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Expression of c-*Myc* in Glucocorticoid-treated Fibroblastic Cells

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Glucocorticoids inhibit proliferation of L929 fibroblastic cells in culture. Inhibition of proliferation is reversible and is not associated with changes in the plating efficiency of the cells. Flow cytometric analysis indicates that glucocorticoid-treated cells exhibit a decrease in the percentage of cells with DNA content >2 N. Thymidine kinase expression is inhibited as cells with 2 N DNA content accumulate. These observations indicate that glucocorticoids arrest proliferation of L929 cells in the G1 phase of the cell cycle. The abundance of c-Myc mRNA does not decrease in glucocorticoid-treated cells, and c-*Myc* protein content in dexamethasone-treated cells is approximately the same as that detected in mid-log phase cells. Nuclear run-on transcription of c-*Myc* is not inhibited by glucocorticoids. These observations indicate that glucocorticoid regulation of fibroblastic cell proliferation does not involve inhibition of c-*Myc* transcription. Although regulation of c-*Myc* expression is central to the mechanism whereby glucocorticoids regulate proliferation of lymphoid cells, it is clear that different mechanisms must be involved in glucocorticoid regulation of fibroblastic cell proliferation.

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INTRODUCTION

Regulation of c-*Myc* expression has been studied in detail in a number of cell lines under a variety of conditions [1–7]. In almost all circumstances, proliferating cells express c-*Myc* mRNA in abundance, whereas non-proliferating cells express the gene to a substantially reduced extent. There are few cell types in which inhibition of proliferation is not associated with inhibition of c-*Myc* expression. Observations of this sort argue that there is a relationship between the capacity for cell proliferation and the ability to express c-*Myc*. For example, inhibition of c-*Myc* expression in HL60 leukemic cells is associated with withdrawal from the cell cycle and differentiation [2, 8–10]. Regulation of c-*Myc* in HL60 cells can be affected by a number of agents that cause G1 arrest and inhibit c-*Myc* transcription. The initial effect appears to be due to an increase in premature termination or attenuation of transcription [2, 11, 12]. The low level of c-*Myc* expression in quiescent T lymphocytes is maintained by premature termination, which is relieved upon mitogen stimulation [13, 14]. Mouse erythroleukemia cells resemble HL60 cells in that differentiation is linked to an increase in the frequency of premature termination and a corresponding decrease in c-*Myc* expression [15]. On the other hand, Burkitt's lymphoma cells do not exhibit attenuation, and the resultant increase in c-*Myc* expression is thought to contribute to the malignant phenotype of these cells [16]. Regulation of transcriptional elongation is by no means the only mechanism that governs c-*Myc* expression (for review see [7]). However, attenuation of c-*Myc* transcription is almost universal and appears to play a significant role in cell proliferation and differentiation, particularly in hematopoietic cells.

Glucocorticoid regulation of c-*Myc* expression has been studied extensively in lymphoid cells. Malignant T cells of both murine [17–19] and human [20, 21] origin exhibit glucocorticoid-mediated inhibition of c-*Myc* expression. The abundance of c-*Myc* mRNA decreases rapidly in glucocorticoid-treated T lymphoma and leukemia cells, and statistically significant changes are detected within 10 min in some cases [19]. In murine lymphoma cells, glucocorticoids rapidly inhibit nuclear run-on transcription of c-*Myc* [18]. However, regulation of mRNA stability may also govern c-*Myc* expression in human leukemia cells [21]. Antisense knockout of c-*Myc* can recapitulate the effects of dexamethasone in CCRF CEM-C7 human T

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leukemia cells, and transient expression of c-*Myc* from chimaeric expression vectors can attenuate the effects of dexamethasone in the same cell line [22]. These observations indicate that glucocorticoid regulation of c-*Myc* expression may be a common mechanism to account for the many and varied effects of glucocorticoids on cell proliferation.

Glucocorticoids play an important physiological and pharmacological role in regulating proliferation of fibroblastic cells [23, 24]. However, glucocorticoid regulation of fibroblast proliferation has not been studied as thoroughly as has glucocorticoid effects on lymphoid cells. Both lymphoid and fibroblastic cells are of connective tissue origin, and it is not unreasonable to suppose that the manner in which these cells respond to glucocorticoids may share common features. For example, glucocorticoids inhibit proliferation of papovavirus-transformed C129 mouse fibroblasts; and glucocorticoids inhibit expression of c-*Myc* in papovavirus-transformed C129 cells [24]. On the other hand, glucocorticoid regulation of c-*Myc* in untransformed C129 fibroblastic cells was difficult to demonstrate.

This manuscript describes a series of experiments that were designed to examine glucocorticoid regulation of L929 mouse fibroblastic cell proliferation and to determine the role of c-*Myc* in this process. L929 fibroblasts were selected because the proliferation of these cells is known to be inhibited by natural and synthetic glucocorticoids [25, 26]. The response is mediated by the glucocorticoid receptor [26, 27]. Preliminary data indicated that L929 cells are much more sensitive to glucocorticoids than are C129 cells, and it was hoped that a more robust response might facilitate analysis of gene regulation. Furthermore, there is a preliminary report that glucocorticoids may regulate expression of c-*Myc* in L929 cells [28]. The initial experiments were undertaken to define the growth response to glucocorticoids and to test the hypothesis that glucocorticoid inhibition of L929 cell proliferation is attributable to inhibition of expression of c-*Myc*. The data are not consistent with this hypothesis. It has been determined that the abundance of c-*Myc* mRNA does not decrease as L929 cells withdraw from the cell cycle. Furthermore, attenuation of c-*Myc* transcription does not occur in L929 cells. The data suggest that these cells are defective in an important regulatory mechanism, which may account for high basal expression and failure to regulate c-*Myc*.

MATERIALS AND METHODS

Reagents

Restriction endonucleases and enzymes for modification of DNA were purchased from BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA) and were used according to the manufacturer's suggested procedures. Proteinase K was purchased from Boehringer Mannheim (Indianapolis, IN). All [^{32}P]-labeled nucleoside triphosphates were obtained from DuPont New England Nuclear (Wilmington, DE). Fetal bovine serum was obtained from Flow Laboratories (McLean, VA). All other chemicals were purchased from U.S. Biochemical Corp. (Cleveland, OH), Sigma Chemical Co. (St Louis, MO) or Fisher Scientific (Houston, TX). Nitrocellulose (BA85, 0.45 μM) was manufactured by Schleicher and Schuell (Keene, NH).

Cell cycle analysis

L929.06 cell line is a subclone of the mouse L cell (NCTC 929) selected for sensitivity to dexamethasone. L929.06 cells were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 5% fetal bovine serum (FBS) and subcultured by treatment with trypsin. L929.06 cells were plated at a density of 8000 cells/cm^2. After 24–48 h, when in mid-logarithmic growth, the cells were treated with either vehicle (ethanol to a final concentration 0.1%) or dexamethasone (to a final concentration, 0.1 μM). The cells were harvested with trypsin and washed with phosphate-buffered saline containing 1 mM $MgCl_2$. The cells were suspended in 1.0 ml of phosphate-buffered saline plus 3 ml of 95% ethanol, sedimented by centrifugation at 200 *g* for 5 min and suspended in a modified Krishan's propidium iodide solution containing 0.005% propidium iodide, 0.002% RNase A, 0.3% Nonidet P40, and 0.1% sodium citrate [29]. The cells were lysed by a 30 min incubation at 4°C, and the resulting nuclei were collected by centrifugation at 200 *g* for 5 min. Nuclei were suspended in fresh modified Krishan's propidium iodide solution and analyzed on a Coulter Epics V Flow Cytometer (Coulter Electronics, Hialeah, FL) equipped with an argon laser source. Dye excitation was at 488 nm with fluorescence emission measured through a 515 nm long pass interference, a 515 nm long pass absorption and a 590 nm dichroic and 590 nm long pass absorption filters. DNA histograms were derived from an analysis of 25,000 cells.

Northern blot analysis

Total RNA from control and dexamethasone-treated cells was extracted with proteinase K and sodium dodecyl sulfate as previously described [30]. Total RNA was denatured with 6% formaldehyde and 50% formamide, fractionated on a 1% agarose gel containing 2.2 M formaldehyde and blotted onto nitrocellulose membranes according to Thomas [31]. The size of the mRNAs was estimated by comparison with ethidium bromide-stained 28S and 18S rRNA. Nitrocellulose filters were hybridized overnight at 55°C in the presence of 10% dextran sulfate, 50% formamide, 5 × SSC, 5 × Denhardt's solution and 100 μg/ml calf thymus DNA. All hybridization filters were washed three times at 65°C for 1 h (each wash), as described previously [32]. Plasmid p5B, corresponding to a fragment of mouse 18 S rRNA [33], was used as an internal

control for Northern analysis of mRNA. Full length mouse c-*Myc* clone pMc-*Myc*54 was obtained from Kenneth Marcu [34].

Thymidine kinase expression

The abundance of thymidine kinase mRNA was estimated by Northern blot analysis, as described above. The probe was a nick-translated restriction fragment from the mouse *Tk*-1 cDNA clone pMTK4 [35]. Thymidine kinase enzyme activity was estimated by measuring the rate of phosphorylation of TMP in extracts from L929 cells, as described elsewhere [32].

Ribonuclease protection assay

Total RNA from control and dexamethasone-treated cells was extracted, as described above, and 10 μg of sample RNA was dissolved in 30 μl hybridization buffer containing 5×10^5 ^{32}P cpm of probe RNA. The mixture was incubated for 3–7 min at 85–90°C to denature RNA and incubated at 60°C overnight. Then 160 μl of RNase digestion buffer, containing 10 mM Tris–HCl (pH 7.5), 0.3 M NaCl, 5 mM EDTA, 15 μg RNase A, and 40–50 U RNase T_1 was added and incubated at 30°C for 20–60 min. Proteinase K (200 μg/ml) and sodium dodecyl sulfate (0.5% w/v) were added and incubated for 15 min at 37°C. The reaction was extracted with phenol:chloroform, carrier tRNA (10 μg) was added to the aqueous phase which was diluted by addition of 1 vol of H_2O, and nucleic acids were precipitated with ethanol. The RNA pellet was dissolved in 4 M urea, 90 mM Tris, 90 mM boric acid, 0.05% (w/v) sodium dodecyl sulfate, 0.025 (w/v) xylene cyanol, 0.025 (w/v) bromphenol blue. The double-stranded RNA:RNA hybrids were resolved on 5% polyacrylamide by electrophoresis in TBE buffer (100 mM Tris, 2 mM EDTA and 100 mM boric acid). The gels were dried and exposed to X-ray film. The relative amount of each transcript was quantified by densitometry using an Applied Imaging BV14000 digital image analysis system.

The c-*Myc* probe was a 547 nucleotide antisense probe prepared by T7 RNA polymerase transcription of a c-*Myc* subclone containing a *ScaI*/*NotI* fragment. This is expected to protect fragments of 342 and 178 nucleotides, corresponding to transcripts initiated at the P1 or P2 c-*Myc* promoters respectively. The internal standard is a 136 nucleotide-sense probe prepared by T7 RNA polymerase transcription of a subclone containing an *HindIII*/*PvuII* fragment that lies between P1 and P2 (+24 to +139 relative to P1). The expected protected fragment is 121 nucleotides and was used to control for efficiency of sample recovery and hybridization. Experimental details are described elsewhere [19].

Measurement of c-Myc protein

The protocol is described in detail in *Antibodies: A Laboratory Manual* [36]. Cells were harvested by scraping and washed by centrifugation in phosphate-buffered saline. The cell pellet was lysed in 100 μl of RIPA lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing aprotinin (2 μg/ml), leupeptin (0.5 μg/ml), PMSF (35 μg/ml) and pepstatin A (0.7 μg/ml) for 30 min. Cell debris were sedimented by centrifugation (10 min in an Ependorf microcentrifuge) and 40 μg of protein was resolved by electrophoresis on 7.5% polyacrylamide gel containing SDS. Proteins were transferred to nitrocellulose by electroblotting. Filters were probed with a c-*Myc* monoclonal antibody (Monoclonal C33, catalog #SC42, Santa Cruz Biotechnology) diluted 1:75. A horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Chemicon) was used at a dilution of 1:4000 and detection was achieved using the Amersham ECL kit.

Nuclear run-on transcription assay

Nuclear run-on transcription was carried out as described by Mahajan and Thompson [37]. Briefly, nuclei were isolated from control and dexamethasone-treated cells and transcription was carried out in the presence of [α^{32}-P]UTP. RNA was isolated after digestion with DNase plus proteinase K in the presence of $CaCl_2$. Labeled RNA was washed by precipitation with trichloroacetic acid. Aliquots were withdrawn after transcription and before hybridization to calculate the recovery of labeled RNA, which was 30–60%. To compare control and dexamethasone-treated cells, equal nuclear equivalents of labeled RNA were hybridized to 3 μg of the appropriate DNA probes, immobilized on nitrocellulose.

The c-*Myc* probes used in the run-on experiments were M13 subclones derived from pMc-*Myc*54 as previously described [18]. The probe for exon I was a 379 bp *HindIII*-*XhoI* fragment and the probe for exon II was a *PstI* fragment of 417 bp subcloned into M13mp8. The M13 recombinant containing the RNA-like strand was used to control symmetric transcription and nonspecific hybridization to the RNA complementary strand. The Syrian hamster 5S gene [38] was used as an internal control for hybridization efficiency in nuclear run-on transcription experiments [18, 32, 37].

RESULTS

Growth of L929.06 cells in the presence of glucocorticoids

L929 cells were obtained from ATCC and 11 subclones were isolated. The data presented below summarize the observations derived from a number of experiments undertaken to characterize the manner in which dexamethasone influences the proliferation of the L929.06 subclone. The behavior of L929.06 is indistinguishable from that of the other clones or the uncloned parental population. Proliferation of these cells was severely inhibited by addition of 0.1 μM dexamethasone, as shown in Fig. 1. Less than one doubling was observed within 96 h after addition of the

steroid. Over the course of the experiment shown in Fig. 1, control cultures doubled every 34 h on average, whereas the glucocorticoid-treated cultures doubled every 113 h. In six independent experiments the mean population doubling time (over a period of 96 h) of control cultures was calculated to be 35(±10)h (range 32–46 h). The population doubling time of parallel, dexamethasone-treated cultures was estimated to be 106(±54)h (range 74–282 h, $P < 0.001$ by unpaired t). (Standard deviations are given in parentheses.)

The ability of L929.06 cells to adhere to the plates and form colonies was not affected, as shown in Fig. 2. Plating efficiency was not reduced when dexamethasone was added at the time of plating or 24 h thereafter (cross-hatched bars). However, a significant reduction in the number of cells/colony was observed (open bars).

The effects of glucocorticoids are reversible. Cells were exposed to glucocorticoids for various periods of time, and the plating efficiency was measured thereafter. As shown in Fig. 3, the plating efficiency was not reduced when L929 cells were exposed to dexamethasone for up to 4 days. In a separate experiment, the rates of proliferation were determined for cells that had been rescued after 96 h in dexamethasone. The population doubling times of such cultures was 34(±4)h and was not significantly different from that of naive cultures. These data indicate that glucocorticoids do not kill L929 cells and do not cause irreversible differentiation.

Glucocorticoids and G1 arrest of L929.06 cells

Nuclei from L929 cells were stained with propidium iodide and analyzed for DNA content by flow cytometry. Representative data are shown in Fig. 4. Glucocorticoids caused a decrease in the number of cells having DNA content >2 N. Quantitative data

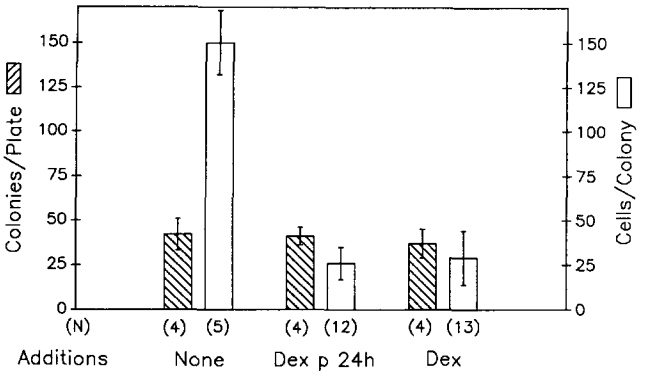


Fig. 2. The effect of dexamethasone on the plating efficiency of L929.06 cells. A suspension of cells was prepared and used to inoculate 3 cm tissue culture plates with 102(±3) cells in 3 ml of complete medium. One set of plates (Dex) received 0.1 μM dexamethasone. Control plates received vehicle alone. A third set of plates was maintained in complete medium for 24 h prior to addition of 0.1 μM dexamethasone (Dex p 24 h). After 6 days, the medium was decanted and the plates were washed with phosphate-buffered saline. The cells were fixed with 1% glutaraldehyde and stained with 1% cytodiachrome. The number of colonies/plate was counted under a dissecting microscope and the number of cells/colony under an inverted microscope. The error bars represent one standard deviation and the number of plates or colonies counted is expressed in parentheses below each bar.

are presented in Table 1. The data indicate that glucocorticoids caused a decrease in the percentage of cells in S, G2, and M phases of the cell cycle. A corresponding increase in G1 phase cells was observed. This situation is reversible and the percentage of cells in S, G2 and M increased following removal of dexamethasone from arrested cultures. These data are consistent with the conclusion that L929 cells accumulate in G1 in the presence of glucocorticoids.

Glucocorticoid-treated L929 cells exhibit decreased incorporation of [^{3}H]thymidine [39]. This is due, in

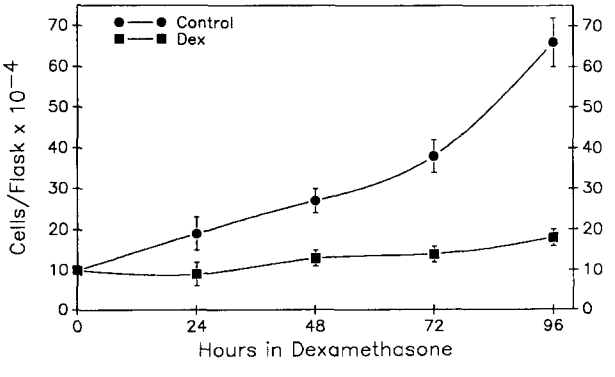


Fig. 1. The effect of dexamethasone on proliferation of L929.06 cells. A series of 25 cm^2 T flasks were inoculated with 1.0(±0.07) × 10^5 cells in 5 ml of complete medium. One series received 0.1 μM dexamethasone and the second an equivalent volume of vehicle (70% ethanol). Cells were harvested by treatment with trypsin at 24 h intervals. Cell number and viability were determined as described in Materials and Methods. Viability in all cases was >95%. Each time point was assayed in triplicated and the error bars represent one standard deviation.

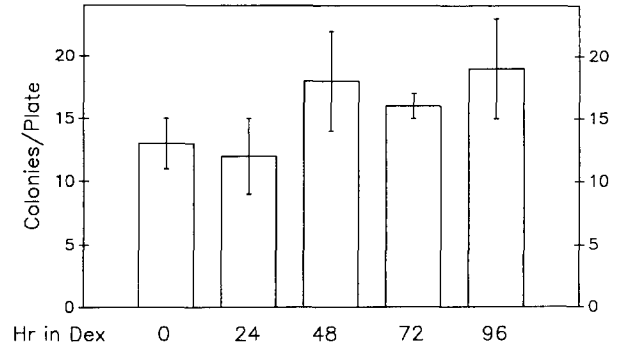


Fig. 3. Viability of dexamethasone-treated cells. L929 cells were exposed to 0.1 μM dexamethasone for periods of time corresponding to those shown below each bar. Thereafter, the cells were disaggregated and washed in phosphate-buffered saline to remove residual dexamethasone. Aliquots of 50 cells in 3 ml of complete medium were used to inoculate 3 cm plates. Colonies were counted (as described in the legend to Fig. 2) 1 week after inoculation. The error bars represent one standard deviation from the mean of 5 or more plates.

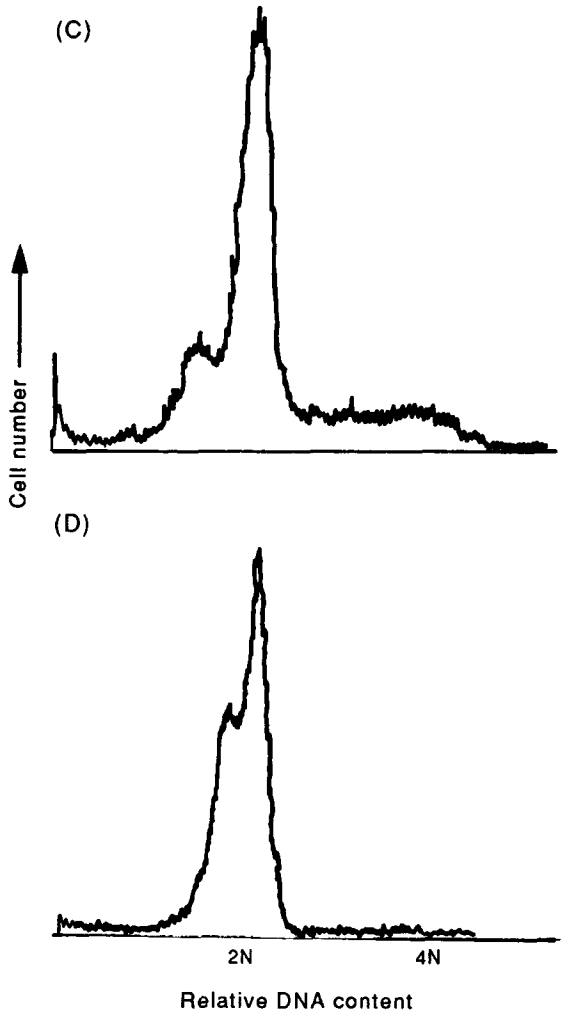


Fig. 4. Fluorescent cytometry of L929 cells. Cytofluorographic analysis of the DNA content from L929.06 cells exposed to solvent (C) or 0.1 μM dexamethasone (D) for 24 h. Quantitative data are given in Table 1.

part, to inhibition of DNA synthesis, as shown in Table 1. In addition, glucocorticoid-treated L929 express very low levels of thymidine kinase [39]. Mid-log phase L929 cultures were treated with dexamethasone for 24 h and RNA was extracted and assayed for the abundance of thymidine kinase (TK) mRNA. In addition, cultures were treated with dexamethasone for 24 h. Thereafter the hormone-containing medium was removed and replaced with fresh medium. RNA was isolated 12 and 24 h after rescue from glucocorticoids. In parallel, extracts were prepared from cultures, treated as described above. These extracts were assayed for thymidine kinase activity. The data are summarized in Fig. 5. Lane C (lane 1) contains RNA from control cells. Lane D (lane 2) contains RNA from dexamethasone-treated cells. Lanes R12 and R24 (lanes 3 and 4) contain RNA from cells that had been rescued for 12 and 24 h, respectively. Quantitative data from TK mRNA analysis and TK enzymatic activity in the corresponding samples are given below the autoradiogram. The abundance of TK mRNA in glucocorticoid-treated cells was <5% of that observed in control cells, with a corresponding decrease in thymidine kinase activity (TK Act.). The effects of dexamethasone are reversible, and both TK mRNA and activity increased rapidly after removal of dexamethasone. Both activity and mRNA abundance at 24 h after withdrawal of dexamethasone were 5–10 times higher than control. This observation indicates that when L929 cells are rescued from dexamethasone, they undergo a synchronous entry into S phase within 24 h after removal of the steroid. Thymidine kinase expression is known to be expressed in late G1/early S phase and repressed in cells that are arrested in G1 by serum starvation [40] and glucocorticoids [32, 39]. The data shown in Fig. 5 are consistent with the conclusion that glucocorticoids cause G1 arrest of L929 cells.

Table 1. *Cell cycle distribution of glucocorticoid-treated cells*

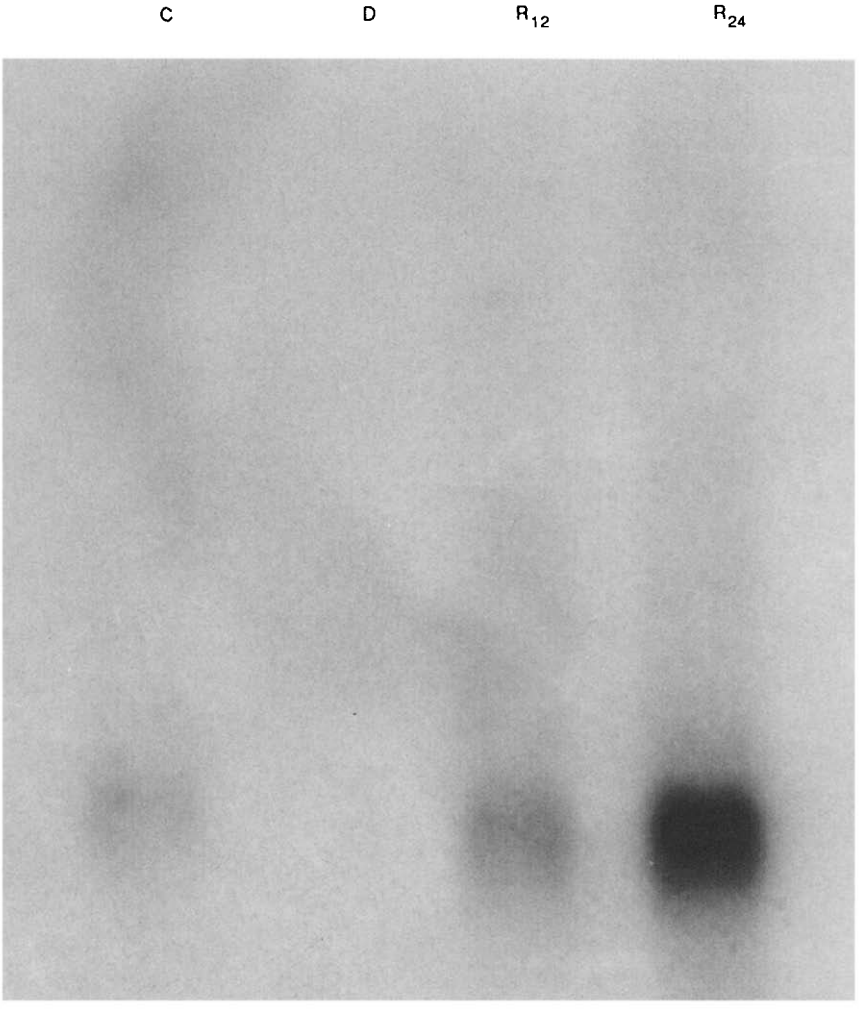
Cell cycle phase:	G1	S	G2 + M	
DNA content:	2N	> 2N < 4N	4N	
	Percent of cells			Total[a] Number
Control	68.5	5.6	17.2	18,816
24 h Dexamethasone	93.0	0.6	2.8	23.608
24 h Dex▶24 h rescue	47.0	7.8	16.6	18,464

[a]Total number of particles counted

Glucocorticoid effects upon expression of c-Myc

Like thymidine kinase, c-*Myc* expression is also reduced in cells that are arrested in G1 [reviewed in 4–7]. Expression of c-*Myc* was analyzed using an RNase protection assay to quantify the transcripts arising from the two major c-*Myc* promoters, P1 and P2. The results of a representative study are shown in Fig. 6. The RNA that was analyzed in this experiment was that obtained in the experiment described in Fig. 5. Lane C contains control RNA; lane D, RNA from dexamethasone-treated cells; and lanes R12 and R24 contain RNA from cells that had been rescued for 12 and 24 h. There was no significant change in the abundance of the P1 or P2 transcripts in glucocorticoid-treated cells and the abundance of neither P1 nor P2 mRNA changed as cells re-entered the cell cycle following removal of dexamethasone. The data shown in Fig. 6(B) indicate that the P2 transcript is about 80 times more abundant than the transcript arising from P1. (See the legend to Fig. 6 for an explanation of the calculations involved in comparing the abundance of the two mRNAs.)

Immunoblotting ("Western blotting") protocols were used to measure expression of c-*Myc* protein in L929 cells. The results of a typical experiment are shown in Fig. 7. The c-*Myc* antibody used in these experiments recognizes primarily a protein of about 64 K. The abundance of this protein did not decrease in glucocorticoid-treated L929 cells (compare lanes 3



	1	2	3	4
TK Act.	120	14	248	1560
TK mRNA	460	8	868	2688

Fig. 5. Glucocorticoid regulation of thymidine kinase (*Tk*-1) expression in L929 cells. L929 cells were treated for 24 h with 0.1 μM dexamethasone and RNA was extracted as described in Materials and Methods. Parallel cultures were treated for 24 h. Thereafter, the dexamethasone-containing medium was removed and replaced with warm (37°C) medium. This medium was removed after 30 min and replaced with fresh, warm medium. RNA was extracted 12 h and 24 h after removal of glucocorticoids. Total RNA (10 μg) was resolved by electrophoreses, blotted onto nitrocellulose, and hybridized with nick-translated TK cDNA probes. The filters were exposed overnight. The intensities of the autoradiographic signals were determined as described in Materials and Methods. The integrated results of quantification are shown below each lane (TK mRNA) in units of $mm^2 \times OD$. In parallel, cytoplasmic extracts were prepared from control, treated and rescued cultures. These extracts were assayed for thymidine kinase activity, as described in Materials and Methods. The activity data (TK Act.) given below each lane are expressed as initial velocity of thymidine phosphorylated in units of cpm of [^{3}H]TMP/min/mg protein.

and 4 of Fig. 7). Expression of c-*Myc* is inhibited in glucocorticoid-treated P1798 T lymphoma cells (18, 19). The c-*Myc* antibody recognizes a P1798 protein of the appropriate size, and the abundance of this protein was reduced in glucocorticoid-treated P1798 cells (compares lanes 1 and 2). The c-*Myc* mRNA in P1798 lymphoma is 3–5 times more abundant than in L929 cells (not shown). This is consistent with the observation that the presumptive c-*Myc* protein is also more abundant in extracts from P1798 cells (compare lanes 1 and 3).

The observation that the abundance of c-*Myc* mRNA does not change in glucocorticoid-treated cells suggests that transcription of c-*Myc* persists in G1-arrested L929 cells. Run-on transcription of c-*Myc* was measured in nuclei isolated from L929 cells. As shown

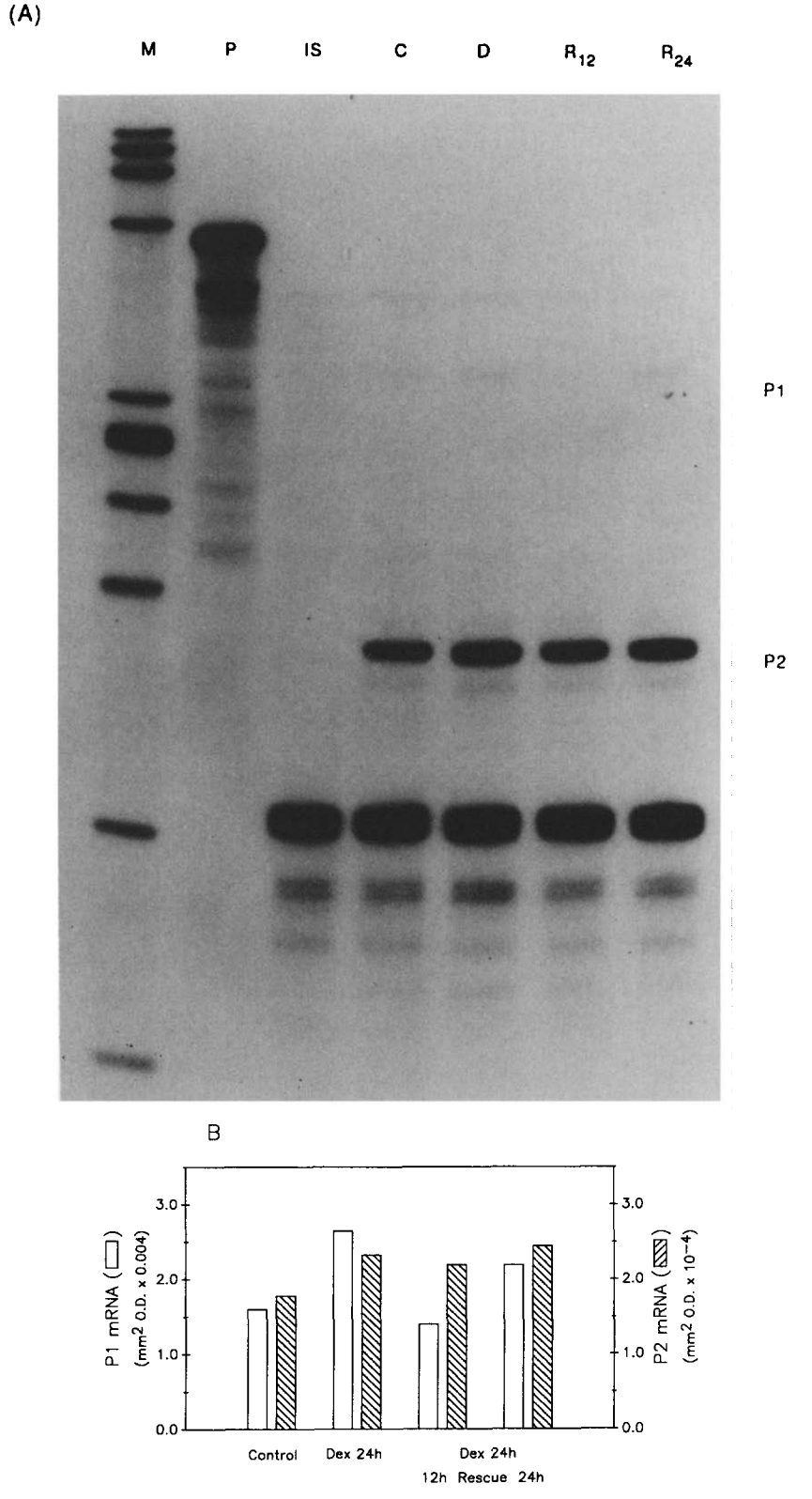


Fig. 6. Abundance of c-*Myc* mRNA in L929 cells. Panel A contains the results of an RNase protection assay. Lane M contains ^{32}P-labeled restriction fragments derived by digestion of bacteriophage phiX174 with *HaeIII*. Lane P contains the ^{32}P-labeled antisense RNA probe. This was mixed with an unlabeled c-*Myc* internal standard mRNA (transcribed from pMIS as described in Materials and Methods). The 117nt protected fragment derived from hybridization of the probe and the internal standard RNA is shown in lane IS. Total RNA was isolated from mid-log phase cells (lane C) as well as cells that had been treated with 0.1 μM dexamethasone for 24 h (lane D). In addition, cells were treated with dexamethasone for 24 h. The agonist was removed and RNA was isolated 12 h and 24 h after rescue (lanes R12 and R24, respectively). The positions of the fragments protected by the P1 and P2 transcripts are as indicated. Panel B contains quantitative data derived by densitometric analysis of Panel A. Note that the scales for P1 and P2 differ by 40-fold ($P2 \times 10^{-4} = P1 \times 0.004$). The protected fragments differ in [^{32}P]UMP content by a factor of 1.9. Consequently, if the autoradiographic intensity of the P2 fragment is 40 times that of the P1, the abundance of the P2 fragment is almost 80 times greater.

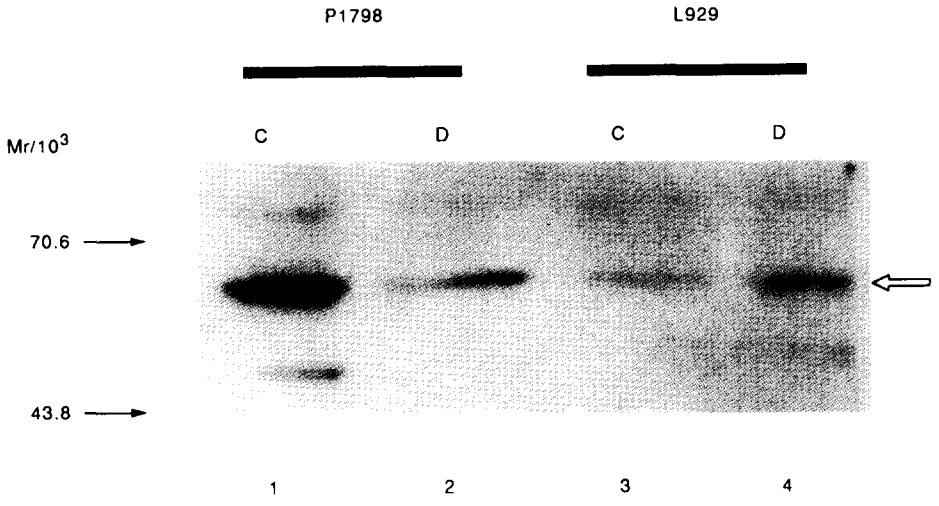


Fig. 7. Glucocorticoid regulation of c-*Myc* protein in L929 and P1798 cells. Extracts were prepared from mid-log phase and glucocorticoid-treated cultures of P1798 T lymphoma cells (lanes 1 and 2, respectively) and from L929 cells (lanes 3 and 4, respectively). Proteins were resolved on SDS–polyacrylamide gels and blotted on nitrocellulose filters. The filters were probed with a monoclonal antibody against c-*Myc* protein, and immunoreaction was visualized by enhanced chemiluminescence. Technical details are given in Materials and Methods.

in Fig. 8(A), several single-stranded probes were used to assess transcription of the entire c-*Myc* gene [pMc-*Myc* 54 and c-*Myc* (+)] as well as exon I and exon II. The probes that correspond to exons I and II are approximately the same size (379 and 417 bp, respectively) and both transcribed (+) and RNA-like (−) strands are included to assess asymmetry of transcription. Several interesting phenomena are observed. Initially, glucocorticoids had no discernible effect upon transcription, as one would predict from the data shown above. Furthermore, transcription of exon I was equivalent to that of exon II, as illustrated by the quantitative data shown in Fig. 8(B). (The data shown in Fig. 8(B) are corrected for length of the probes and for UMP content.) These data suggest that attenuation of transcription does not prevail, to any significant extent, in mid-log phase L929 cells or in cells that have been arrested in G1 by addition of dexamethasone. Figure 8(B) also contains the results of nuclear run-on experiments carried out with P1798 lymphoma cells, which are known to exhibit attenuation [18]. The transcriptional activity of exon II in P1798 cells was only about 20% of that of exon I. This observation indicates that only one RNA polymerase molecule in five is capable of reading through the first exon in P1798 T lymphoma cells. The extent of attenuation is L929 cells is strikingly reduced.

DISCUSSION

Regulation of c-*Myc* is central to proliferation of hematopoietic cells. Cell cycle arrest of HL60 cells is thought to be due to a decrease in the abundance of c-*Myc* mRNA [2, 8–11, 41, 42]. Proliferation of erythroleukemia cells is also directly related to c-*Myc* expression [15, 43, 44]. Activated splenic lymphocytes express high levels of c-*Myc* mRNA [45, 46] and glucocorticoids inhibit c-*Myc* expression and block mitogen stimulation of such cells (unpublished data from this laboratory). Glucocorticoids inhibit expression of c-*Myc* in human CCRF-CEM leukemic cells [20, 21, 28] as well as mouse T lymphoma S49 [17, 28] and P1798 cells [18, 19]. These data indicate that mitotic activity of hematopoietic cells is dependent upon c-*Myc* expression and that glucocorticoid inhibition of lymphoid cell proliferation involves regulation of c-*Myc*.

As in hematopoietic cells, regulation of c-*Myc* is also tightly linked to fibroblast proliferation (reviewed in 7). Serum starvation imposes a GO arrest on untransformed fibroblasts, and c-*Myc* mRNA abundance decreases. The abundance of c-*Myc* mRNA increases rapidly following serum stimulation of quiescent fibroblasts. It has been reported that activation of a *Myc*-estrogen receptor chimaeric protein is sufficient to drive serum-starved fibroblasts into S phase [47]. Glucocorticoids inhibit fibroblast proliferation in culture and *in vivo* [23, 24], although the mechanism(s) have not been studied in great detail. L929 fibroblasts undergo G1 arrest in the presence of glucocorticoids. It is not known if G1 arrest occurs in glucocorticoid-treated papovavirus-transformed C129 fibroblasts, but the proliferation of such cells is strikingly inhibited by glucocorticoids [24]. Growth arrest of transformed C129 cells is associated with a decrease in c-*Myc* mRNA [24]. However, untransformed fibroblasts exhibit a different response. The abundance of c-*Myc* mRNA and c-*Myc* protein does not decrease as glucocorticoid-treated L929 cells cease to proliferate. Glucocorticoids also inhibit proliferation of untransformed

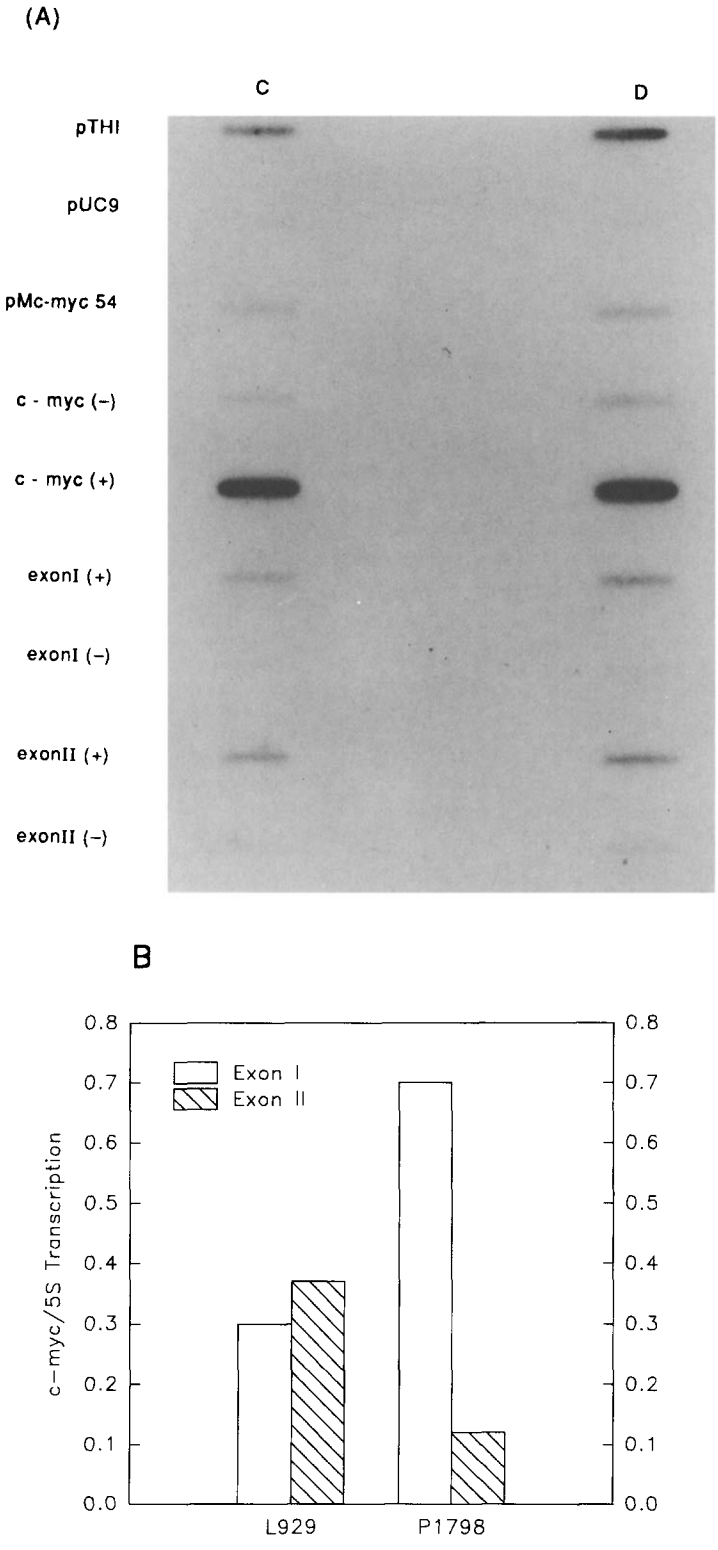


Fig. 8. Nuclear run-on transcription of c-*Myc* in L929 cells. Nuclei were isolated from control cells and from those that were exposed to 0.1 μM dexamethasone for 24 h. Nuclear run-on transcription was carried out with 10^7 nuclei and the products of transcription were isolated and hybridized to probes corresponding to the 5S RNA gene (pTH1), vector (pUC9), or various derivatives of c-*Myc*, as shown in Panel A. The derivatives include double-stranded, full-length cDNA (pMc-*Myc*54), M13 clones corresponding to both strands of full-length c-*Myc* cDNA [c-*Myc*(+) and c-*Myc*(−)] and both strands of subclones from exons I and II (as indicated). Lane C contains the products of hybridization from control nuclei and lane D those from dex-treated nuclei. The intensity of the autoradiographic signals from exons I and II were quantified, and are presented in Panel B, relative to transcription of the 5S RNA gene. All data are normalized for length of the single stranded probe and UMP content. The data shown in this figure are the average of two experiments. Run-on transcription of exons I and II was also analyzed in nuclei isolated from P1798 lymphoma cells.

C129 cells, with very little effect on the abundance of c-*Myc* mRNA [24]. The abundance of c-*Myc* protein was not assayed in glucocorticoid-treated C129 cells, so one cannot say for certain that glucocorticoid-arrested, untransformed C129 cells continue to express c-*Myc*. Examples of G1-arrested cells that express c-*Myc* are very rare [for review see 7]; and, when one considers the evidence that links glucocorticoid regulation of lymphoid cell proliferation to inhibition of c-*Myc*, it is unanticipated that glucocorticoid-inhibited, untransformed fibroblasts fail to turn off c-*Myc*.

It is also surprising that L929 cells do not exhibit attenuation of c-*Myc* transcription. Almost all cells that have been examined exhibit significant attenuation of c-*Myc* [2, 12, 44, 48–50]. The process is best observed in nuclear run-on transcription assays, which reveal that the RNA polymerase II packing density of c-*Myc* exon I is often >10 times that of exon II. Loss of attenuation contributes to activation of c-*Myc* in Burkitt's lymphoma cells [16, 48]. It has been proposed that failure of attenuation is due to the predominant utilization of the P1 promoter in Burkitt's lymphoma cells [16, 48]. According to this hypothesis, only those transcripts initiated at P2 are susceptible to premature termination, which might therefore be avoided by a promoter switch. This situation cannot account for failure of attenuation in L929 cells, which appear to use the P2 promoter almost exclusively. The lack of attenuation in L929 cells could reflect some change in the sequence or context of the c-*Myc* gene. Alternatively, L929 cells may harbor a defect in the expression of one or more substances that affect attenuation in *trans*. The information that is currently available is insufficient to discriminate between these alternative hypotheses.

Fibroblastic and lymphoid cells share a common ontological origin, and both types of cells exhibit growth inhibition in the presence of glucocorticoids. One might be inclined to speculate that glucocorticoid-mediated growth arrest in closely related cell types might reflect similar, if not identical, mechanisms. This is clearly not the case. Inhibition of c-*Myc* expression is critical to the anti-proliferative and lytic responses that are triggered by glucocorticoids in lymphoid cells of thymic origin [17–22]. The response is rapid [19], due to inhibition of transcription [18], and insensitive to inhibition of protein synthesis (T. Ma and E. A. Thompson, unpublished observation). Although it has not been directly demonstrated, the data strongly suggest that the glucocorticoid receptor interacts with the c-*Myc* promoter so as to inhibit transcription. One is inclined to speculate that regulation of c-*Myc* might be a common mechanism whereby glucocorticoids influence proliferation of cells of many different types, but the data presented above are inconsistent with this conclusion. Regulation of c-*Myc* does not account for glucocorticoid inhibition of proliferation of the best characterized, glucocorticoid sensitive fibroblastic cell line, L929. Alternative mechanisms must be considered to account for glucocorticoid effects on fibroblast proliferation.

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