

Novel Detection and Differential Utilization of a *c-myc* Transcriptional Block in Colon Cancer Chemoprevention¹

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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. $1\alpha,25$ -Dihydroxyvitamin D₃ also induced transcriptional blockage. 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 through elevated β -catenin/TCF³ signaling (2, 3). We have shown that the short-chain fatty acid, butyrate, and the nonsteroidal anti-inflammatory drug, sulindac, both of which have been ascribed chemopreventive activity in the colon (4–6), stimulate β -catenin/TCF activity in colon cancer cells (7). However, butyrate clearly reduces steady-state *c-myc* RNA levels in these cells (8, 9).

We hypothesized that resolution of this issue may be linked to an important mechanism of regulation of *c-myc* expression identified in a variety of cell types, *i.e.*, changes in the ability of RNA polymerase II to progress through specific DNA sites within the first exon/intron border of *c-myc*, at which transcriptional elongation can be blocked (8, 10). To address how elevated β -catenin/TCF signaling could be linked to reduced *c-myc* expression, we used a novel method of FISH to visualize *c-myc* nuclear transcription sites and interrogate transcriptional initiation and elongation (11–13). We show that butyrate-mediated stimulation of *c-myc* transcriptional initiation was efficiently abrogated by a block to transcriptional elongation. This block was also

induced by another physiological inducer of colon cell maturation, $1\alpha,25$ -dihydroxyvitamin D₃ (14, 15), but not by sulindac. We have identified differential regulation of expression of a key gene controlling colonic tumorigenesis by chemopreventive agents with physiological and pharmacological relevance to the colonic epithelium. These results concerning the effects of chemopreventive agents on *c-myc* transcription are particularly significant in light of the recent report that even brief inactivation of *c-myc* is sufficient to induce a sustained loss of the transformed phenotype in murine osteogenic sarcoma cells that overexpress the gene (16).

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 deoxythymidine (amino-allyl dT) at defined positions in the sequence, purified by reverse chromatography, and the activated fluorophores, Cy3, Cy5 (both from Amersham, Piscataway, 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.⁴ Probes were as follows: human *c-myc* exon 1 HCM5, 5'-TTTTATACT-CAGCGGATCCCTCCCTCCGTTCTTTTTTCCCGCCAAGCCT; human *c-myc* exon 1 HCM6, 5'-AAAAAATCCAGCGTCTAAGCAGCTG-CAAGGAGAGCCCTTCAGAGAAGCG; human *c-myc* exon 3 HCM3, 5'-TGACCTTTTGCCAGGAGCCTGCCTCTTTTGACAGAAACAACATCGATTT; human *c-myc* exon 3 HCM4, 5'-CTGGTGCATTTTCGGTT-GTTGCTGATCTGTCTCAGGACTCTGACACTGTC; human β -actin HBA1, 5'-GTGAACCTTTGGGGGATGCTCGCTCCAACCGACTGCTGT-CACCTTCACCGT; human β -actin HBA2, 5'-TCCTTAGAGAGAAGT-GGGGTGGCTTTTAGGATGGCAAGGACTTCTGTGA; human β -actin HBA3, 5'-CTTTTATTCAACTGGTCTCAAGTCAGTGACAGGTAAG-CCCTGGCTGCCT.

In Situ Hybridization. SW837 colon carcinoma cells (CCL-235; American Type Culture Collection, Manassas, VA) were maintained by serial passage at 37°C, 5% CO₂ in RPMI 1640 supplemented with 10% FBS, 1× antibiotic/antimycotic (100 units/ml streptomycin sulfate, 100 units/ml penicillin G sulfate, and 0.25 μ g/ml amphotericin B), 100 μ M nonessential amino acids, and 10 mM HEPES buffer solution (all from Invitrogen, Carlsbad, CA). The cells were seeded onto glass coverslips (Fisher, Pittsburgh, PA) coated with 0.5% gelatin (Sigma Chemical Co., St. Louis, MO). After the cultures reached ~60% confluency, they were treated with 5 mM butyrate, 10⁻⁷ M $1\alpha,25$ -dihydroxyvitamin D₃, 1.6 mM sulindac, or 1 μ M TSA (all from Sigma) for a time period of 0, 2, 4, 6, 8, 12, 16, or 24 h.⁵

Image Acquisition and Analysis. Images were captured with a Photometrics CoolSNAP HQ digital CCD camera (Roper Scientific, Tucson, AZ) mounted on an AX-70 Provis microscope using a PlanApo 60X, 1.4 NA objective, and 100W mercury lamp for epi-illumination (Olympus, Melville, NY). A Uniblitz model VS35S2ZM0 shutter with model VMM-D1 electronic

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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.

⁴ A detailed protocol of the method is available at <http://protocols.singerlab.org/>.

⁵ The description of the protocol used for hybridization, preparation, and storage of slides is available at <http://protocols.singerlab.org/>.

controller (Vincent Associates, Rochester, NY) was used for the capturing of images in three dimensions. These images were acquired using #31000 (DAPI), #41001 (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 RNeasy kit (Qiagen, Valencia, CA), and first-strand cDNA was synthesized from 3 μ g 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 acted as an internal control, were also used. Primer sequences were as follows: human *c-myc* forward, 5'-CGTCTCCACACATCAGAGCACAA; human *c-myc* reverse, 5'-TCTTG-GCAGCAGGATAGTCCTT; human *GAPDH* forward, 5'-TCGGAGT-CAACGGATTTGG; and human *GAPDH* reverse, 5'-CAACAATATC-CACTTTACCAGAGTTAAAA.

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 $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ is the difference in C_T (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). $P \leq 0.05$ 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. 1*a*. 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. 1*a*.

Fig. 1*b* 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. 1*b*, 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. 1*a*, there was a significant increase in the number of nuclei with detectable *c-myc* transcription sites within 12 h of stimulation (Fig. 2*a*). 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 $\sim 70\%$ (Fig. 2*b*). 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. 2*b*).

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. 2*c*. 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 G_0 - G_1 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 nuclei with active *c-myc* transcription sites (Fig. 3*a*). However, in contrast to butyrate, there was no decrease in the number of sites that completed elongation of *c-myc* RNA throughout the 24-h incubation (Fig. 3*b*).

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. 4*a*), 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

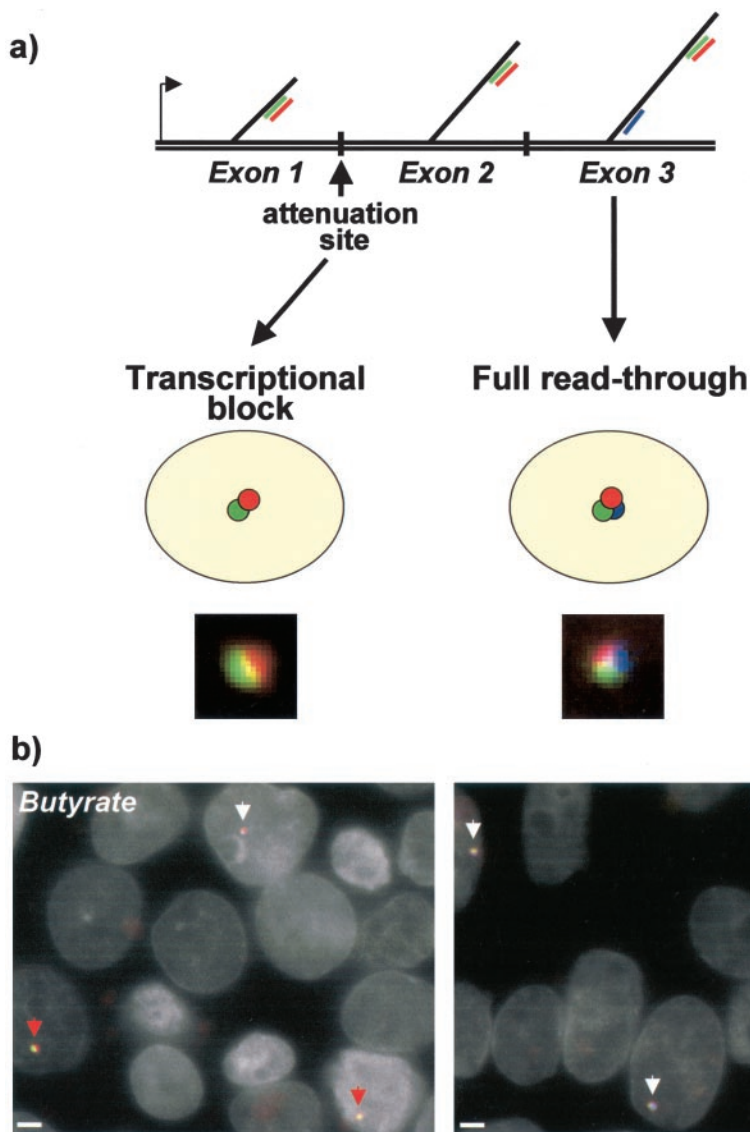


Fig. 1. *a*, schematic illustration of the experimental FISH strategy used to assay transcriptional initiation and to demonstrate transcriptional blockage in *c-myc*. The structure of the *c-myc* gene is shown, with introns indicated by a vertical bar; a site that can function in transcriptional attenuation is located as indicated. *c-myc* RNA was interrogated by probes labeled with FITC (green) and Cy3 (red) that recognized exon 1 and probes labeled with Cy5 (blue) that recognized exon 3. Magnified examples of a *c-myc* transcription site with truncated or full-length transcripts are shown. *b*, low-power photomicrographs of transcription sites detected in fields of butyrate and sulindac-treated cells in relation to DAPI-stained nuclei. The red arrows indicate two-color sites, and the white arrows indicate three-color sites. Scale bar, 3 μm .

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 $1\alpha,25$ -dihydroxyvitamin D_3 , another physiologically relevant inducer of G_0 - G_1 arrest and apoptosis in colon cancer cells *in vitro* (14, 15), on *c-myc* transcription. We identified two mechanisms by which 10^{-7} M $1\alpha,25$ -dihydroxyvitamin D_3 reduced *c-myc* expression (Fig. 4*b*). Consistent with its ability to reduce β -catenin/TCF activity in colon cancer cells (15), $1\alpha,25$ -dihydroxyvitamin D_3 significantly reduced the number of *c-myc* transcription sites detected at 8 h by $\sim 50\%$, and this reduction persisted to 24 h (Fig. 3*a*). 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. 3*b*). Our demonstration of a $1\alpha,25$ -dihydroxyvitamin D_3 -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 $1 \mu\text{M}$ TSA also induced a significant increase in the number of *c-myc* transcription sites detected (Fig. 3*a*). Furthermore, the number of *c-myc* transcripts undergoing full read-through was significantly reduced by TSA (Fig. 3*b*), 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, $1\alpha,25$ -dihydroxyvitamin D_3 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-

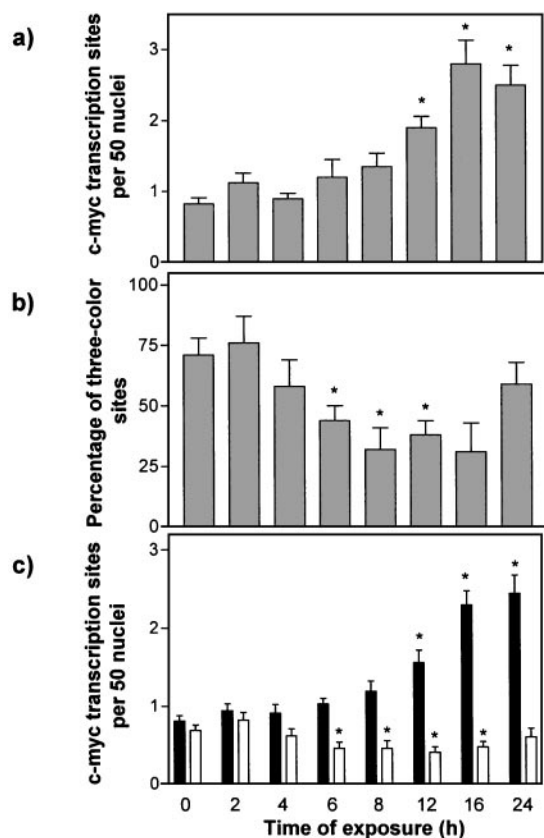


Fig. 2. Butyrate stimulated initiation of *c-myc* transcription but induced a block to transcriptional elongation in *c-myc* in SW837 cells. The number of nuclear *c-myc* transcription sites (a) and the percentage of three-color transcription sites (b) were detected in cells treated with butyrate for the indicated times after simultaneous interrogation of initiated and elongated *c-myc* transcripts. c, the number of *c-myc* transcription sites detected in butyrate-treated cells when initiated (■) and elongated (□) transcripts were interrogated individually using, respectively, exon 1 or exon 3 probes separately labeled with FITC, and Cy3 and Cy5. Values are means; bars, SE. *, $P < 0.05$, Student's *t* test.

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 anti-tumorigenic 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 $1\alpha,25$ -dihydroxyvitamin D_3 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.

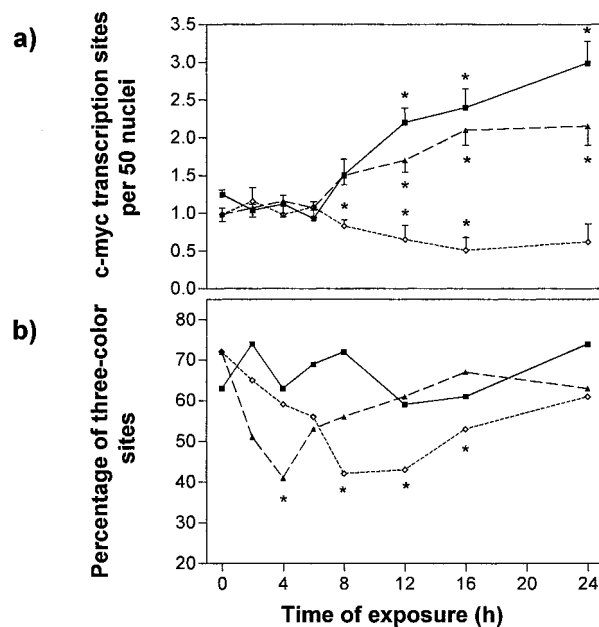


Fig. 3. Effects of sulindac (■), $1\alpha,25$ -dihydroxyvitamin D_3 (◇), and TSA (▲) on *c-myc* transcription in SW837 cells. a, the number of nuclear *c-myc* transcription sites detected in cells treated as indicated after simultaneous interrogation of initiated and elongated transcripts. Values are means; bars, SE. b, the percentage of nuclear three-color transcription sites detected after simultaneous interrogation of initiated and elongated transcripts. Values show the means (bars were omitted for clarity). *, $P < 0.05$, Student's *t* test.

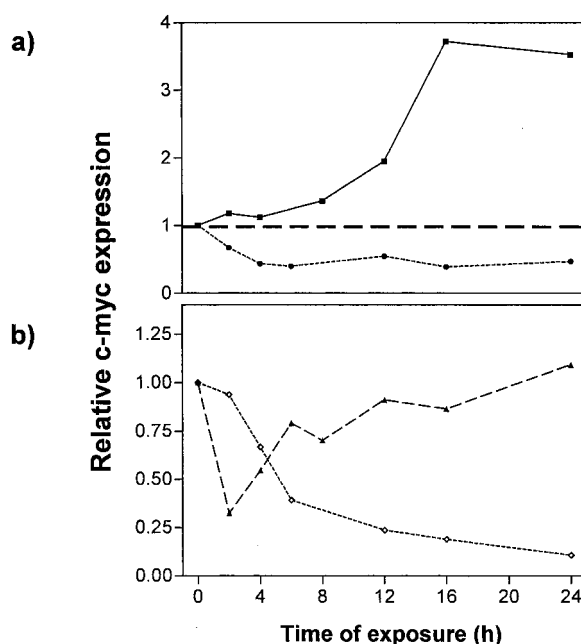


Fig. 4. Modulation of steady-state *c-myc* RNA levels in SW837 cells. Real-time PCR analysis of the effects of 5 mM butyrate (●) and 1.6 mM sulindac (■), and 10^{-7} M $1\alpha,25$ -dihydroxyvitamin D_3 (◇) and 1 μ M TSA (▲); b) on *c-myc* expression is shown. Values show the mean of triplicate measurements and are expressed relative to expression in untreated cells after normalization to GAPDH steady-state mRNA reference levels.

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