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Transforming growth factor β1 suppression of c-*myc* gene transcription: Role in inhibition of keratinocyte proliferation

(antisense/growth factor/keratinocytes/transcriptional regulation)

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ABSTRACT **Transforming growth factor β1 (TGF-β1) is a potent growth inhibitor for many cell types, including most epithelial cells. However, the mechanism of growth inhibition is unknown. In skin keratinocytes, TGF-β1 has been shown to inhibit growth and to rapidly reduce c-*myc* expression. It has been demonstrated that protein synthesis is required for TGF-β1 regulation of c-*myc* in keratinocytes. Here we present evidence that treatment of mouse BALB/MK keratinocyte cells with either antisense c-*myc* oligonucleotides or TGF-β1 inhibited cell entry into S phase. These results suggest that TGF-β inhibition of c-*myc* expression may be essential for growth inhibition by TGF-β1. The block in c-*myc* expression by TGF-β1 occurred at the level of transcriptional initiation. Studies with a series of 5′ deletion c-*myc*/chloramphenicol acetyltransferase constructs indicated that a cis regulatory element(s), which resides between positions −100 and +71 relative to P1 transcription start site, is responsible for the TGF-β1 responsiveness. Based on these data, it is proposed that the mechanism of TGF-β1 growth inhibition involves synthesis or modification of a protein that may interact with a specific element(s) in the 5′ regulatory region of the c-*myc* gene, resulting in inhibition of transcriptional initiation.**

Transforming growth factor β1 (TGF-β1) is the prototype of a very large family of ubiquitous factors important in growth control, development, and differentiation (for review, see refs. 1 and 2). TGF-β1, TGF-β2, and TGF-β3 are the most potent growth-inhibitory polypeptides known for a wide variety of cell types in culture, including most epithelial, endothelial, and lymphoid cells and many myeloid cells (for review, see ref. 2). The growth-inhibitory effect of TGF-β1 on epithelial cells has also been demonstrated *in vivo* (3, 4).

We are interested in determining how the TGF-βs regulate normal epithelial cell proliferation. A continuous line of mouse keratinocytes (BALB/MK cells) cultured in low-calcium serum-containing medium has been utilized in these studies (5). The keratinocytes require epidermal growth factor/transforming growth factor α for proliferation and retain the ability to differentiate under high-calcium conditions (6). The cells were found to be reversibly inhibited in their growth by TGF-β (7–9), with the majority of the cells blocked in the G_1 phase of the cell cycle (9). Induction of terminal differentiation, although reported for bronchial epithelial cells (10), does not appear to be a general phenomenon and did not occur in the keratinocytes (9).

The mechanism by which TGF-β inhibits cell proliferation is poorly understood. TGF-β1 does not appear to interfere with growth factor–receptor interactions or with transduction of at least some growth factor signals in skin keratinocytes (5, 8). However, TGF-β1 was found to selectively reduce c-*myc* expression (5). In view of previous data indicating that expression of c-*myc* plays an important role in cell proliferation (11), we investigated whether TGF-β1 inhibition of epithelial cell growth occurs as a consequence of down-regulation of c-*myc* gene expression. The results presented here demonstrate that c-*myc* expression is necessary for proliferation of the keratinocytes. Furthermore, we have shown that TGF-β1 inhibition of c-*myc* expression occurs at the level of transcriptional initiation and involves a cis-acting element in the 5′ region of the c-*myc* gene.

MATERIALS AND METHODS

Cell Culture. BALB/MK cells were passaged in low-calcium minimal essential medium (MEM) supplemented with epidermal growth factor (4 ng/ml) and 8% (vol/vol) dialyzed fetal calf serum as described (6).

Antisense Experiments. BALB/MK cells were plated in low-calcium MEM (4×10^4 cells per well), and experiments were performed when the dishes were 60–80% confluent, 24 hr after plating. Cells were treated with TGF-β1 (10 ng/ml) or c-*myc* oligonucleotides (30 μg/ml). The cells were labeled with [^{3}H]thymidine at 8 μCi/ml (1 Ci = 37 GBq) for 2 hr from 24 to 26 hr after addition of reagents. At least 750 nuclei per well were counted and the percentage of labeled nuclei was determined. To overcome degradation of oligonucleotides by nucleases the oligonucleotides have been modified on the backbone to phosphorothioates (12). Phosphorothioates were synthesized by a modification of the H-phosphonate procedure as described (13) and purified by ion-exchange chromatography and ethanol precipitation. Phosphorothioate oligonucleotides were 5′-labeled with T4 polynucleotide kinase and characterized by gel electrophoresis. The antisense phosphorothioate oligonucleotides were a racemic mixture; therefore, it is difficult to determine an accurate concentration of the active form. As a result the effective concentration is probably lower than that used.

Nuclear Run-On Transcription. BALB/MK cells were seeded at 9.5×10^5 cells per 100-mm plate; after 2 days the medium was changed, and TGF-β1 (10 ng/ml) was added for the indicated times. Nuclei from 2.0×10^7 cells were isolated per experimental point. Isolation of nuclei and nuclear run-on transcription assays were performed as described (14). Equivalent amounts (1.0×10^7 cpm) of trichloroacetic acid-precipitable deproteinized [^{32}P]RNA were used in the hybridization to Nytran filters on which 5.0 μg of the indicated denatured plasmids had been immobilized. [^{32}P]RNA·DNA filter hybridizations were as described (14). The following double-stranded probes were used: pSp70c-fos (coding region of c-fos cDNA), pGEM4Zc-jun (coding region of c-jun cDNA), pKC (15), and pSVc-myc (16).

Plasmids. To construct 5′ flanking deletion mutants of the human c-*myc* gene (*MYC* in human gene nomenclature)

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Abbreviations: TGF-β, transforming growth factor beta; CAT, chloramphenicol acetyltransferase.

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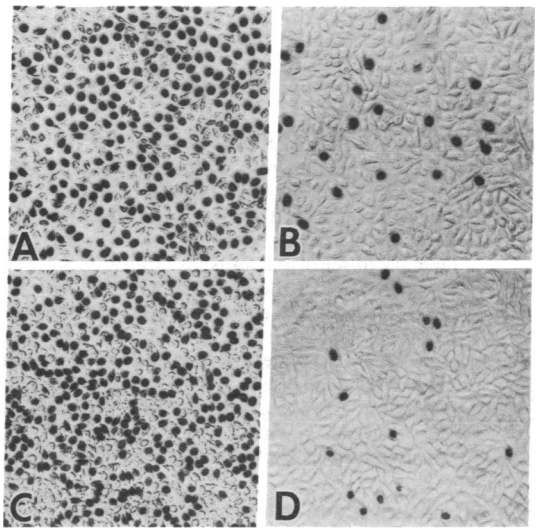

FIG. 1. Inhibition of DNA synthesis in rapidly growing BALB/MK cells treated with antisense c-*myc* oligonucleotides. The oligonucleotides used were identical or complementary to sequences at the beginning of the coding region of c-*myc* in exon 2 (5′-ATGCCCGTGAACGTT-3′). The effect of antisense c-*myc* oligonucleotide treatment in comparison to sense oligonucleotide and TGF-β1 treatment was determined by assaying changes in percent of labeled nuclei after incorporation of [^{3}H]thymidine during a 2-hr pulse period 24–26 hr after treatment, as visualized by autoradiography. (*A*) Control cultures of rapidly growing BALB/MK cells (58% labeled nuclei). (*B*) TGF-β1 (10 ng/ml)-treated rapidly growing BALB/MK cells (7% labeled nuclei). (*C*) Sense c-*myc* oligonucleotide (30 μg/ml)-treated rapidly growing BALB/MK cells (66% labeled nuclei). (*D*) Antisense c-*myc* oligonucleotide (30 μg/ml)-treated rapidly growing BALB/MK cells (6% labeled nuclei).

restriction sites in the 5′ flanking region of the gene plus a *Pvu* II site at nucleotide 510 relative to the P1 start site of transcription were used. Isolated fragments were subcloned into the chloramphenicol acetyltransferase (CAT) expression vector, pPLFCAT (17). The 5′ flanking restriction enzyme sites, *Hin*dIII, *Cla* I, *Pvu* II, *Sma* I, and *Xho* I, were used to generate the pPLFCAT/−2290, −1248, −350, −100, and +71 chimeras, respectively. To construct the SP6 c-myc RNA probe, a 635-base-pair *Hin*dIII–*Bgl* II fragment from pPLFCAT/−100 was subcloned into pSP73.

Cell Transfection and CAT Protein Analysis. BALB/MK cells were plated 1 day prior to transfection at 9.5×10^5 cells per 100-mm plate. Calcium phosphate DNA precipitates containing 20 μg of c-*myc* promoter/CAT plasmids and 5 μg of Rous sarcoma virus β-galactosidase plasmid were prepared by the method of Graham and van der Eb (18). After 6 hr of incubation with calcium-precipitated DNA, the cultures were treated with medium containing 25% (vol/vol) dimethyl sulfoxide for 3 min, washed with isotonic phosphate-buffered saline three times, and refed with growth medium. Twelve hours later, the cells were treated with TGF-β1 (10 ng/ml). Cell extracts were prepared 18–24 hr after treatment and assayed for CAT by using a nonradioactive CAT assay (19). The amount of protein extract utilized in the CAT assay was normalized to β-galactosidase activity.

RNase Protection Analysis. BALB/MK cells were plated and transfected with 20 μg of pPLFCAT/−100 and 5 μg of OVEC REF, a simian virus 40-enhancer-driven rabbit β-globin expression plasmid (20). Twelve hours after transfection, the cells were treated with TGF-β1 (10 ng/ml) for 24 hr and total cellular RNA was isolated by the guanidinium thiocyanate procedure, followed by centrifugation through CsCl (21). RNA was analyzed by the RNase protection procedure as described (22). Transcription of the SP6 c-myc RNA probe by SP6 RNA polymerase produced an RNA corresponding to the region between positions −100 and +610 of the c-*myc* gene (relative to the site of initiation for P1 being at position +1) and 14 nucleotides of 3′ flanking polylinker sequence from pPLFCAT/−100 from which it was subcloned. Total RNA (40 μg) plus 5×10^5 cpm of labeled SP6 c-myc RNA probe were hybridized at 45°C for 12–14 hr. Samples were treated with RNase A and proteinase K as described (22). RNase-resistant fragments were analyzed by PAGE on a 5% polyacrylamide denaturing gel.

RESULTS

Treatment of BALB/MK Cells with Antisense c-*myc* Oligonucleotides Inhibits Cell Proliferation. Rapidly growing BALB/MK cells were treated with antisense c-*myc* oligonucleotides; sense oligonucleotides were used as a control. The untreated control cells had 58% labeled nuclei. Cultures treated with sense c-*myc* oligonucleotides had a labeling index of 66%. TGF-β1 and antisense c-*myc* oligonucleotides markedly reduced the labeling index to 7% and 6%, respectively (approximately 90% inhibition) (Fig. 1). Thus, both TGF-β1 and antisense c-*myc* oligonucleotides inhibited the entry of BALB/MK cells into S phase with similar efficacy. In addition, c-myc protein synthesis was reduced in BALB/MK cells treated with either TGF-β1 or antisense c-*myc* oligonucleotides (J.A.P., J.T.H., H.L.M., and R. M. Lyons, unpublished data). These results suggest that the expression of c-*myc* is necessary for keratinocyte proliferation. Furthermore, the data support the hypothesis that the effects of TGF-β1 are mediated through c-*myc*. The mechanism by which TGF-β1 reduces c-*myc* expression was examined in more detail.

Transcriptional Regulation of Specific Oncogenes by TGF-β1. The effect of TGF-β1 on the transcription of c-*myc* was compared to three other growth-related genes: c-*fos*, c-*jun*, and KC (15). TGF-β1 dramatically reduced c-*myc* and KC transcription; however, it had little or no effect on c-*fos* or c-*jun* transcription (Fig. 2). The kinetics of inhibition of c-*myc* and KC transcription were similar; however, only the mechanism of TGF-β1 inhibition of c-*myc* gene transcription was further investigated.

Inhibition of c-*myc* Transcriptional Initiation by TGF-β1. The regulation of c-*myc* transcription can occur at two levels, initiation and elongation (23–26). To determine whether the

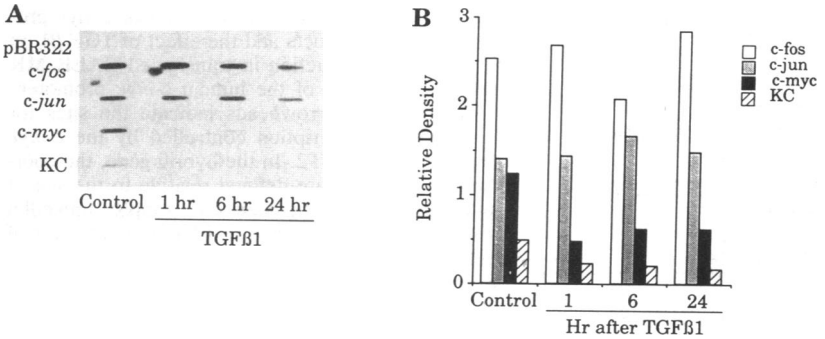

FIG. 2. Effect of TGF-β1 on oncogene transcription in isolated BALB/MK nuclei from rapidly growing BALB/MK cells. (*A*) A time-course experiment examining the effect of TGF-β1 treatment on oncogene transcription. Nuclear run-on transcripts were hybridized with the following double stranded-probes: c-*fos*, c-*jun*, c-*myc*, and KC. (*B*) Laser densitometry of the nuclear run-on shown in *A*.

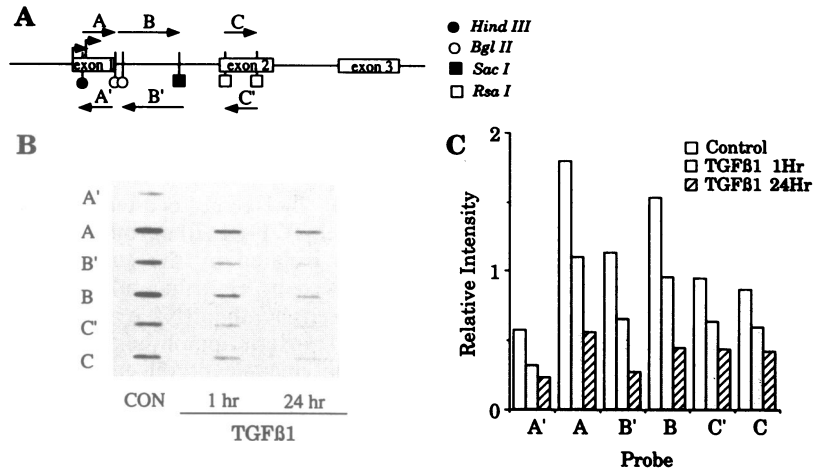

FIG. 3. Effect of TGF-β1 on c-*myc* transcription in isolated nuclei from rapidly growing BALB/MK cells. (*A*) The locations of murine c-*myc* DNA fragments cloned into M13 vectors are indicated; only the pertinent restriction sites are shown. Probes complementary to sense and antisense transcripts are shown above and below the map, respectively. (*B*) A time-course experiment examining the effect of TGF-β1 treatment on c-*myc* transcription by using strand-specific c-*myc* gene DNA probes. The number of uridine residues present in the region by each probe is as follows. Probes: A and A′, 94 residues; B and B′, 199 residues; C and C′, 102 residues. (*C*) Laser densitometry of nuclear run-on is shown in *B*.

reduction of c-myc mRNA expression after TGF-β1 treatment of BALB/MK cells was due to inhibition of transcriptional initiation or elongation, nuclear run-on transcripts were hybridized with single-stranded DNA probes derived from various portions of the c-*myc* locus. If TGF-β1 blocked the elongation of c-myc mRNA, we would have expected to detect less transcription across the first intron and second exon relative to the first exon (23). In contrast, an inhibition of transcriptional initiation would result in a similar decrease in signal across the first exon, first intron, and second exon. Within 1 hr after TGF-β1 treatment of BALB/MK cells, there was a sustained decrease of transcriptional activity of the c-*myc* gene across the first exon, first intron, and second exon [Fig. 3 *B* and C, compare the transcription signals with probe A (first exon), probe B (first intron), and probe C (second exon)]. TGF-β1 treatment also reduced antisense transcription across the first exon, first intron, and second exon of the c-*myc* gene [Fig. 3 *B* and *C*, see the results in which RNA was hybridized with the antisense probe A′ (first exon), B′ (second exon), and C′ (third exon)]. However, the effect of TGF-β1 was selective since transcription of 1B15 [a cyclosporin A binding protein (27) used as a control], as detected by single-stranded probes, was not significantly affected by TGF-β1 (data not shown). The results obtained with the probes that detect sense transcription suggest that TGF-β1 reduction of c-*myc* expression occurred primarily at the level of transcriptional initiation and not through a block to transcriptional elongation.

TGF-β1 Inhibits Transcriptional Initiation from the P2 Promoter. To further investigate specific sequences in the c-*myc* gene that are necessary for TGF-β1 repression of transcriptional initiation, deletion mutagenesis was performed in which various portions of the 5′ flanking region of the human c-*myc* gene were subcloned into a bacterial CAT expression vector (Fig. 4*A*). The c-*myc* promoter/CAT chimeras, pPLFCAT/−2290, −1248, −350, −100, and +71 were designated according to the 5′ nucleotide position relative to the c-*myc* P1 transcription start site (+1); all deletion mutants had 3′ flanking c-*myc* gene sequence to position +510. These expression vectors did not contain the sequences in exon 1 shown to be necessary for attenuation (28); therefore, we were studying regulation of transcriptional initiation. The effect of TGF-β1 treatment on CAT protein expression from 5′ flanking mutants extending to positions −2290, −1248, −350, −100, and +71 was determined in transfected BALB/MK cells (Fig. 4*B*). The protein expression from the chimeras was compared in TGF-β1-treated and untreated cells. CAT protein production arising from the chimeras with upstream sequences from positions −2290 to −100 was reduced by 50%–60% after treatment with TGF-β1. In contrast, TGF-β1 did not reduce CAT protein levels from pPLFCAT/+71. TGF-β1 did not affect CAT mRNA or protein stability, since CAT protein produced from expres-

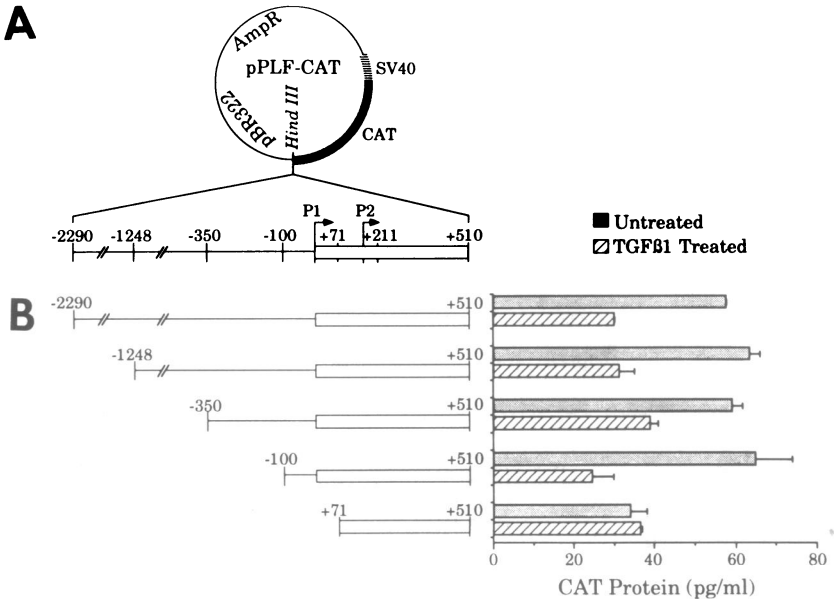

FIG. 4. Schematic representation of c-*myc* promoter/CAT constructs and the effect of TGF-β1 on CAT protein production in transfected BALB/MK cells. (*A*) Diagram of the human c-*myc* promoter/CAT chimeras. Arrowheads indicate the sites for initiation of transcription controlled by the c-*myc* promoters, P1 and P2. In the hybrid gene, the coordinates for c-*myc* are defined relative to the site of initiation for P1 (designated +1). AmpR, ampicillin resistance; SV40, simian virus 40. (*B*) Quantitation of CAT protein production in untreated and TGF-β1-treated transfected cells. These results are from three experiments and are expressed as mean with the standard deviation depicted by the error bars.

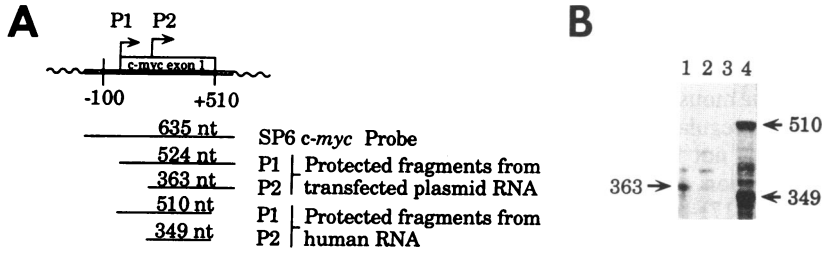

FIG. 5. TGF-β1 inhibition of transcription from the c-*myc* P2 promoter in pPLFCAT/−100. (*A*) The diagram illustrates the RNA probe constructed from pPLFCAT/−100 and the size of its RNase-protected fragments. The RNA probe contains a sequence of the c-*myc* gene from position −100 to position +510 and 14 base pairs of 3′ flanking polylinker sequences. The predicted size of the protected mRNA fragments for pPLFCAT/−100 and human c-*myc* gene is shown in nucleotides (nt). (*B*) BALB/MK cells were transfected with pPLFCAT/−100 to +510. A 635-nucleotide RNA probe encompassing the region between positions −100 and +510 region of the human c-*myc* gene was hybridized as follows: Lanes: 1, total RNA from pPLFCAT/−100 transfected BALB/MK cells; 2, total RNA from pPLFCAT/−100 transfected BALB/MK cells treated with TGF-β1; 3, tRNA alone; 4, total RNA from HeLa cells.

sion vectors driven by the Rous sarcoma virus and the simian virus 40 enhancers were unaffected by TGF-β1 treatment (data not shown).

To establish that transcription initiated correctly within the transfected plasmids and that the decrease in CAT protein levels after TGF-β1 treatment was due to a decrease in the levels of c-myc/CAT RNA, steady-state RNA was measured by quantitative RNase protection analysis. An RNA probe encompassing the region from position −100 to position +510 of the human c-*myc* gene was utilized (Fig. 5*A*). The effect of TGF-β1 treatment on myc/CAT mRNA initiating from the P1 and P2 promoters in pPLFCAT/−100 was measured (Fig. 5*B*). The total myc/CAT RNA levels in treated and untreated cell were normalized to the β-globin RNA levels obtained from a cotransfected simian virus 40-enhancer-driven rabbit β-globin expression plasmid (20). TGF-β1 treatment of BALB/MK cells reduced initiation of transcription from the P2 in pPLFCAT/−100 by a factor of 3.5 (Fig. 5*B*). This result corroborates the reduction of CAT protein expression seen after TGF-β1 treatment of BALB/MK cells transfected with pPLFCAT/−100 (Fig. 4*B*). The relative steady-state mRNA levels produced in the transfected BALB/MK cells mirrored the CAT protein levels shown in Fig. 4. The level of P1-initiated mRNA was low in these cells as compared to P2, and as a result it was difficult to determine if P1 transcription was also repressed by TGF-β1.

The sequence similarity between the human and murine c-*myc* genes from nucleotides −100 to +71 is ≈70% (29). To test whether the corresponding region of the murine c-*myc* gene was also necessary in TGF-β1 regulation, we constructed murine c-*myc* promoter/CAT chimeras and analyzed the effect of this region in repression. We found that the corresponding sequences of the murine c-*myc* gene were necessary for TGF-β1 repression (data not shown). Thus, the regulatory mechanism(s) involved in TGF-β1 repression appeared to be conserved between mouse and human c-*myc* genes.

DISCUSSION

One of the goals of this study was to understand the molecular mechanisms through which TGF-β1 inhibits cellular proliferation. A major clue, provided by previous findings (5), was that TGF-β1 selectively reduced mRNA expression of a well-studied growth-controlling gene: the c-*myc* nuclear protooncogene. TGF-β1 reduction of c-*myc* has been detected in a variety of epithelial cells and many resistant phenotypes that fail to respond to TGF-β1 by c-*myc* reduction are not growth-inhibited (30–32). This led us to hypothesize that TGF-β1 repression of c-*myc* may play an important role in growth inhibition. The role of c-*myc* in keratinocyte proliferation and the mechanism of TGF-β1 regulation of c-*myc* expression were, therefore, examined.

The mouse keratinocytes utilized in this study display cell-cycle-specific arrest in the G_1 phase in response to TGF-β1. TGF-β1 down-regulated c-*myc* mRNA levels and this reduction required protein synthesis (5). To further investigate the role of c-*myc* in the regulation of BALB/MK cell growth, c-*myc* antisense oligonucleotides were utilized. Previously, antisense c-*myc* oligonucleotides and expression vectors have been utilized to inhibit a variety of nonepithelial cell types (11). Treatment of the BALB/MK cells with the antisense c-*myc* oligonucleotides resulted in keratinocyte arrest to the same degree as observed in the TGF-β1-treated cultures. These data provide additional evidence in support of a causative role for TGF-β1 reduction of c-*myc* in the growth-inhibitory response.

Nuclear run-on analysis demonstrated that TGF-β1 selectively down-regulated c-*myc* and KC transcription. Actinomycin D chase experiments performed on the keratinocytes failed to reveal any significant change in the c-myc mRNA turnover (data not shown). The half-life of c-myc mRNA before and 30 min after TGF-β1 treatment was 20–25 min. An earlier study concluded posttranscriptional inhibition based on nuclear run-on assays (5). However, these analyses were carried out with double-stranded exon 2–3 probes and did not distinguish between inhibition of transcriptional initiation and attenuation or between effects on sense and antisense transcription. In the present study, we have used single-stranded probes specific for detection of sense and antisense transcription across exon 1, intron 1, and exon 2 to distinguish between two potential levels of regulation of c-*myc* transcription: initiation and elongation (23). TGF-β1 rapidly reduced c-*myc* transcription in both the sense and antisense directions across exon 1, intron 1, and exon 2 in rapidly growing BALB/MK cells. Previous studies have demonstrated (26, 33) both regulation and a lack of regulation of antisense c-*myc* transcription, depending on the cell type and physiological condition. The significance of antisense c-*myc* transcription generally and its regulation by TGF-β1 specifically is unknown.

Through the use of 5′-deletion mutants and RNase protection analyses, it was determined that sequences present within the region from position −100 to position +71 of the c-*myc* promoter were necessary for TGF-β1 repression of c-*myc* P2 transcriptional initiation. It is of interest to note that CAT expression in BALB/MK cells transfected with pPLFCAT/+71 was not only unregulated by TGF-β1 but was also lower than that produced in cells transfected with all the other deletion mutants. This result suggests that in addition to a negative regulatory element being present in the region between positions −100 and +71 region, there also may be an element necessary for positive modulation of c-*myc*. In fact, other groups have suggested that both positive and negative modulators do not act separately on c-*myc* promoter but can

compete or act synergistically to regulate gene expression (34, 35).

Negative upstream elements have been identified (28, 34–38) in both the human and the mouse c-*myc* gene that have been shown to be important in regulation of its expression. However, these elements have not been shown to be involved in growth-factor regulation of c-*myc*, and none of these elements are present in the 171-base region identified in this study, except for a negative regulatory region described by Lipp *et al.* (35). However, our study is unique in that we demonstrate that specific sequences in the c-*myc* gene are necessary for regulation by a growth factor, TGF-β1.

Similar to this report, TGF-β1 regulation of the type I collagen gene (39), the TGF-β1 gene (40), and the transin gene (41) has been demonstrated to occur at the level of transcription through potential trans-acting factors that interact with specific promoter elements. None of the specific elements identified in these reports are present within the 171-base region identified in our studies.

Protein synthesis has been shown to be required for TGF-β1 repression of c-*myc* expression (5), suggesting stimulation of the synthesis of a cellular protein or modification of a preexisting factor that may interact in a direct or indirect manner with the DNA. Two extreme models of several can be proposed to explain a potential mechanism(s) of TGF-β1 regulation of c-*myc* expression. In one model TGF-β1 could function by repressing or modifying a positive factor required for c-*myc* expression. In a similar manner as reported in this study, TGF-β1 may down-regulate other proliferation-associated genes that may be involved in regulating c-*myc*. In a second model, TGF-β1 functions in a direct or indirect manner to induce or modify factors involved in negative regulation of cell growth. We have presented evidence suggesting involvement of the retinoblastoma gene product in the pathway of TGF-β1 inhibition of c-*myc* expression (42).

In conclusion, our studies present strong evidence that expression of c-*myc* is necessary for proliferation of an epithelial cell model, the skin keratinocyte, and that TGF-β1 reduction of c-*myc* expression is due to inhibition of transcriptional initiation. Another finding of these studies is the identification of a regulatory region in the c-*myc* gene, which is necessary for TGF-β1 repression of c-*myc* transcriptional initiation. These results may be important in the general mechanisms of TGF-β1 regulation of gene expression and inhibition of cell proliferation.

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