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Influence of Protein Tyrosine Phosphorylation on the Expression of the c-*myc* Oncogene in Cancer of the Large Bowel

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Abstract We tested the potential impact of tyrosine phosphorylation on the expression of the c-*myc* gene in two colon cancer cell lines, HCT8 and SW837. We found that the protein tyrosine kinase inhibitor genistein causes a decrease in the abundance of c-*myc* RNA and an inhibition of proliferation with a similar dose response. Geldanamycin, a mechanistically different tyrosine kinase inhibitor, also causes a decrease in both the expression of c-*myc* RNA and proliferation. Genistein has also been found to inhibit topoisomerase II, but the topoisomerase II inhibitor novobiocin did not lower the expression of c-*myc*. The most likely interpretation is that inhibition of protein tyrosine kinase activity caused a decrease in c-*myc* expression in these cells. The impact of tyrosine phosphorylation on the expression of the c-*myc* gene is further supported by the finding that inhibition of phosphotyrosine phosphatase using orthovanadate causes an increase in the level of c-*myc* RNA. The effect of genistein on HCT8 cells is not dependent on the synthesis of new protein and does not involve an alteration in the stability of the message. Analysis of transcription in the c-*myc* gene reveals a more complicated picture with a decrease in initiation and an increase in elongation but no net change in transcription. We speculate that the genistein induced reduction in *myc* expression is the result of a posttranscriptional intranuclear event(s).

Key words: colorectal cancer, tyrosine phosphate, tyrosine kinase, genistein, geldanamycin, RNA stability, transcription

The proto-oncogene c-*myc* encodes a nuclear protein which binds to DNA with sequence specificity and appears to function as a transcription factor. Several lines of experimentation have shown a relationship between deregulation of c-*myc* expression and neoplastic transformation [for recent reviews of *myc* see DePinho et al., 1991; Marcu et al., 1992; Rothberg and Heruth, in press; Spencer and Groudine, 1991]. We and others have demonstrated that malignancies of the large bowel and cell lines derived from these malignancies frequently contain elevated expression of c-*myc* RNA and protein, compared with nonmalignant mucosa, in the absence of amplification or rearrangement of the gene [Dolcetti et al., 1988; Erisman et al., 1985, 1988; Finley et al., 1989; Guillem et al., 1990; Stewart et al., 1986; Tsuboi et al., 1987; Untawale and Blick, 1988]. In addition, we have shown that the exon 1/intron 1 boundary region, which is found mutated in most endemic Burkitt's lymphomas and some diffuse large cell lymphomas [Bradley et al., 1993; Morse et al., 1989; Pelicci et al., 1986; Taub et al., 1984], has a germline sequence in a panel of cell lines derived from colorectal carcinomas [Heruth et al., 1993]. The lack of a *cis* alteration suggests that the elevated expression of the c-*myc* gene in this disease is due to a *trans* event. In support of this, the overexpression of c-*myc* and the tumorigenic phenotype were suppressed by fusion of colon carcinoma cells with cells that express low levels of c-*myc* RNA and also by transfer of a normal chromosome 5 [Erisman et al., 1989; Rodriguez-Alfageme et al., 1992; Tanaka et al., 1991].

Our research has been directed toward an understanding of how the c-*myc* gene is regulated in cells of the colorectal mucosa. In previous work we have found that sodium butyrate, a natural differentiation agent for this tissue, causes a decrease in the expression of c-*myc*

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RNA in SW837 colorectal carcinoma cells due to an increase in transcriptional attenuation [Herold and Rothberg, 1988; Heruth et al., 1993].

Evidence from several lines of inquiry suggests a role for protein tyrosine kinases (PTKs) in signal transduction to the nucleus that regulates expression of the c-*myc* gene. 1) Many agents including platelet derived growth factor, epidermal growth factor, insulin, and interleukin 3 cause an increase in *myc* expression in susceptible cells [Conscience et al., 1986; Greenberg et al., 1985; Kelly et al., 1983; Taub et al., 1987]. The receptors for these ligands are PTKs and/or activate other PTKs. 2) Infection of the murine myeloid cell line FDC-P1 with any one of several different recombinant retroviruses that express one of the PTK oncogenes *abl, fms, trk,* or *src* abrogates the need for exogenous stimulation with interleukin 3 to cause cell growth and increased *myc* expression [Cleveland et al., 1989]. In this system a temperature sensitive v-*abl* PTK was shown to conditionally cause an increase in *myc* expression that was not dependent on synthesis of new protein [Cleveland et al., 1989]. 3) Elevated expression of c-*myc* RNA was seen in chick or quail fibroblasts transformed by Rous sarcoma virus, whose oncogene is the *src* PTK [Kuchino et al., 1985; Sovova et al., 1993]. 4) The PTK inhibitors geldanamycin and herbimycin inhibit the expression of c-*myc* RNA in the murine lymphoblastoma cell line L5178Y [Yamaki et al., 1989].

In this study we made use of inhibitors to assess the role of tyrosine phosphoproteins in regulating the expression of the c-*myc* gene in two colon cancer cell lines: SW837, derived from an adenocarcinoma of the rectum, and HCT8, which was derived from an adenocarcinoma of the ileocecum. We provide evidence that one or more PTKs is involved in regulating the expression of the c-*myc* gene.

MATERIALS AND METHODS

Cell Culture and Treatment

HCT8 and SW837 cells were obtained from the American Type Culture Collection (Rockville, MD). They were grown in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 units/ml), and fungizone (0.25 μg/ml). Cells were grown in 175 cm^2 flasks at 37°C in a humidified atmosphere with 5% CO_2. Experiments were initiated when monolayers reached 70–80% of confluence by adding the indicated reagents from concentrated sterile solutions. Tissue culture reagents were obtained from JRH Biosciences (Lenexa, KS). Genistein and geldanamycin were purchased from Life Sciences, Inc. (Bethesda, MD), and dissolved at 100 mg/ml and 1 mg/ml, respectively, in dimethylsulfoxide. At this concentration the dimethylsulfoxide did not interfere with measurements of *myc* expression or with the MTT assay for proliferation (data not shown). Novobiocin was obtained from Sigma (St. Louis, MO). Sodium orthovanadate was obtained from Aldrich (Milwaukee, WI). Actinomycin D was obtained from Boehringer Mannheim (Indianapolis, IN).

RNA Preparation, Northern Analysis, and DNA Probes

RNA was prepared using the RNAzol method as described by the manufacturer (Cinna/Biotecx, Friendswood, TX) using 4 ml of RNAzol-B and 600 μl of chloroform for each T175 flask. Gel electrophoresis, transfer, and hybridization of Northern blots was done as described previously [Herold and Rothberg, 1988], except for the 18S ribosomal RNA gene probe which was hybridized for 2 h at room temperature, followed by washing at 68.5°C instead of our usual 65°C. The altered conditions were necessitated by the great excess of ribosomal RNA on the Northern blots of unselected RNA. The conditions were chosen to maintain pseudo first-order kinetics during the hybridization reaction. The following probes were used for blotting analysis: for c-*myc,* E3-*myc,* a 1.4 kbp ClaI-EcoRI fragment from the third exon of human c-*myc* [Rothberg et al., 1984]; for the p53 gene, a 1.3 kbp PvuII-PvuII fragment derived from a human cDNA clone [Matlashewski et al., 1984], which was generously provided by Dr. S. Benchimol; for glyceraldehyde-3-phosphate dehydrogenase, a 1.2 kbp PstI-PstI fragment from the human cDNA clone pHcGAP [Tso et al., 1985], which was obtained from the ATCC; for 18S ribosomal RNA, RM17, a 1 kbp BamHI-EcoRI genomic DNA fragment [Miesfeld and Arnheim, 1982], which was generously provided by Dr. N. Arnheim. Northern blot intensities were quantitated using Imagequant software and a Molecular Dynamics Phosphorimager (Sunnyvale, CA), followed by autoradiography. Autoradiography was carried out by exposure of the blot to X-ray film (Kodak XAR-5) at −70°C with a Dupont Cronex (Wilmington, DE) intensifying screen.

Northern blot autoradiographs are shown in the figures, except when noted in the legend.

MTT Cell Proliferation Assay

HCT8 and SW837 cells were plated in growth medium at a density of 3,000 and 5,000 cells per well, respectively, in 96-well microtiter plates. After 24 h the PTK inhibitors were added and the incubation continued for 3 days. The number of metabolically active viable cells was determined using the MTT tetrazolium dye assay [Alley et al., 1988; Mosman, 1983]. Briefly, the medium was replaced with fresh medium (without serum) containing 0.5 mg/ml MTT (Sigma), and the cells were incubated for 3 more h. The medium was then aspirated off, and the insoluble formazan product in the cells was dissolved with 150 μl dimethylsulfoxide and quantitated spectrophotometrically at a wavelength of 550 nm using an ELISA plate reader (model 7520; Cambridge Technology, Inc., Watertown, MA). All determinations were done in triplicate.

Nuclear Runoff Assays

The isolation of nuclei and in vitro elongation reactions were done according to the protocol of Linial et al. [1985], with modifications as previously described [Heruth et al., 1993]. Radioactive transcripts were purified by the RNAzol method using 400 μl RNAzol B and 80 μl chloroform per reaction. The following DNAs were used as probes for runoff transcription assays: single-stranded M13 clones corresponding to the template strand (to detect sense transcripts) and the coding strand (to detect antisense transcripts) of P0, a 535 bp SmaI fragment, of exon 1, a 443 bp XhoI-PvuII fragment, of intron 1, a 606 bp SstI fragment, and of exon 2, a 414 bp PstI fragment [Bentley and Groudine, 1988]. The M13 clones were kindly provided by Dr. M. Groudine. Single-stranded DNA probes to detect sense (+) and antisense (−) transcripts specific for exon 3 were prepared as described previously [Heruth et al., 1993]. Single-stranded DNA from pGEM-3Zf(−) (Promega, Madison, WI), which contains phage f1 and plasmid sequences, was used to detect hybridization to vector. Denatured double-stranded DNA from the TPI cDNA clone pHTPI-5a [Maquat et al., 1985], subcloned into pSP65, was used as a control probe for runoff transcriptions. Hybridization and washing of blots was done as previously described [Heruth et al., 1993]. Values from exon 3+ and TPI were corrected for cross-hybridization to the vector by subtracting the value for the pGEM signal. Similar correction for the other probes was not necessary because the appropriate vector did not generate a signal (data not shown). Estimates of transcriptional elongation were calculated using signals adjusted for the uridine content of the transcripts. The uridine content of transcripts homologous to the sense (+) DNA probes is as follows: exon 1, 74; intron 1, 136; exon 2, 69; and exon 3, 373 [Bentley and Groudine, 1988; Heruth et al., 1993]. Signal intensities were quantitated using Imagequant software and a Molecular Dynamics Phosphorimager.

RESULTS

PTK Inhibitors Lower c-*myc* Expression in SW837 and HCT8 Cells

Genistein is a well-characterized inhibitor of PTK enzymes. It is an isoflavone which was originally isolated from *Pseudomonas* cultures [Akiyama et al., 1987]. Genistein inhibition of the v-*src* PTK in vitro has been shown to be competitive with respect to ATP [Akiyama et al., 1987]. We used this inhibitor to evaluate the potential impact of PTK activity on expression of the c-*myc* gene in colorectal cancer. Figure 1 shows a Northern blot analysis of the effect of genistein on the expression of the c-*myc* gene. The blots were prepared using total cellular RNA isolated from SW837 rectal carcinoma cells and HCT8 ileocecal carcinoma cells which had been exposed to the indicated concentration of the inhibitor for 6 h. The blots were rehybridized with a p53 gene probe (Fig. 1) and an 18S ribosomal RNA gene probe (data not shown) to control for differences in RNA quantity between the wells. The results clearly show that genistein caused a decrease in the quantity of c-*myc* RNA. The dose response was similar to that seen in experiments that demonstrated a role for tyrosine phosphorylation in activation of T-lymphocytes triggered by the antigen-specific T cell receptor–CD3 complex [Mustelin et al., 1990] and in experiments implicating a PTK in the prolactin-stimulated proliferation of Nb2 lymphoma cells [Fan et al., 1993]. As seen in the graphical presentation in Figure 2, the HCT8 cells were slightly more sensitive to this effect of genistein than were the SW837 cells. Time course experiments revealed that the reduction in expression of c-*myc* RNA in both lines was apparent as early as 1 h after exposure and maximal by 2–6 h (data not shown). These re-

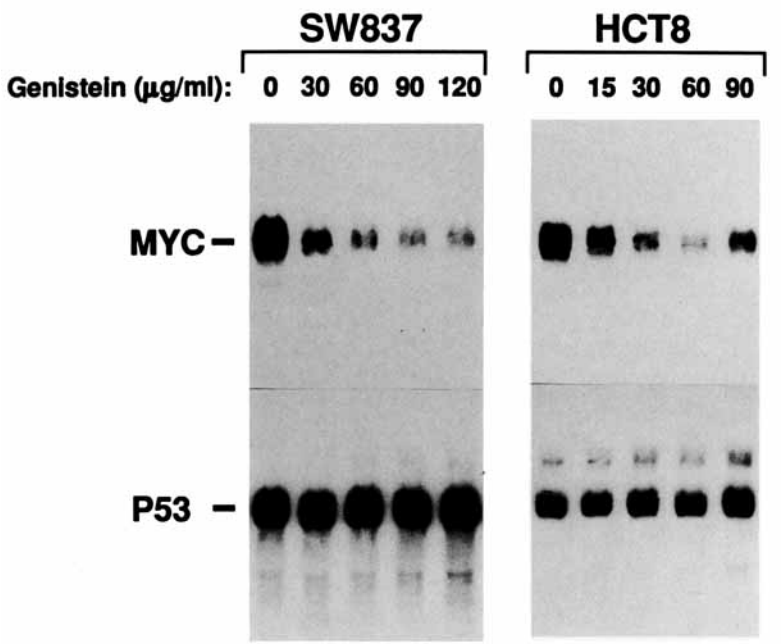


Fig. 1. Northern blot analysis of the effect of genistein on the level of c-*myc* RNA in SW837 and HCT8 cells. The cells were incubated with the indicated concentration of genistein in complete medium for 6 h before harvesting cells for RNA preparation. The level of *myc* RNA was measured by Northern blot hybridization using a probe from the third exon of the human c-*myc* gene. The blot was rehybridized with a human p53 cDNA probe to adjust for the actual quantity of RNA in each lane.

sults suggest that a PTK(s) plays a role in regulating the expression of the c-*myc* gene in these lines.

The effect of genistein on cellular proliferation was evaluated using the MTT assay by quantifying the relative number of viable cells after 3 days of continuous exposure to genistein. The results, shown graphically in Figure 2, reveal that genistein inhibited cellular proliferation in both cell lines examined. Again, the HCT8 cells showed slightly more sensitivity than the SW837 cells.

Genistein has also been shown to be an inhibitor of topoisomerase II [Constantinou et al., 1990; Okura et al., 1988]. Thus, it is possible that the effect of genistein on c-*myc* expression is due to some other effect than inhibition of a PTK. In order to address this question we employed geldanamycin, a chemically different inhibitor of PTK. Geldanamycin is a benzenoid ansamycin antibiotic which was isolated from *Streptomyces hygroscopicus* [Uehara et al., 1986]. This class of antibiotic differs from genistein in being able to inhibit the v-*src* PTK in vivo but not in vitro [Uehara et al., 1986]. We exposed the HCT8 cells to geldanamycin for 6 h and measured the expression of the c-*myc* gene. Figure 3 shows that geldanamycin also caused a decrease in the expression of c-*myc* RNA. The dose response was similar to that used to cause reversion of transformation in Rous sarcoma virus infected NRK cells [Uehara et al., 1986]. Geldanamycin also inhibited proliferation as measured by the MTT assay (Fig. 3B). In this experiment there was a discrepancy in the extent of the reduction in c-*myc* RNA quantity depending upon the control probe used for normalizing the *myc* signals for unequal loading of RNA. The extent was greater when we used an 18S ribosomal RNA gene probe than when we used a p53 gene probe. For this reason we present both curves in Figure 3B. This may indicate an effect of geldanamycin on p53 gene expression, an effect which we have not yet attempted to confirm. Geldanamycin also caused a decrease in expression of the c-*myc* gene in the SW837 cells (data not shown).

Effects of Novobiocin, Orthovanadate, and Cycloheximide on c-*myc* Expression

In another approach to evaluating the potential impact of inhibiting topoisomerase II on the expression of the c-*myc* gene, we incubated HCT8 cells with the topoisomerase II inhibitor novobiocin [Ali-Osman et al., 1993; Gellert et al., 1976] and evaluated its effect on the expression of the

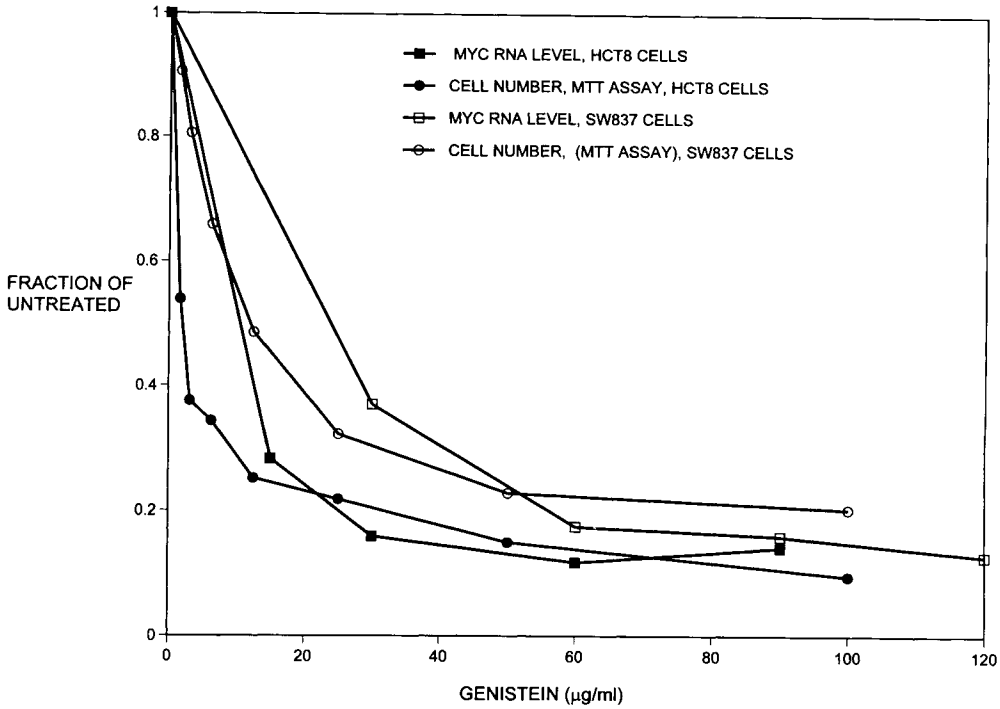
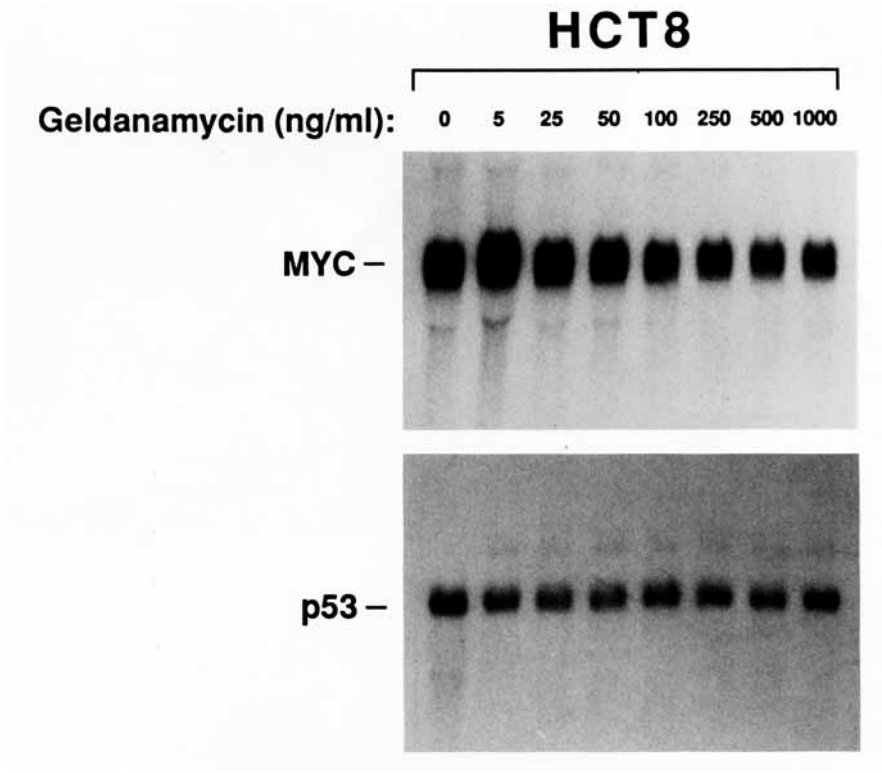


Fig. 2. Dose-response curves for the effect of genistein on the level of c-*myc* RNA and cellular proliferation. The level of *myc* RNA was determined by Northern blot hybridization as shown in Figure 1. The ratio of *myc* signal to p53 signal in each lane was used as a measure of the relative quantity of *myc* RNA. The results were very similar when the signal from 18S ribosomal RNA was used as a control for variations in RNA quantity. The relative number of viable cells was determined by MTT assay after a 3 day incubation with the indicated concentration of genistein. All values are expressed as a fraction of the same measurements on untreated cells.

c-*myc* gene. As seen in Figure 4, novobiocin caused a slight increase in c-*myc* expression in these cells. Thus, it is unlikely that the inhibition of topoisomerase II by genistein caused its effect on the expression of the c-*myc* gene, as another PTK inhibitor, geldanamycin, inhibited expression, while the topoisomerase II inhibitor novobiocin did not. The finding of increased expression of the c-*myc* gene in response to incubation with novobiocin has been seen previously in rodent fibroblasts [Aller and Baserga, 1986].

While PTKs are responsible for increasing the quantity of phosphotyrosine in proteins, phosphotyrosine phosphatases lower the level of phosphotyrosine. Orthovanadate is a specific inhibitor of phosphotyrosine phosphatases [Klarlund, 1985; Leis and Kaplan, 1992; Nelson and Branton, 1984; Swarup et al., 1982a,b]. We reasoned that if decreasing phosphotyrosine with the PTK inhibitors genistein and geldanamycin cause a decrease in c-*myc* expression, then increasing the level of phosphotyrosine using the phosphatase inhibitor orthovanadate would cause an increase in the expression of c-*myc* RNA. As shown in Figure 5, that is indeed the case. The expression of c-*myc* RNA increased with increasing dose of vanadate in the range of up to 100 μM, the highest dose tested. The difference in *myc* expression between the untreated and the 100 μM dose is statistically significant ($P < 0.01$; Student's *t*-test). Similar results were obtained with the SW837 cell line (data not shown). Vanadate has previously been shown to cause an increase in expression of the c-*myc* oncogene in an ovarian carcinoma cell line [Itkes et al., 1990].

We wondered whether genistein has a direct effect on the expression of c-*myc* RNA or if the effect is mediated by the induction of a new protein(s). We used the protein synthesis inhibitor cycloheximide to explore this aspect. As can be seen in the Northern blot in Figure 6A and the graphical presentation of results from this blot and two others in Figure 6B, the level of *myc* expression was reduced in cells treated for 3 h with genistein, regardless of the presence or absence of cycloheximide. The synthesis of new protein was not necessary for genistein to cause



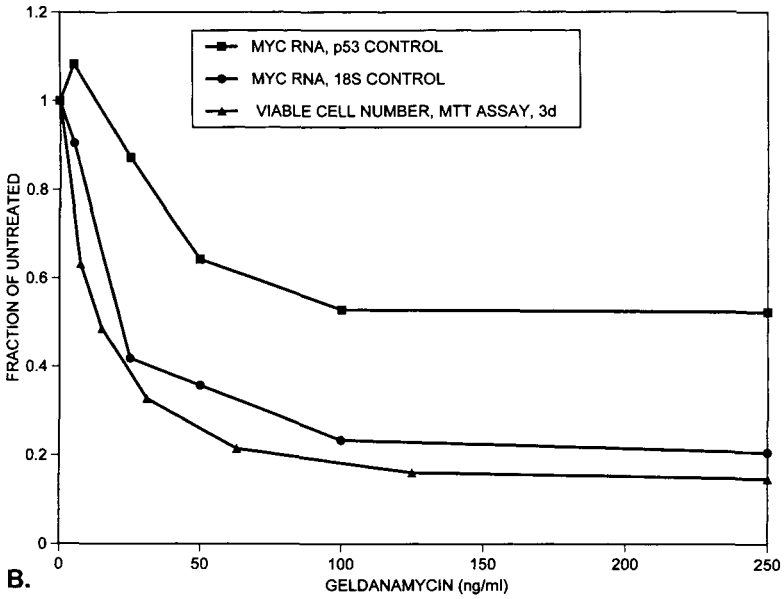


Fig. 3. *The effect of geldanamycin on the expression of c-myc* RNA and cellular proliferation. **A:** HCT8 cells were incubated with the indicated concentration of geldanamycin for 6 h in complete medium before harvesting the cells for RNA purification and Northern blot analysis. The blot was hybridized with an exon 3 human *c-myc* probe and then rehybridized with a human p53 cDNA probe to adjust for the actual quantity of RNA in each lane. **B:** The ratio of *myc* signal to the signal from p53 RNA or 18S ribosomal RNA (Northern blot autoradiograph not shown) in each lane was used as a measure of the relative quantity of *c-myc* RNA. The relative number of viable cells was determined by MTT assay after a 3 day incubation with the indicated concentration of geldanamycin. All values are expressed as a fraction of the same measurements on untreated cells.

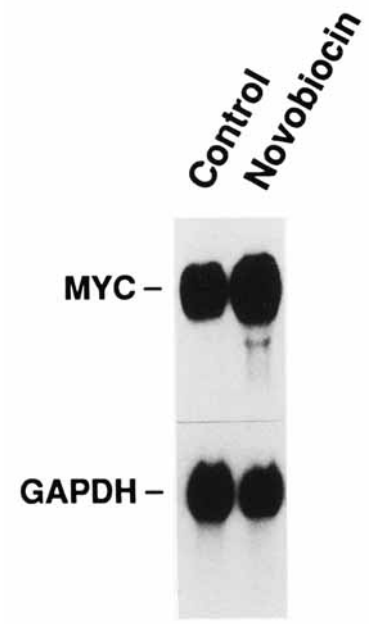


Fig. 4. Northern blot analysis of the effect of novobiocin on c-*myc* expression in HCT8 cells. The cells were incubated with 200 μg/ml of the topoisomerase II inhibitor novobiocin in complete medium for 3 h. The blot was hybridized with a c-*myc* exon 3 probe and then rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

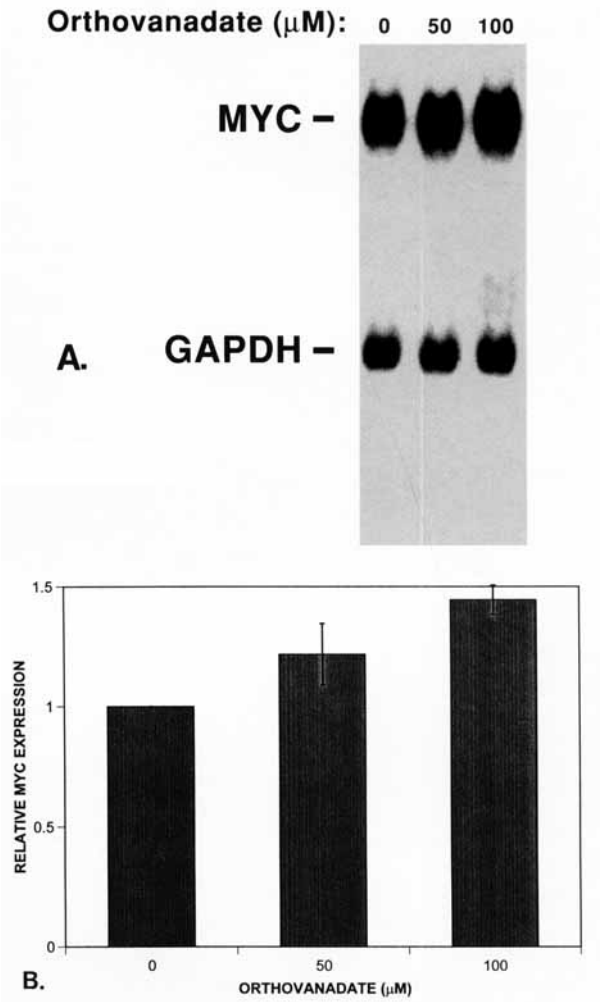


Fig. 5. Northern blot analysis of the effect of orthovanadate on the expression of c-*myc* RNA in HCT8 cells. **A:** The cells were incubated with the indicated concentration of orthovanadate in complete medium for 90 min. The Northern blot was probed with a c-*myc* exon 3 probe and then rehybridized with a glyceraldehyde-3-phosphate dehydrogenase probe (GAPDH). A phosphorimager printout is shown. **B:** A graphical representation of the average results from the Northern blot in A and one other analysis is shown. The signal from the *myc* hybridization was adjusted for differential loading of RNA and expressed as a multiple of the untreated cells. The error bars show the standard deviation.

a reduction in the expression of c-*myc* RNA. Interestingly, HCT8 is similar to other colorectal cancer cell lines in which we and others have shown that protein synthesis inhibitors do not cause an increase in c-*myc* RNA [Herold and Rothberg, 1988; Mulder and Brattain, 1988; Souleimani and Asselin, 1993]. In fact, at time points after 3 h of treatment with cycloheximide the level of c-*myc* RNA decreased in this cell line (data not shown).

Synthesis and Stability of c-*myc* RNA in Genistein-Treated Cells

A key question concerns the step in control of gene expression which is altered by genistein. To determine if the effect of genistein on the expression of c-*myc* RNA is due to an alteration in RNA stability, we determined the half-life of c-*myc* RNA in both untreated and genistein-treated HCT8 cells. The cells were left alone or exposed to 60 μg/ml genistein for 6 h before addition of actinomycin D (5 μg/ml) and then harvested after varying lengths of time for RNA preparation. The expression of c-*myc* RNA was measured by Northern blot hybridization and adjusted for unequal loading by rehybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene probe (Fig. 7A). The half-lives of the RNAs were determined from the slope of the lines in Figure 7B. There was no significant difference in c-*myc* RNA half-life between untreated (19.3 min) and genistein-treated cells (17.4 min). This indicates that the genistein-induced decrease in the abundance of c-*myc* RNA is not the result of a posttranscriptional increase in its degradation.

To determine if an alteration in transcription is responsible for the effect of genistein on the

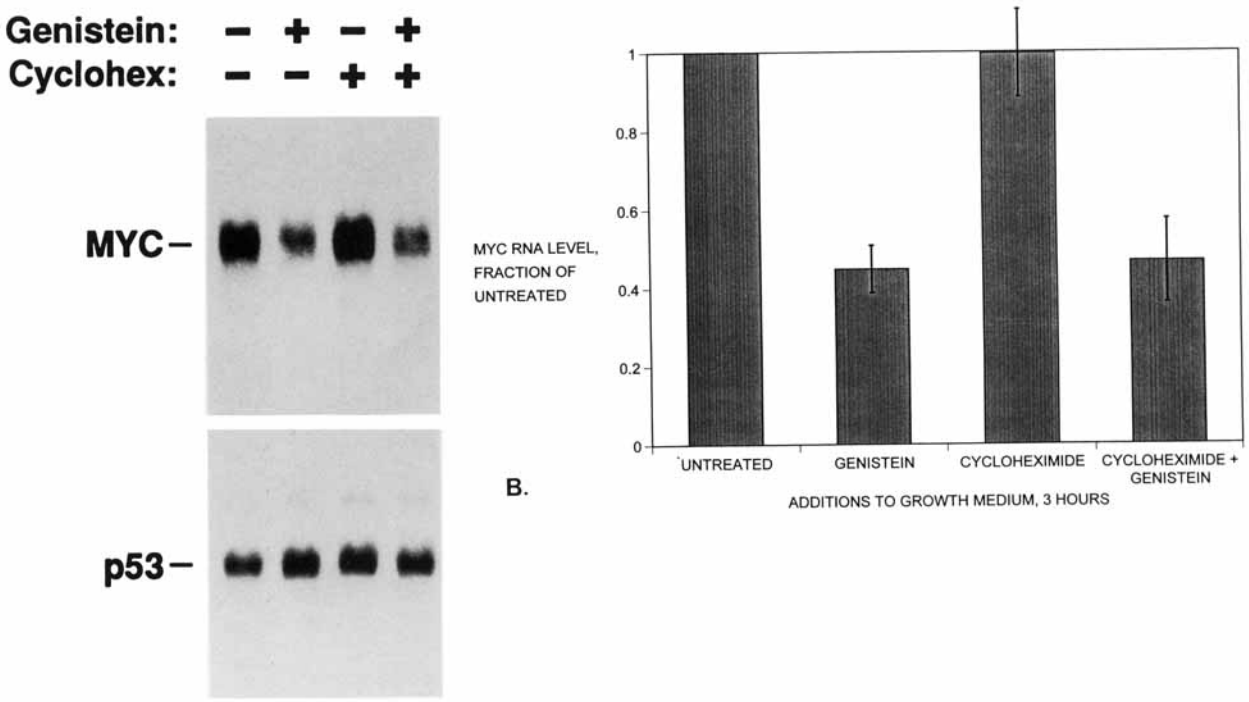


Fig. 6. Northern blot analysis of the influence of the protein synthesis inhibitor cycloheximide on the genistein-induced reduction in expression of c-*myc* RNA. HCT8 cells were incubated in complete medium for 3 h with the indicated addition: genistein (60 μg/ml), cycloheximide (10 μg/ml), or both. The quantity of *myc* RNA was measured by Northern blot hybridization and adjusted for total RNA quantity by rehybridization with a p53 probe. Autoradiographs of the Northern blot hybridized with the indicated probe is shown in **A. B:** Graphical representation of the data from three Northern blots including the one shown in the autoradiograph. The level of *myc* RNA is expressed as a fraction of the results from untreated cells. The error bars show the standard deviation.

expression of c-*myc* RNA in HCT8 cells, we performed runoff transcription assays employing nuclei isolated from untreated cells (nine assays), and cells treated with 60 μg/ml genistein for 2 h (six assays) or 6 h (seven assays). In each experiment 4×10^7 nuclei were allowed to elongate nascent RNA chains, that had been initiated in intact cells, in the presence of [^{32}P]UTP. The radioactive transcripts were hybridized to single-stranded DNAs containing appropriate fragments of the c-*myc* gene and controls. A representative experiment is shown in Figure 8. Antisense transcription was detected for exon 2 but did not vary between nuclei isolated from treated and untreated cells, so this data is not shown in the figure. Antisense transcription of exon 1, intron 1, and exon 3 was very low (data not shown).

Transcription of the c-*myc* gene has been shown to be controlled at the level of initiation and elongation. The genistein-treated cells consistently had a decreased rate of transcriptional initiation as well as an increased rate of transcriptional elongation. The rate of transcriptional initiation, calculated from the ratio of signal from the sense strand of exon 1 to the signal from the control gene triose phosphate isomerase (TPI) and the amount of radioactive RNA applied to the filter, was decreased to one-third in the genistein-treated cells. The rate of elongation was calculated from the ratio of signal from exon 3 to exon 1, adjusted for background, hybridization to vector sequences, and the number of uridine residues in the RNA hybridizing to each probe. Elongation increased threefold in the genistein-treated cells, from about 15% readthrough in untreated cells to about 45% readthrough in the genistein-treated cells. The two alterations, decreased initiation, and increased elongation, canceled out and left almost no net change in transcription in the genistein-treated cells. Thus, we do not have direct evidence for an alteration in transcription or RNA stability in causing the decrease in *myc* expression induced by genistein.

DISCUSSION

We have found that two mechanistically distinct PTK inhibitors, genistein and geldanamycin, cause a decrease, and the tyrosine phospha-

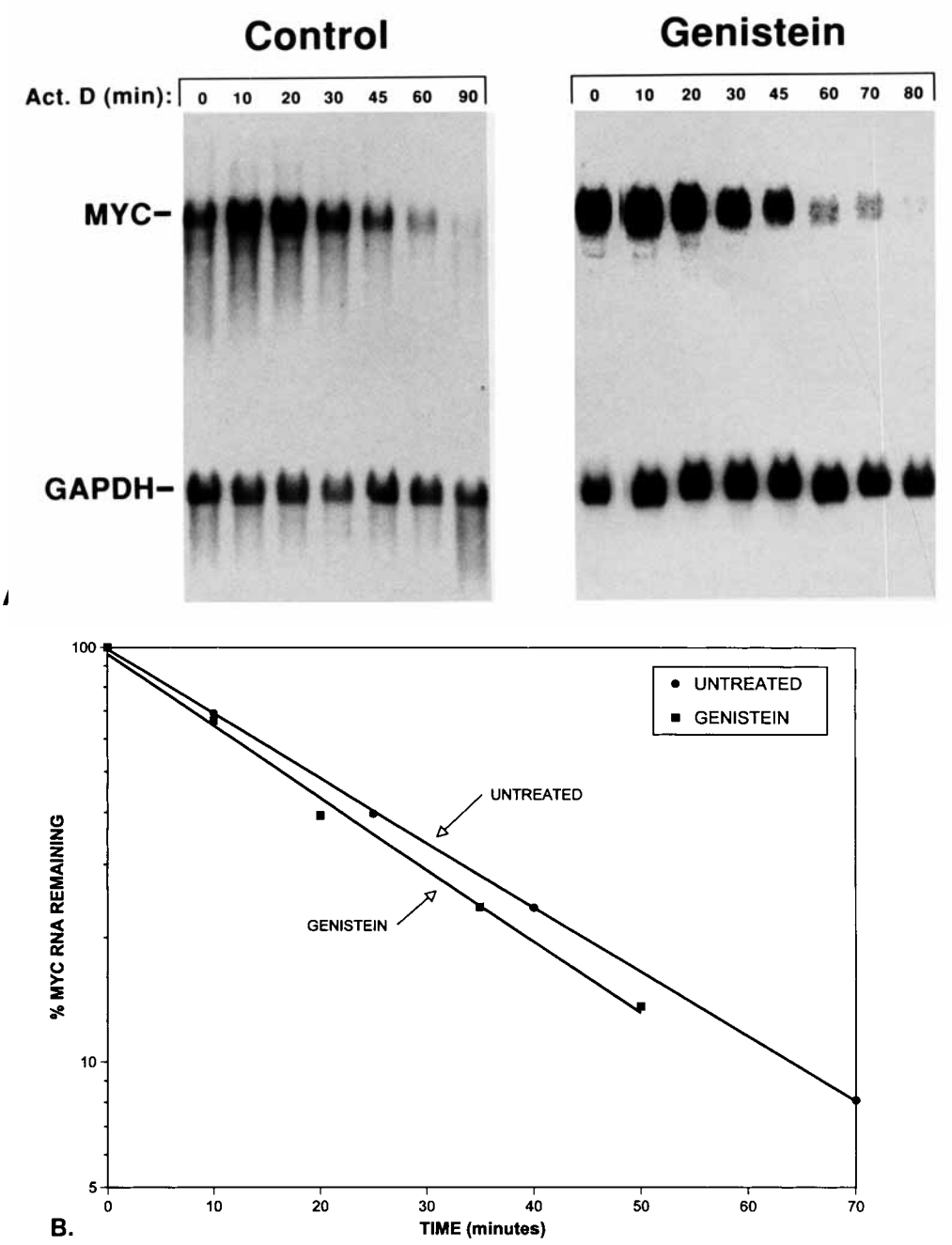


Fig. 7. Analysis of c-*myc* RNA stability in untreated and butyrate-treated HCT8 cells. **A:** Actinomycin D (5 μg/ml) was added to HCT8 cells, untreated or pretreated with 60 μg/ml genistein for 6 h, and total cellular RNA was isolated at the indicated time points. Northern blots were prepared and hybridized with the third exon of the c-*myc* gene and then rehybridized with a glyceraldehyde-3-phosphate dehydrogenase gene probe (GAPDH) as indicated. A phosphorimager printout is shown. **B:** The graph shows the percentage of c-*myc* RNA (normalized to the signal for GAPDH) remaining at the indicated time points. We used the time point with the highest level of c-*myc* RNA, after which the decay follows first order kinetics, as the starting point of the decay curves. The data analyzed corresponds to the 20–90 min and 10–60 min actinomycin D treatments for the untreated and genistein-treated curves, respectively. The y axis is plotted in logarithmic scale. Lines are linear regressions of time vs. the logarithm of relative c-*myc* RNA level fit by computer, using Delta Graph Professional for Windows. The data used to create the graphs and determine the RNA half-lives was taken from two tissue culture experiments and three Northern blots for the untreated cells and from one tissue culture experiment and two Northern blots for the genistein-treated cells.

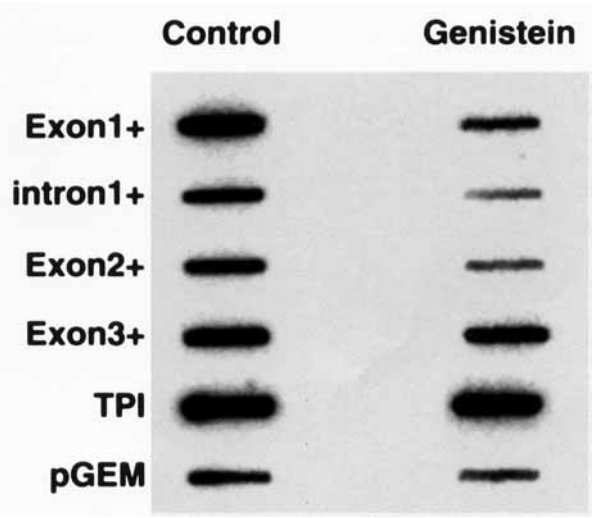


Fig. 8. Nuclear runoff analysis of the effect of genistein on transcription of the c-*myc* gene in HCT8 cells. Each filter was hybridized with labeled RNA elongated in vitro using nuclei isolated from untreated cells (72 million cpm) or cells treated with 60 μg/ml genistein for 2 h before isolation of nuclei (120 million cpm). Single-stranded DNA probes marked + detect sense transcripts. The antisense results are not shown. Denatured double-stranded DNA from the triose phosphate isomerase (TPI) gene was included as a control for the quantity of radioactive RNA applied to the filters. A phosphorimager printout is shown.

tase inhibitor orthovanadate causes an increase, in the abundance of c-*myc* RNA in two colorectal cancer cell lines. Genistein is also known to inhibit topoisomerase II, but another topoisomerase II inhibitor, novobiocin, did not cause a decrease in c-*myc* expression. These experiments provide evidence that one or more proteins phosphorylated on tyrosine is involved in regulating the expression of the c-*myc* gene in this system. Of course, we cannot exclude the possibility that other effects of these inhibitors are responsible for our observations. However, our findings are in accord with evidence from other systems, described above (Introduction), that suggest a role for PTK activity in regulation of expression of the c-*myc* gene.

Genistein and geldanamycin also cause a reduction in cellular proliferation with a dose response similar to that for the reduction in *myc* expression. The PTK inhibitor herbimycin A had previously been shown to inhibit proliferation in a panel of colon cancer cell lines [Garcia et al., 1991]. It is unlikely that the reduction in proliferation is the cause of the effect on *myc*. The effect of genistein and geldanamycin on the level of *myc* RNA was rapid and was not blocked by the protein synthesis inhibitor cycloheximide, implying a fairly direct route not involving the activation of other genes. Considering the evidence for the importance of the c-*myc* gene in cellular proliferation, the reduction in its expression may be the cause of the reduction in proliferation. However, except for the similar dose responses, we provide no direct evidence in support of that conclusion.

We have shown that sodium butyrate causes a decrease in transcriptional elongation in SW837 rectal cancer cells [Heruth et al., 1993]. A similar investigation of RNA degradation and synthesis failed to result in an understanding of which step in control of RNA quantity is responsible for the effects of genistein on *myc* RNA abundance in HCT8 cells. The rate of degradation of the bulk of c-*myc* RNA, which is cytoplasmic, was unchanged in genistein-treated cells. However, there were a number of alterations in transcription of the c-*myc* gene in the treated cells. The rate of initiation was decreased, but there was a compensating increase in elongation. The net result was no significant change in transcription of the complete c-*myc* gene. This analysis reveals that the effect of genistein on *myc* expression is complicated, which is probably the result of a number of different PTKs being inhibited, some of which have an impact on transcription of the c-*myc* gene. Also, one PTK may have multiple effects on the expression of the c-*myc* gene. The finding of multiple alterations in *myc* gene transcription with little net effect has been seen previously in studies on liver regeneration in mice [Morello et al., 1990]. Multiple effects of a PTK on transcription have also been seen in a murine myeloid cell line in which the v-*abl* oncogene PTK caused an increase in expression of the c-*myc* gene due to both increased initiation and increased elongation [Cleveland et al., 1989]. In our system a different step is altered that we did not directly measure. It is likely that an increased rate of degradation of intranuclear c-*myc* RNA and/or decreased processing or transport of the RNA is responsible.

Which PTK(s) is responsible for the effects of the inhibitors on c-*myc* expression and proliferation? Genistein has been shown to inhibit the PTK activity of the epidermal growth factor receptor, v-*src*, and v-*fes* [Akiyama et al., 1987]. The c-*src* and c-*yes* genes have been shown to be overexpressed and overactive in many colorectal cancers, and cell lines derived from them, compared to normal colorectal mucosa and other cell

lines [Bolen et al., 1987; Cartwright et al., 1989; DeSeau et al., 1987; Park et al., 1993]. These protooncogene products are a good starting point in investigating the key targets for the PTK inhibitors.

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