Novel Detection and Differential Utilization of a c-myc Transcriptional Block in Colon Cancer Chemoprevention

Andrew J. Wilson, Anna Velcich, Diego Arango, Amy R. Kurland, Shailesh M. Shenoy, Rossanna C. Pezo, Jeffrey M. Levsky, Robert H. Singer, and Leonard H. Augenlicht

Department of Oncology, Albert Einstein Cancer Center, Montefiore Medical Center, Bronx, New York 10467 [A. J. W., A. V., D. A., L. H. A.], and Department of Anatomical and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461 [A. R. K., S. M. S., R. C. P., J. M. L., R. H. S.]

Abstract

Mutations in the adenomatous polyposis coli (APC) gene, which initiate almost all human colon cancers, directly target the proto-oncogene, c-myc, by elevating β-catenin/T-cell factor (TCF) signaling. We have shown that agents ascribed chemopreventive activity for colon cancer in fact also stimulate β-catenin/TCF activity in vitro. Their effects on c-myc transcription were assayed using a novel variant of fluorescence in situ hybridization that detects c-myc transcription sites in intact nuclei. Increased transcriptional initiation of c-myc induced by the short-chain fatty acid, butyrate, consistent with elevated β-catenin/TCF activity, was efficiently abrogated by a block to transcriptional elongation, resulting in decreased c-myc expression. 1α,25-Dihydroxyvitamin D3 also induced transcriptional elongation. In contrast, the nonsteroidal anti-inflammatory drug, sulindac, increased c-myc expression, an effect attributable at least in part to its failure to induce transcriptional blockage. We have described a novel approach for evaluating the effects of chemopreventive agents on the expression of a gene critical in colonic tumorigenesis.

Introduction

Elevated expression of the c-myc proto-oncogene product is characteristic of various human malignancies, including colon cancer (1). Almost all human colon cancers are initiated by mutations in the APC gene, which directly induce the c-myc gene and three separate regions of the c-myc mRNA encoded by exon 1 or exon 3 of the c-myc gene and three separate regions of human β-actin mRNA. The probes were synthesized with deoxynucleoside (amino-alloy 1T) at defined positions in the sequence, purified by reverse chromatography, and the activated fluorophores, Cy3, Cy5 (both from Amersham, Pisacatay, NJ), and FITC (Molecular Probes, Eugene, OR) were chemically coupled to these primary amines (17). Each probe was labeled with a total of five identical fluorophore molecules.

Materials and Methods

Oligonucleotide Probe Synthesis. DNA probes consisting of 50 nucleotides were designed complementary to two separate regions of c-myc mRNA encoded by exon 1 or exon 3 of the c-myc gene and three separate regions of human β-actin mRNA. The probes were synthesized with deoxynucleoside (amino-alloy 1T) at defined positions in the sequence, purified by reverse chromatography, and the activated fluorophores, Cy3, Cy5 (both from Amersham, Pisacatay, NJ), and FITC (Molecular Probes, Eugene, OR) were chemically coupled to these primary amines (17). Each probe was labeled with a total of five identical fluorophore molecules.

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2 To whom requests for reprints should be addressed, at Albert Einstein Cancer Center, Department of Oncology, Montefiore Medical Center, Hofheimer 509, 111 East 210th Street, Bronx, NY 10467. Phone: (718) 720-2993; Fax: (718) 882-4464; E-mail: awilson@montefiore.org

3 The abbreviations used are: TCF, T-cell factor; DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence in situ hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TSA, trichostatin A; APC, adenomatous polyposis coli.
controller (Vincent Associates, Rochester, NY) was used for the capturing of images in three dimensions. These images were acquired using #31000 (DAPI), #31001 (FITC), #41007a (Cy3), and #41008 (Cy5) fluorescence filter sets (Chroma Technology, Brattleboro, VT) and viewed and superimposed with IPLab Windows version 3 software (Scanalytics, Fairfax, VA). Images acquired by the FITC, Cy3, and Cy5 filters were assigned green, red, and blue colors, respectively, for visualization.

To be scored as a true transcription site, our experimental strategy required the simultaneous detection of at least two colors at a site, independent hybridization events highly unlikely to be attributable to chance. Only sites in nonoverlapping, apparently intact nuclei were counted. This counting was performed with the scorer blinded to the identity of the slide; at least 20 transcription sites were counted/slide. Transcription sites were scored as three-color (red/green/blue) or two-color (red/green) based on the intensity of signal in the Cy5 channel above background. The majority of sites were readily classified as having either high or negligible Cy5 intensity. A small percentage of sites with intermediate Cy5 intensity were detected but were not included in the analysis. The number of nuclei in each field examined was also counted (DAPI image), including those in fields where transcription sites were not detected, to determine the relative frequency of occurrence of these sites.

**Measurement of c-myc mRNA Levels.** The steady-state level of c-myc mRNA in SW837 cells was measured by real-time PCR analysis. Cells were grown and treated in a similar fashion to those used in the FISH experiments. RNA was prepared using an RNaseasy kit (Qiagen, Valencia, CA), and first-strand cDNA was synthesized from 3 μg of total RNA by the Superscript II RT method provided (Invitrogen). cDNA was amplified using the SYBR Green Core Reagents kit and 7900HT real-time PCR apparatus (Applied Biosystems, Foster City, CA). A region within exon 3 of human c-myc cDNA was amplified using primers designed by Primer Express software (ABI) and purchased from Sigma-Genosys (The Woodlands, TX). Primers specific for human GAPDH cDNA, the expression of which as an internal control, were also used. Primer sequences were as follows: human c-myc forward, 5'-CGTCTCCACATCAGACGACAA; human c-myc reverse, 5'-CTCTTG-GAGCAGGATGCTCTT; human GAPDH forward, 5'-TCCGAGGT-CAACGAGTTGG; and human GAPDH reverse, 5'-CAAAATATC-CATTITACCAGGTAAAA.

A dissociation reaction was added at the conclusion of the amplification to ensure that a single product was obtained. Expression of c-myc was assayed in triplicate and was standardized using GAPDH as a reference. Relative levels were quantified by calculating 2^ΔΔCT, where ΔΔCT is the difference in CT (cycle number at which the amount of amplified target reaches a fixed threshold) between target and reference.

**Statistical Analysis.** Statistical significance was assessed by Student’s t test (Minitab release 8 for the Macintosh; Minitab Inc., State College, PA). P ≤ 0.05 compared with untreated cells was considered statistically significant.

**Results and Discussion.**

The experimental strategy to demonstrate transcriptional blockage used a novel method of imaging of transcription sites (11–13), as illustrated in Fig. 1a. Oligonucleotide probes recognizing exon 1 of c-myc RNA interrogated initiation of c-myc transcription, because these probes recognize regions of the transcripts encoded by DNA sequences upstream of a site at the exon 1/intron 1 border that can function in transcriptional attenuation (8, 10). In addition, the unsuccessful completion of c-myc transcription was evaluated using probes that recognize exon 3 of c-myc mRNA, because these probes only detect transcripts that undergo full elongation. The exon 1 probes were labeled with FITC and Cy3, and the exon 3 probes were labeled with Cy5. Therefore, when initiated and elongated transcripts were detected simultaneously, full-length and truncated c-myc RNA transcripts were detected as three-color (red/green/blue) and two-color (red/green) transcription sites, respectively. A magnified example of each type of site obtained is shown in Fig. 1a.

Fig. 1b shows lower magnification pictures of fields of nuclei in which transcription sites (arrows) are detected after treatment with butyrate or sulindac. The red arrows indicate that two two-color sites were detected in the field of butyrate-treated cells shown, whereas the remaining four sites detected in these fields were three-color (white arrows). There are three important points regarding these data:

(a) The nuclei were visualized by staining with DAPI and demonstrated that each transcription site, as expected, had a nuclear localization.

(b) At the magnification shown in Fig. 1b, it is difficult to resolve whether the transcription site is two-color or three-color. Therefore, this evaluation of the computer image was always made at a higher magnification (∼5-fold higher).

(c) Finally, because some individual genes are activated transiently (13), the distribution of detectable transcription sites at a point in time is heterogeneous in the culture. It was, therefore, essential, to score the number and type (color) of transcription sites to achieve a statistically valid representation of the results. This was done blinded from the treatment of the cells.

We have shown that butyrate increases β-catenin/TCF activity in colon cancer cells cultured in vitro (7), and c-myc is a direct target of this signaling pathway (3). Therefore, we first determined the effect of 5 mM butyrate on the initiation of c-myc transcription, using SW837 colon cancer cells. When initiated transcripts were detected as outlined in Fig. 1a, there was a significant increase in the number of nuclei with detectable c-myc transcription sites within 12 h of stimulation (Fig. 2a). This time course is consistent with our previously reported kinetics of butyrate-mediated induction of β-catenin/TCF (7). By 16–24 h, a 2.5-fold increase was observed.

Simultaneous interrogation of initiated and elongated transcripts revealed that the percentage of c-myc transcription sites in untreated subconfluent cells scored as three-color sites was ∼70% (Fig. 2b). This observation demonstrated that full read-through of the gene was the predominant event under basal conditions. However, butyrate induced an ∼2-fold reduction in the number of elongated transcripts detected at c-myc transcription sites, with significant effects observed between 6 and 12 h after its addition (Fig. 2b).

To confirm these results, we used a related, but different, approach. Three spectrally distinct probes recognizing either the 5’ or 3’ end of the transcript were used to detect initiated and elongated transcription, respectively, in parallel experiments. The results of these experiments are shown in Fig. 2c. Interrogation of initiated and elongated transcripts individually confirmed, first, a butyrate-induced initiation of c-myc transcription, and, second, that it significantly decreased the number of full-length transcripts detected compared with untreated cells. Therefore, the stimulatory effect of butyrate on c-myc transcriptional initiation was abrogated by recruitment of a block to transcriptional elongation.

We extended these experiments to the nonsteroidal anti-inflammatory drug, sulindac, because this drug has significant chemopreventive activity for colon cancer (6). In addition, we have shown that, similar to butyrate, it induces G1/G0 arrest and apoptosis and increases β-catenin/TCF activity in colon cancer cells in vitro (7). As expected, therefore, 1.6 mM sulindac significantly increased the number of full-length transcripts detected compared with untreated cells. Therefore, the stimulatory effect of butyrate on c-myc transcriptional initiation was abrogated by recruitment of a block to transcriptional elongation.

Alteration of steady-state c-myc mRNA levels by butyrate and sulindac were consistent with their relative effects on c-myc transcription sites. Butyrate, as has been demonstrated (8, 9), decreased c-myc expression (Fig. 4a), whereas sulindac increased c-myc RNA levels, again consistent with a previous observation (18). Therefore, the recruitment of the block to transcriptional elongation by butyrate efficiently abrogated the stimulation of c-myc initiation by increased β-catenin/TCF activity, whereas the failure of sulindac to recruit this
block resulted in elevated c-myc levels. The relative effects of butyrate and sulindac on c-myc expression in SW837 cells are consistent with our previous observation in another colon cancer cell line, SW620 (19), although it is unknown whether their effects are mediated by the same mechanism in these cells.

It was striking that a physiological regulator of colonic epithelial maturation, butyrate, induced a block to transcriptional elongation in c-myc, whereas a pharmacological regulator, sulindac, did not. We therefore examined the effect of 1α,25-dihydroxyvitamin D₃, another physiologically relevant inducer of G₀-G₁ arrest and apoptosis in colon cancer cells in vitro (14, 15), on c-myc transcription. We identified two mechanisms by which 1α,25-dihydroxyvitamin D₃ reduced c-myc expression (Fig. 4b). Consistent with its ability to reduce β-catenin/TCF activity in colon cancer cells (15), 1α,25-dihydroxyvitamin D₃ significantly reduced the number of c-myc transcription sites detected at 8 h by ~50%, and this reduction persisted to 24 h (Fig. 3a). This sustained inhibition of c-myc transcriptional initiation was a likely explanation for the persistent decrease in c-myc expression observed, although the transcriptional block to the gene did not persist past 16 h (Fig. 3b). Our demonstration of a 1α,25-dihydroxyvitamin D₃-induced block to c-myc transcriptional elongation is a novel finding in colon cancer cells.

To gain insight into the mechanisms underlying the transcriptional block, we investigated the effect of TSA, which, similar to butyrate, is an inhibitor of histone deacetylase (20). TSA induces similar effects to butyrate in stimulating β-catenin/TCF activity (7). This was confirmed in the present study, because 1 μM TSA also induced a significant increase in the number of c-myc transcription sites detected (Fig. 3a). Furthermore, the number of c-myc transcripts undergoing full read-through was significantly reduced by TSA (Fig. 3b), suggesting that the butyrate-induced transcriptional block in c-myc was mediated, at least in part, through its ability to alter chromatin structure through histone hyperacetylation. However, this effect of TSA was observed at an earlier time point than that of butyrate and was not as sustained. A similar pattern of effect was also observed in the relative effects of butyrate and TSA on steady-state c-myc RNA levels (Fig. 4). These observations are consistent with our previous finding that TSA induces more rapid histone acetylation than butyrate, but that the resultant hyperacetylation is transient (19).

Parallel experiments using probes that recognized three regions within human β-actin mRNA were also performed. Neither butyrate, TSA, 1α,25-dihydroxyvitamin D₃ or sulindac induced a significant change in the number of β-actin transcription sites detected by probes separately labeled with FITC, Cy3, and Cy5 (data not shown), indi-
cating that the results obtained with the c-myc probes was not a consequence of nonspecific effects on transcription by these factors. Through high resolution profiling of the transcriptional activity of c-myc, we have identified a striking difference in regulation of its expression between physiological regulators of colonic epithelial maturation that are chemopreventive for colon cancer and/or exert antitumorigenic effects in colon cancer cells cultured in vitro and a pharmacological agent with similar actions. Sulindac failed to induce a block to transcriptional elongation after stimulation of transcriptional initiation, leading to elevated c-myc mRNA levels. Because high levels of c-myc induces apoptosis in cells that are blocked in the cell cycle (21, 22), inappropriately high expression may underlie, at least in part, the long-term toxic effects of sulindac therapy observed in colon cancer patients, such as mucosal ulceration (6).

It has been demonstrated recently that even brief inactivation of c-myc leads to a sustained regression of a neoplastic phenotype in a mouse model of osteogenic sarcoma (16). This emphasizes the potential importance of the mechanism described here, through which butyrate and 1α,25-dihydroxyvitamin D₃ may act as chemopreventive agents for colon cancer. Furthermore, the technology described here, which permits highly sensitive detection of transcriptional events that result in down-regulated expression, is more generally a novel and potentially important approach for evaluating the effects of chemopreventive and chemotherapeutic treatments on the expression of c-myc and other genes critical in tumorigenesis. The use of spectrally distinct probes, especially when used in different combinations, can permit both the evaluation of multiple genes at a single-cell level (13) and multiple parameters of the transcriptional regulation of these genes, such as initiation and elongation as demonstrated here. Rapid, high-throughput transcriptional monitoring of drug effects could, when correlated with specific outcomes, become a valuable prognostic indicator for patient material.
References


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