

Transcriptional Regulation of the Transforming Growth Factor β 1 Promotor by *v-src* Gene Products Is Mediated through the AP-1 Complex

MARIA C. BIRCHENALL-ROBERTS,^{1,2*} FRANCIS W. RUSCETTI,¹ JAMES KASPER,² HY-DE LEE,³
ROSALIND FRIEDMAN,² ANDREW GEISER,³ MICHAEL B. SPORN,³ ANITA B. ROBERTS,³
AND SEONG-JIN KIM³

Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute, Frederick Cancer Research Facility, P.O. Box B,¹ and Biological Carcinogenesis Development Program, Program Resources, Inc., National Cancer Institute, Frederick Cancer Research Facility,² Frederick, Maryland 21701, and Laboratory of Chemoprevention, National Cancer Institute, Bethesda, Maryland 20892³

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Growth factor-independent 32D-*src* and 32D-*abl* cell lines, established by infecting the interleukin-3-dependent myeloid precursor cell line (32D-123) with retroviruses containing the *src* or *abl* oncogene, were used to study transcriptional regulation of transforming growth factor β 1 (TGF- β 1) mRNA. Analysis of different TGF- β 1 promoter constructs regulated by pp60^{v-*src*} indicated that sequences responsive to high levels of *src* induction contain binding sites for AP-1. Both *src* and serum induced expression of the *c-fos* and *c-jun* genes in myeloid cells, resulting in transcriptional activation of the TGF- β 1 gene. We found that serum treatment increased TGF- β 1 mRNA levels in 32D-123 cells and that the v-Src protein could replace the serum requirement by stimulating binding to the AP-1 complex of the TGF- β 1 promoter, thereby mediating the induction of TGF- β 1 transcription.

Transforming growth factor β 1 (TGF- β 1) is a polypeptide hormone that exerts pleiotropic effects during the growth, differentiation, and function of nearly all cell types. Recent studies favor the possibility that autocrine regulation may be partially responsible for the regulation of cell growth by TGF- β 1. Van Obberghen-Schilling et al. (20) have shown that TGF- β 1 positively regulates its own expression in normal and transformed cells. Several attempts to understand the mechanisms of this regulation suggest that TGF- β 1 expression is not governed by the classical pathways of growth factor signal transduction (e.g., those utilizing cyclic AMP as a second messenger or the inositol phosphate pathway, which involves protein kinase C activation) (4, 16).

To elucidate the biochemical pathways that signal the regulation of TGF- β 1 gene expression, we have used a unique system in which an interleukin-3 (IL-3)-dependent murine myeloid cell line [(32D-123; 8)] was made growth factor independent by infection with Moloney murine leukemia virus containing *src* (32D-*src*) and *abl* (32D-*abl*) (11a). Total RNA was isolated from the cell lines (5) and was further subjected to Northern (RNA) blot analysis (7). In the presence of serum, levels of TGF- β 1 mRNA expression were high in both cell lines; however, 32D-*src* cells expressed significantly higher levels of TGF- β 1 mRNA than did the parental 32D-123 and *abl*-transformed cell lines (32D-*abl*) when the cells were grown without serum (Fig. 1, lanes 3, 6, and 9). Although the expression of TGF- β 1 mRNA in these cell lines is not novel (12, 18), our finding that this mRNA was expressed at high levels in the absence of serum in 32D-*src* cells, but not in 32D-123 and 32D-*abl* cells, suggests that the *src* protein may be involved in the transcriptional regulation of the TGF- β 1 gene.

Activation of the TGF- β 1 promoter by pp60^{v-*src*}. Plasmids

phTG5 and phTG16, which contain the first and second promoters of the TGF- β 1 gene linked to the chloramphenicol acetyltransferase (CAT) gene (11), were transiently expressed in 32D-123 and A549 (human lung adenocarcinoma) cells (Fig. 2A) so that we could analyze their activity when cotransfected in the presence or absence of the pM5HHB5 (pp60^{c-*src*} protein) or pMvsrc (pp60^{v-*src*} protein) expression vectors (8). Cotransfection with the pp60^{v-*src*} expression vector increased expression of the phTG5 promoter 17- and 31-fold in 32D-123 and A549 cells, respectively, whereas expression of the phTG16 promoter was increased 20-fold in both cell lines (Fig. 2A). In contrast, when the phTG5 or phTG16 construct was cotransfected with the pM5HHB5

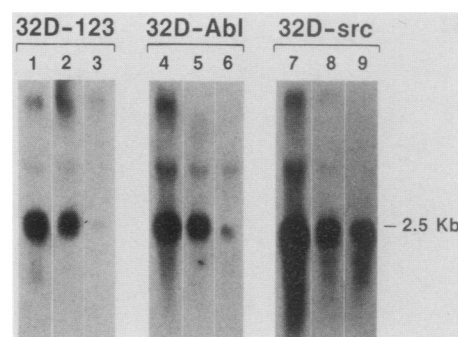


FIG. 1. Induction of TGF- β 1 mRNA by serum. Total RNA was isolated from 32D-123 (IL-3, 100 U/ml), 32D-*abl*, and 32D-*src* cells growing in the presence of 10% fetal calf serum at two cell densities (1.5×10^5 cells per ml [lanes 2, 5, and 8] and 2.5×10^5 cells per ml [lanes 1, 4, and 7]) and from cells maintained under serum-free conditions (2.5×10^4 cells per ml [lanes 3, 6, and 9]) for 48 h. Northern blots (15 μ g of RNA per lane) from 32D-123, 32D-*abl*, and 32D-*src* cells were hybridized by using the TGF- β 1 probe. Ethidium bromide staining of the rRNA indicated that similar levels of undegraded RNA were present in all of the samples (data not shown).

* Corresponding author.

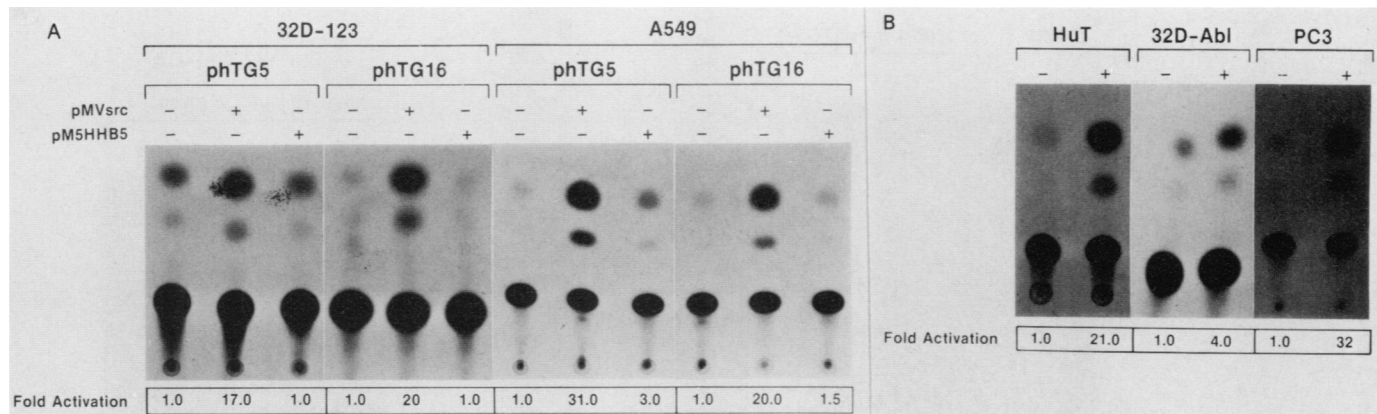


FIG. 2. Activation of plasmids pH TG5 and pH TG16 by pp60^{v-src} in different cell lines. Within the first promoter, construct pH TG5 (-453 to +11) has been shown to stimulate the greatest level of CAT activity in different cell types (8), because it contains no negative regulatory elements and includes an AP-1 (-374 to -361)-binding site that is commonly transcriptionally induced. The second promoter contains two AP-1 regulatory regions (+155 to +170 and +247 to +289) (9). 32D-123, 32D-src, 32D-abl, and human T (HuT) cells (10⁷/250 μl) were transfected by electroporation. After the addition of DNA (10 μg of TGF-β1 promoter and 1 μg of v-src expression vector or pUC18), the cells sat on ice for 10 min. They were then electroporated in a Bio-Rad gene pulser system (250 V, 960 μF) and sat on ice for 10 min. Stimulating agents were added after 1 h of culture. A549 and PC3 cells were transfected by the calcium phosphate coprecipitation method as previously described (9). Extracts were prepared by freeze-thaw disruption of cell pellets. Equal amounts of protein were used to assay for the CAT enzyme, according to the method of Gorman et al. (10). (A) Activation in 32D-123 and A549 cells. Cells were cotransfected with the TGF-β1 promoters (pH TG5 and pH TG16) plus pMvsrc or pM5HHB5 (c-src). (B) Activation of the TGF-β1 promoters by pp60^{v-src} in other cell lines. PC3, 32D-abl, and human T cells were cotransfected with the pH TG5 TGF-β1 promoter in the presence (+) or absence (-) of pMvsrc. The numbers represent fold activity of stimulated vectors over control levels; the control consisted of each TGF-β1 promoter CAT construct expressed without further stimulation. Results are averages of at least three transfections. To observe the differences in the intensity of induction of pH TG5 by v-src, the CAT assay for v-abl was exposed for 6 h instead of overnight. (C) Identification of the TGF-β1 promoter regions required for activation by pp60^{v-src}. Top, Structure of the TGF-β1 promoter deletion constructs and activation by pMvsrc. The structure of the human TGF-β1 promoter region, indicating the two major transcription initiation sites (P1 and P2) and the AP-1-binding sites, is shown. Middle, Plasmids pH TG2 through pH TG7 (5' deletion mutants) were cotransfected in A549 cells with pMvsrc (+) or pUC18 (-). Fold activation refers to the ratio between CAT activity in A549 cells cotransfected with the TGF-β1 promoter plus pMvsrc constructs and CAT activity in cells transfected with the TGF-β1 promoter; values represent averages of three independent experiments. Bottom, Plasmids pH TG16 through pH TG28 were cotransfected in A549 cells with pMvsrc (+) or pUC18 (-). Values represent averages of three independent experiments.

(pp60^{c-src}) vector in either cell line, the levels of CAT expression were comparable to the basal levels observed with either promoter alone.

We further examined the regulation by v-src of expression of the first TGF-β1 promoter in normal cells (human T cells transfected 3 days after the addition of phytohemagglutinin [0.5 μg/ml] and IL-2 [200 U/ml]) and transformed cells (32D-abl and PC3, a prostate adenocarcinoma cell line). In these cells, we detected 21-, 4.8-, and 32-fold increases, respectively, in TGF-β1 promoter expression in the presence of the pp60^{v-src} protein (Fig. 2B). v-Abl protein, which is highly expressed in these cells (11a), was also a potent transactivator of the TGF-β1 promoter, so that v-src did not increase the transcription as markedly as in other cell lines. Overall, these results suggest that pp60^{v-src} can regulate TGF-β1 expression in various cell types and that these effects are mediated through the two previously described promoters of the TGF-β1 gene (11).

Identification of the sequences in the TGF-β1 upstream region responsible for transactivation by pp60^{v-src}. Deletion plasmids (containing sequences located 5' to the upstream transcriptional start site [the first promoter] and between the two major transcriptional initiation sites [the second promoter] of the TGF-β1 gene [10; Fig. 2C]) were cotransfected into A549 cells, in the presence and absence of the pMvsrc expression vector, in order to measure the levels of their expression. Deletion analysis of the first promoter CAT constructs revealed an increase in CAT activity when the plasmids (pH TG2, pH TG3, pH TG4, pH TG5, pH TG6, and

pH TG7) were cotransfected with plasmid pMvsrc (Fig. 2C). The induction level of CAT expression of these vectors varied between 11-fold (pH TG7) and 28-fold (pH TG5). The highest level of expression was observed with the pH TG5 CAT construct, which contained an AP-1 site (AP-1⁺), suggesting that the AP-1 site (-374 to -361) is involved in the regulation of CAT expression by the v-src gene product. In contrast, the pH TG7 CAT construct, which does not contain an AP-1 site (AP-1⁻), showed an 11-fold increase in CAT induction. This finding suggests that other elements are involved in the regulation by v-src of the TGF-β1 promoter.

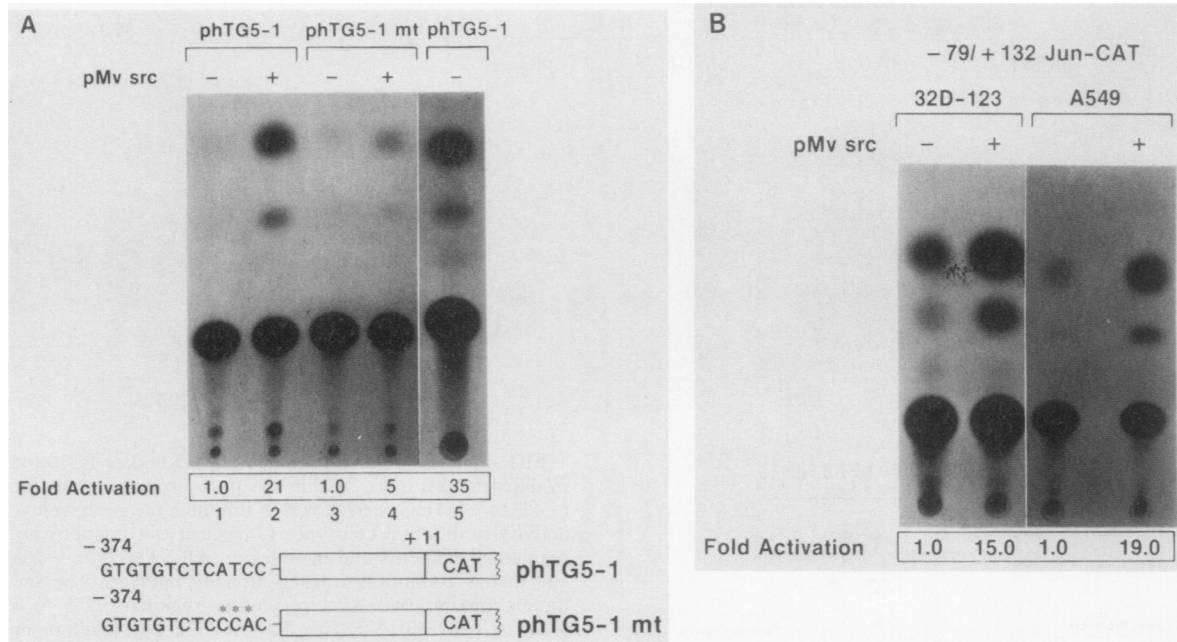


FIG. 3. Further characterization of TGF- β 1 promoter sequences involved in transactivation by *v-src*. (A) The phTG5-1 and phTG5-1mt constructs were prepared as described in the text. A549 cells were cotransfected with phTG5-1 or phTG5-1mt in the presence or absence of the pMvsrc construct. Values represent averages of at least three transfections. Lane 5 shows an increase in AP-1 activity of the TGF- β 1 promoter (phTG5-1) when transfected in the 32D-src cell line in the absence of the pMvsrc expression vector. (B) Stimulation of the *jun* promoter by *v-src* as shown by activation of plasmids -79/+132-jun-CAT by pMvsrc in the 32D-123 and A549 cell lines. Each plasmid was cotransfected into the cells in the presence or absence of the pMvsrc construct. After transfection, CAT activities were assayed as described in the legend to Fig. 2. The numbers indicate fold increases in activity of the stimulated vectors over control levels and represent averages of at least three transfections.

Similarly, CAT activity increased when second promoter-CAT constructs (phTG16, phTG18, phTG22, and phTG26) were cotransfected with the pMvsrc construct (Fig. 2C). The levels of CAT induction by the *v-src* gene varied between 14-fold (phTG26) and 20-fold (phTG16 and phTG18). However, induction by pp60^{*v-src*} dropped almost to the basal level when the sequences between +145 and +173 were deleted (phTG22). CAT expression was induced again when the deletion reached +247, suggesting the presence of a negative regulatory element. The 14-fold increase in CAT induction with the phTG26 construct correlates with the presence of an AP-1-binding site. With use of the phTG28 construct (AP-1⁻), CAT expression was only 1.4-fold above basal levels. These results show that in the second promoter of TGF- β 1, the AP-1 site is essential for *v-src* induction. Several plasmid controls, including the Moloney murine leukemia virus vector pEVX, pATV-8, the *c-src* vector pM5HHB5, and pUC18, induced significantly lower levels of TGF- β 1 promoter expression (data not shown). The finding of high levels of CAT expression with phTG5 (one AP-1 site), phTG18 (two AP-1 sites), and phTG26 (one AP-1 site) strongly supports the notion that AP-1 proteins participate in the regulation of the TGF- β 1 promoter by the pp60^{*v-src*} protein.

Since phTG5 demonstrated the greatest level of CAT induction for the first TGF- β 1 promoter (28-fold) by the *v-src* gene product, we further characterized the sequences of this construct responsible for *v-src* transactivation. Plasmids phTG5-1 and phTG5-1mt were generated by polymerase chain reaction amplification, using oligonucleotides designed to generate ends with the *Hind*III and *Xba*I restriction sites, and subcloned into the CAT vector cut with the same enzymes. Plasmid phTG5, which contains the sequence identified as the TGF- β 1 AP-1-binding site (11), was used as

a template for the polymerase chain reaction, and the primers were GTGTGTCTCATCCCCCGGA (wild type; -374 to -355) or GTGTGTCTCCCACCCCCGGAG (AP-1 mutated) and CGAGGGAGGTGGGAG (-5 to +11). The level of CAT protein expression by the phTG5-1 CAT vector was increased 21-fold by the *v-src* gene, whereas the level of expression of the phTG5-1mt CAT protein increased by only 5-fold (Fig. 3A). Expression of the phTG5-1 construct in 32D-src cells was greatly augmented over its expression in parental 32D cells (Fig. 3A). These results strongly suggest that the *v-src* gene product induces an element that binds to the AP-1-binding site of the first TGF- β 1 promoter. We are currently completing studies in which we have demonstrated the essentiality of the AP-1 site of the first TGF- β 1 promoter by completely mapping the different elements of the phTG7 CAT construct (unpublished data).

To determine whether AP-1-binding sites are at least partially responsible for transactivation by the *v-src* gene, we next studied the transactivation of constructs containing *c-jun* promoter sequences (-79 to +132, AP-1⁺), which contain only an AP-1 sequence (1). Expression of -79/+132-jun-CAT was increased 15- and 19-fold when these constructs were cotransfected into 32D-123 and A549 cells, respectively, in the presence of the pMvsrc construct (Fig. 3B). In the absence of pMvsrc, only basal levels of *c-jun* expression were observed in serum-treated 32D-123 cells. These and previously published data demonstrating that pp60^{*v-src*} transactivates the *c-fos* promoter (8) further support our initial findings, which suggest that the induction of TGF- β 1 mRNA expression by the *v-src* gene is due in part to the regulation of *trans* elements (*c-Jun* and *c-Fos* proteins) that bind the AP-1-binding site of the TGF- β 1 promoter.

Relative activity of the two TGF- β 1 promoters. We consid-

ered the possibility that the presence of one AP-1 site in the first promoter and two AP-1 sites in the second promoter may account for the different levels of transcriptional activity (Fig. 2C). Two S1 nuclease-resistant fragments (Fig. 4) were evident after hybridization of total RNA with the 1.7-kilobase *Xba*I-*Bgl*III mouse TGF- β 1 promoter fragment. These two fragments represent the major transcriptional start sites at the 5'-most end of the murine TGF- β 1 cDNA, as described previously (11). The very similar intensities of these two bands (Fig. 4) within each of the cell lines indicate that equal levels of transcriptional activity are associated with each promoter.

***c-fos* and *c-jun*: analysis of mRNA expression and promoter activity.** Because we previously demonstrated that autoinduction of TGF- β 1 expression is mediated by binding of the AP-1 (Jun-Fos) complex (14), we next studied the expression of the *c-fos* and *c-jun* genes in 32D-src and 32D-123 cells. Northern blot analysis of total RNA isolated from 32D-src cells (grown without serum for 48 h) demonstrated the constitutive expression of the *c-fos* and *c-jun* mRNAs (Fig. 5A). RNAs isolated from 32D-123 cells grown without IL-3 or serum were used as a control. *c-fos* and *c-jun* mRNA expression was not detectable on Northern blots (data not shown). TGF- β 1 mRNA expression was also observed (Fig. 1 and 5A). These results are consistent with our analysis of the genes induced by *v-src*, which suggests that the constitutive expression of the *c-fos* and *c-jun* genes is followed by constitutive expression of TGF- β 1. These cells also expressed glyceraldehyde phosphate dehydrogenase mRNA, which was used as a control for the quantification of the RNAs under study.

Expression of *c-fos* by 32D-123 cells was measured by using the *c-fos* promoter (-404/+42-*fos*-CAT) constructs. Serum stimulated a 6-fold increase in basal *c-fos* CAT levels (-404/+42-*fos*-CAT), which were further augmented by IL-3 (25-fold) (Fig. 5B). Interestingly, IL-3 by itself significantly increased *c-fos* CAT expression (30-fold). The *c-fos* mutated promoter CAT construct (-307/+42-*fos*-CAT) induced only a threefold increase over basal levels (no serum or IL-3 induction) of CAT expression.

The work reported here demonstrates two possible ways of regulating TGF- β 1 gene expression. First, the *v-src* gene is capable of stimulating TGF- β 1 mRNA transcription (in hematopoietic progenitor cells); second, serum induces high levels of TGF- β 1 mRNA in 32D-123 cells. Both means of regulating TGF- β 1 mRNA transcription appear to be mediated by *trans*-acting elements of the TGF- β 1 promoter.

Delineation of the *src*-responsive element in TGF- β 1. Detailed analysis of the TGF- β 1 promoters indicated that sequences responsive to high levels of induction by *src* were localized at -453 to +11 and +145 to +289 upstream of the 5' end (Fig. 2C). This region contains a high-affinity binding site for AP-1 (-365 to -371) which, although not identical to the prototype AP-1, has been previously shown to respond to TGF- β 1 autoinduction (12). A mutation introduced in the AP-1-like element abolished the responsiveness of this vector to *src* induction (Fig. 3A). However, we also found that other promoter constructs lacking AP-1-like elements were induced to a lesser extent by *src*, suggesting that an undetermined element(s) also participates in the induction process.

Transcriptional control. (i) Modulation of AP-1 activity by *src*. Other studies (8) demonstrated that the Src protein activates various promoters, including the *c-fos* promoter. The constitutive expression of *c-jun* in 32D-src cells and the activation of the *c-jun*-CAT promoter by pp60^{v-src} in our

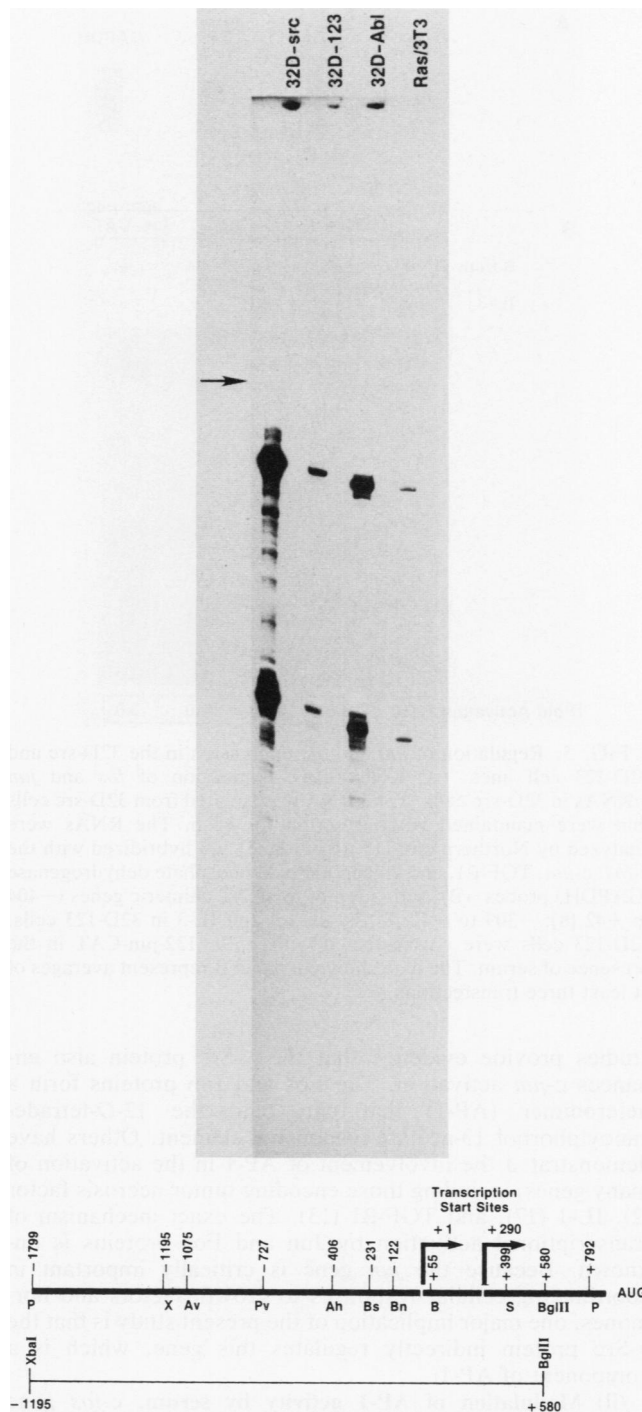


FIG. 4. S1 nuclease protection assay. The activity of the two TGF- β 1 promoters was studied with total RNA isolated from serum-stimulated cells (32D-src, 32D-123, 32D-abl, and control NIH 3T3 *ras*-transformed fibroblasts [Ras/3T3]). The S1 probe was generated by end labeling a 1.7-kilobase *Xba*I-*Bgl*III (-1195 to +577) mouse TGF- β 1 promoter fragment with [γ -³²P]ATP, which was then isolated from an agarose gel and purified on glass beads. The probe (7.5×10^4 cpm) was hybridized with 60 μ g of total RNA for 16 h at 55°C. Probe hybridization and S1 nuclease digestion buffers and conditions were those described by Kim et al. (11). The arrow indicates the migration of the 1.7-kilobase *Xba*I-*Bgl*III fragment. The two S1-resistant fragments (580 and 290 nucleotides) visible on the gel represent transcription initiated at the first (top, larger transcript) and second (bottom, smaller transcript) transcriptional start sites.

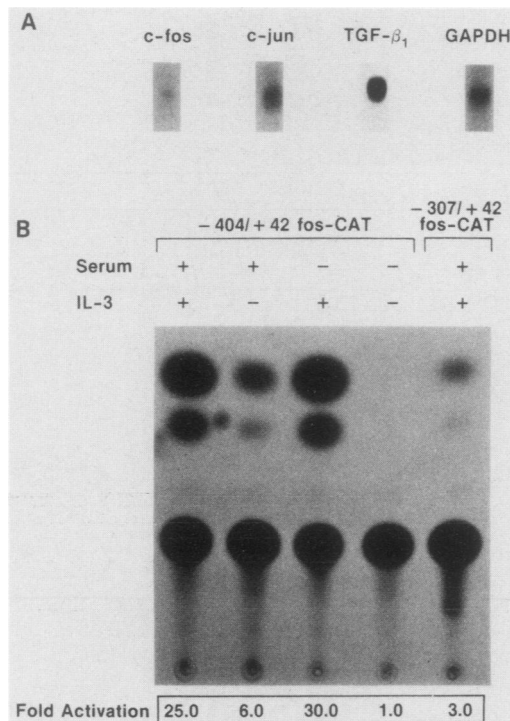


FIG. 5. Regulation of *jun* and *fos* expression in the 32D-src and 32D-123 cell lines. (A) Constitutive expression of *fos* and *jun* mRNAs in 32D-src cells. Total RNA was isolated from 32D-src cells that were maintained without serum for 48 h. The RNAs were analyzed by Northern blot (15 μ g per lane) and hybridized with the *c-fos*, *c-jun*, TGF- β 1, and glyceraldehyde phosphate dehydrogenase (GAPDH) probes. (B) Activation of *fos*-CAT chimeric genes (-404 to +42 [8]; -309 to +42 [8]) by serum and IL-3 in 32D-123 cells. 32D-123 cells were cotransfected with -79/+132-*jun*-CAT in the presence of serum. The data shown in panel B represent averages of at least three transfections.

studies provide evidence that the v-Src protein also enhances *c-jun* activation. The Fos and Jun proteins form a heterodimer (AP-1) that can bind the 12-*O*-tetradecanoylphorbol 13-acetate-responsive element. Others have demonstrated the involvement of AP-1 in the activation of many genes, including those encoding tumor necrosis factor (2), IL-1 (17), and TGF- β 1 (13). The exact mechanism of transcriptional activation by Jun and Fos proteins is unknown. Because the *jun* gene is critically important in coordinating cellular responses to growth factors and hormones, one major implication of the present study is that the v-Src protein indirectly regulates this gene, which is a component of AP-1.

(ii) **Modulation of AP-1 activity by serum.** *c-fos* gene transcription is induced by serum through the distal sequence element (14, 15). As previously shown in *fos* expression (8), we found that the minimum AP-1 element in the *jun* promoter is inducible with serum, albeit at lower levels in the absence of the distal sequence element. Serum, like *src*, was found to induce *c-fos* and *c-jun* gene expression in myeloid cells, resulting in the transcriptional regulation of the TGF- β 1 gene. These results suggest two possibilities. First, the tyrosine kinases (Src) may be a component of the signal transduction pathway responsive to serum. Second, the induction of *fos* and *jun* by serum involves the regulation of the protein kinase C pathway (14, 15), suggesting that protein kinase C is an alternative pathway for TGF- β 1

regulation in these cells. This hypothesis is strengthened by other studies (1), which indicate that TGF- β 1 and epidermal growth factor can regulate TGF- β 1 gene expression via distinct pathways (20).

Our investigations suggest that v-*src* transformation is responsible for the serum independence that maintains steady-state levels of TGF- β 1 mRNA (as a consequence of the constitutive expression of *c-fos* and *c-jun* in 32D-src cells. In 32D-123 cells, serum is required for the induction of *c-fos*, *c-jun*, and, consequently, TGF- β 1. These results favor the idea that pp60^{v-*src*} replaces serum in directly regulating the intracellular pathways that activate *c-fos* and *c-jun*. Additional support for this observation comes from the work of Dutta et al. (6), which indicates that serum independence of transcription (from the viral promoter in v-*src*-transformed 3Y1 cells) is due to the constitutive activation of intracellular pathways responsive to pp60^{v-*src*}.

Deregulation of TGF- β 1 by v-*src*: potential role in carcinogenesis. These studies further support the hypothesis that tyrosine kinases such as Src play important roles during cell growth. It has been proposed that deregulated secretion of growth factors such as TGF- β 1 by tumor cells may stimulate tumor growth (9, 19). The abnormal regulation of *jun* by v-Src, and the resulting constitutive expression of TGF- β 1, could be involved in carcinogenesis. Recently, Cartwright and co-workers (3) identified the abnormal expression of tyrosine kinases as an early event in the genesis of human colon carcinoma. Aberrant expression of tyrosine kinases consequently deregulates cellular responses (for example, to TGF- β 1) that control growth.

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