in mediating the effects of diet. This implies that dietary intervention does not simply counteract the effects of carcinogen. Although very little is known regarding the role of diet on posttranscriptional control, the n-3 PUFA, eicosapentaenoic acid (20:5n-3) has been shown to inhibit protein synthesis at the level of protein initiation (40). Collectively, therefore, these findings are noteworthy because they provide further inroads into how diet regulates global gene expression during the early stages of colorectal tumorigenesis.

Disclosure of Potential Conflicts of Interest

Laurie A. Davidson, Naisyin Wang, Ivan Ivanov, Jennifer Goldsby, Joanne R. Lupton, and Robert S. Chapkin, no conflict of interest.

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References


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