Tissue Factor Gene Transcription in Serum-Stimulated Fibroblasts Is Mediated by Recruitment of c-Fos into Specific AP-1 DNA-Binding Complexes†

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ABSTRACT: Serum stimulation of quiescent mouse fibroblasts results in transcriptional activation of tissue factor (TF), the cellular initiator of the protease cascade leading to blood coagulation. In this study, we demonstrate that two AP-1 DNA-binding elements located 200−220 bp upstream of the transcription start site are both necessary and sufficient to confer serum inducibility to the TF gene promoter in fibroblasts. Analysis of AP-1 DNA-binding complexes indicates that the predominant form of AP-1 activity in quiescent cells consists of an unidentified Fos-related protein and JunD. While c-Fos is notably absent from these preexisting complexes, serum stimulation results in the rapid entry of c-Fos into the TF AP-1 DNA-binding complexes. A similar induction of c-Fos DNA-binding activity occurs in cells treated with recombinant growth factors such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). Importantly, overexpression of JunD and c-Fos abrogates the requirement for serum in the stimulation of TF promoter activity in fibroblasts. Together, these data indicate that the entry of c-Fos into heterodimeric AP-1 DNA-binding complexes with JunD is a key event underlying serum-stimulated transcription of the TF gene in fibroblasts.

Tissue factor (TF)[1] is a transmembrane glycoprotein that serves as the cell-surface receptor and essential cofactor for plasma coagulation factor VII/VIIa. The active bimolecular complex is generally regarded as the most physiologically significant means of initiating the protease blood coagulation cascade (Edgington *et al.,* 1991). The role of constitutively expressed TF as a hemostatic "envelope", poised to activate coagulation upon vascular injury, is well established (Drake *et al.,* 1989). However, much recent interest has been focused on the role of TF in cell types in which expression is tightly regulated by extracellular stimuli such as growth factors, inflammatory cytokines, phorbol esters, components of the extracellular matrix, and antigen−antibody complexes or as a response to tissue injury (Hartzell *et al.,* 1989; Tijburg *et al.,* 1991; Ranganathan *et al.,* 1991; Conway *et al.,* 1989; Bevilacqua *et al.,* 1984; Crossman *et al.,* 1990; Moll *et al.,* 1995; Fan & Edgington, 1991; Schwartz *et al.,* 1982; Marmur *et al.,* 1993). Cell types that have been shown to display highly inducible TF activity include endothelial cells (Tijburg *et al.,* 1991; Conway *et al.,* 1989; Bevilacqua *et al.,* 1984; Crossman *et al.,* 1990; Moll *et al.,* 1995), monocytes and macrophages (Gregory *et al.,* 1989; Brand *et al.,* 1991; Mackman *et al.,* 1991; Oeth *et al.,* 1994), vascular smooth muscle cells (Marmur *et al.,* 1993; Maynard *et al.,* 1977), and fibroblasts (Hartzell *et al.,* 1989; Blatti *et al.,* 1988). Indeed, TF is classified as a cellular "immediate early" gene because its expression is rapidly induced in serum-stimulated fibroblasts independent of prior protein synthesis (Hartzell *et al.,* 1989; Ranganathan *et al.,* 1991). While the physiological significance of inducible TF activity is not known, the lack of identified TF deficiencies (Carson *et al.,* 1985) has prompted speculation that TF may mediate vital biological processes other than hemostasis (Edgington *et al.,* 1991).

Interest in the mechanisms that regulate TF expression has been heightened by the involvement of inappropriately expressed TF in atherosclerosis, disseminated intravascular coagulation, and *Escherichia coli*-induced septic shock (Taylor *et al.,* 1991; Wilcox *et al.,* 1989; Warr *et al.,* 1990; Rao, 1992). In addition, TF activity has been found in association with a variety of solid tumors including carcinomas of the breast, lung, colon, prostate, pancreas, bladder, cervix, and ovary (Callander *et al.,* 1992). Immunoreactive TF has also been detected in both tumor cells and fibroblastic cells associated with tumor stroma (Callander *et al.,* 1992).

The molecular mechanisms that regulate TF gene expression have only recently been examined (Edgington *et al.,* 1991; Moll *et al.,* 1995; Brand *et al.,* 1991; Mackman *et al.,* 1990, 1991; Oeth *et al.,* 1994; Cui *et al.,* 1994). The region between −111 and +14 of the human TF gene promoter has been shown to be sufficient for serum inducibility of a reporter gene in COS-7 cells (Mackman *et al.,* 1990). Similar results were obtained in HeLa cells (Cui *et al.,* 1994). However, this region of the human promoter was unable to confer serum or phorbol ester inducibility onto a heterologous promoter. This region of the human gene promoter contains several putative SP-1 elements, only one of which is positionally conserved in the mouse promoter (Mackman *et al.,* 1992). A more extensively studied system is the lipopolysaccharide (LPS)-induced activation of TF in human monocytic cells (Brand *et al.,* 1991; Mackman *et al.,* 1991; Oeth *et al.,* 1994) and in endothelial cells (Moll *et al.,* 1995). These studies have shown that a highly conserved 56 bp

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[1] Abbreviations: TF, tissue factor; Q, quiescent; FCS, fetal calf serum; CAT, chloramphenicol acetyltransferase; CHX, cycloheximide; TGF-β1, transforming growth factor β1; PDGF, platelet-derived growth factor; FGF_a, acidic fibroblast growth factor; FGF_b, basic fibroblast growth factor.

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DNA element in the TF promoter is both essential and sufficient for LPS induction in both cell types. This LPS-response element consists of two activator protein-1 (AP-1) binding sites and a neighboring nuclear factor κB (NFκB) binding site. Recent studies have demonstrated a rapid increase in NFκB DNA-binding activity upon LPS stimulation of both monocytes and endothelial cells and have further shown that binding of c-Rel/p65 heterodimers to the NFκB element is critical for LPS-induced transcription of the TF gene (Moll *et al.*, 1995; Oeth *et al.*, 1994). AP-1 DNA-binding activity was also detected in both cell types, being induced in monocytic cells (Oeth *et al.*, 1994) and constitutive in endothelial cells (Moll *et al.*, 1995). Nonetheless, in monocytes and endothelial cells, modulation of NFκB composition and binding activity appears to be critical to the stimulation of TF gene transcription. In contrast, AP-1 binding appears less critical but may serve to enhance the stimulatory effect of NFκB.

Although we (Ranganathan *et al.*, 1991; Blatti *et al.*, 1988) and others (Hartzell *et al.*, 1989) have previously demonstrated that serum or serum-derived growth factors stimulate TF gene transcription and procoagulant activity in fibroblasts, the underlying mechanisms have not been delineated. The results presented in this study demonstrate that the AP-1 DNA-binding sites of the previously described LPS-response element are both necessary and sufficient to mediate a response of the mouse TF promoter to serum stimulation in AKR-2B fibroblasts. These observations are in striking contrast to those obtained in serum-stimulated epithelial cells (Mackman *et al.*, 1990; Cui *et al.*, 1994). Also, in a reversal of the pattern observed in LPS-stimulated endothelial cells (Moll *et al.*, 1995), serum stimulation of fibroblasts results in a rapid modulation of AP-1 DNA-binding activity while NFκB binding appears largely constitutive. In particular, our data suggest that the rapid entry of c-Fos into AP-1 DNA-binding complexes is essential to the activation of TF gene transcription in serum-stimulated fibroblasts. These results suggest that differential patterns of assembly of specific AP-1 and NFκB DNA-binding complexes may facilitate cell-type-specific expression of the TF gene in response to a diverse spectrum of extracellular stimuli.

MATERIALS AND METHODS

Cell Culture, Transfection, and CAT Assays. AKR-2B mouse fibroblasts were maintained at 37 °C and 5% CO_2 and grown to confluence in McCoy's 5A medium (Gibco) containing 5% fetal bovine serum (Hyclone) and Pen-Strep (Gibco). For transient transfections, cells were trypsinized and 1×10^6 cells were plated per 100 mm dish in 5 mL of growth medium. After 18−24 h, each plate of cells was washed twice with HBS (21 mM HEPES, pH 7.1, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4, 6 mM glucose) and transfected by the addition of a total of 15 μg of plasmid DNA and 250 μg of DEAE-Dextran (Pharmacia) in a final 1 mL of serum-free medium (MCDB402, JRH Biosciences). After 1 h incubation, the transfection mix was removed, and the cells were shocked with 10% (v/v) DMSO in HBS for 2 min, then washed twice with growth medium containing 5% serum, and allowed to recover in growth medium containing 10% serum for 5−6 h. The cells were then washed and allowed to quiesce in serum-free medium for 36−48 h. Duplicate plates of cells were restimulated for 5 h in fresh MCDB402 medium containing the appropriate stimulus. Cells stimulated with protein synthesis inhibitors were washed twice and incubated in MCDB402 medium for an additional 2 h prior to harvest to allow for recovery of CAT protein synthesis. Cellular extracts were prepared, and equal amounts of cellular protein were assayed for CAT enzymatic activity as described previously (Gorman *et al.*, 1982). Unless stated otherwise, fold inductions are the average of duplicate plates from at least two independent experiments and were calculated after analysis of the percent conversion of [^{14}C]chloramphenicol versus cellular protein ensured that the values for quiescent and stimulated transfectants were within the linear range of the assay.

Growth Factors. TGF-β1, PDGF (BB), and acidic FGF (all human recombinant) were obtained from Austral Biologicals.

DNA Constructs. The mouse tissue factor promoter was isolated from AKR-2B genomic DNA using PCR reaction conditions recommended by the manufacturer (Perkin Elmer) but with the addition of 2% (v/v) formamide and primed with the following oligonucleotides: 5′-AACCCAAAGCTTGCAAGTGA-3′, which corresponds to sequences −897 to −879, and 5′-GAGGCAGCCGAGAAA-3′, which corresponds to the antisense strand of sequences +155 to +169 reported previously (Mackman *et al.*, 1992). After re-amplification, the amplified product was purified (Magic PCR prep, Promega) and its sequence verified by direct sequencing (ds DNA cycle sequencing, BRL). The DNA fragment was digested with *Hin*dIII and *Kpn*I and cloned into pSP72 (Promega). The cloned promoter was then liberated with *Hin*dIII and *Bgl*II for cloning into pBLCAT3 (Gorman *et al.*, 1982). This initial reporter construct (clone A15) contained 890 bp of upstream promoter sequence and 15 bp of 5′ untranslated sequence from the mouse TF gene. This reporter construct was then truncated by digestion with *Hin*dIII and *Bam*HI to remove the intervening 526 bp. The truncated vector was then gel-purified and religated using a synthetic HindXba linker comprised of oligonucleotides 5′-AGCTTCATCTAGAGTG-3′ and 5′-GATCCACTCTAGATGA-3′, which had been annealed together. This clone (A15Δ, or TF264CAT) is diagrammed in Figure 1. TF264luc was constructed by cloning the *Xba*I−*Bgl*II promoter fragment from TF264CAT into *Nhe*I−*Bgl*II-digested pGL_2-basic (Promega).

Site-directed mutations were constructed in TF264CAT by overlap extension (Horton *et al.*, 1989; Stoflet *et al.*, 1992) with the addition of 5% (v/v) formamide to the reactions. Pfu DNA polymerase (Stratagene) was used in some reactions. Fragments AB were generated using HindXba linker (above) as primer A and the following B primers (the specific mutations are underlined): $AP1_{Sal(both)}$, 5′-GGTCGACACCGGGTCGACACAGCGCCGCGGAGCA-3′; $AP1_A$, 5′-GGTCGACACAGCGCCGCGGAGCA-3′; $AP1_B$, 5′-GGTCGACACCGTGATTCAACAGCG-3′; and $p65_{AGA}$, 5′-CTCCGGTCGACGTATCTAACTCCGAGACCCCGAA-3′. Fragments CD were generated with using TFCATBglIIrev as primer D (5′-CTCGAGATCCAGATCTGATATC-3′) and the following C primers: $AP1_{Sal(both)}$, 5′-GTCGACCCGGTGTCGACCCCCCTTTCGGGGTCT-3′; $AP1_A$, 5′-GTCGACCCGGTTGAGTCACCCCTT-3′; $AP1_B$, 5′-GTCGACCCCCCTTTCGGGGTCT-3′; and $p65_{AGA}$, 5′-GTCTCGGAGTTAGATACGGGAGGGGAGTG-3′.

The corresponding AB and CD fragments (1 μL from each PCR reaction) were "SOEn" together using HindXba linker

and TFCATBglIIrev primers. The final products were resolved on 2% agarose gels, purified from the gel slice with Qiaex (Qiagen), digested with *Xba*I and *Bgl*II, and ligated into the similarly digested and purified vector fragment from TF264CAT.

Specific deletions were constructed by PCR as described previously (Stoflet *et al.*, 1992) using 55 °C as the annealing temperature. TF264CAT served as the template DNA, and TFCATBglIIrev was used as the reverse primer. The following primers were used to generate the deletion (lower case letters denote XbaI restriction site and extra nucleotides to facilitate cloning of the final products): TF195CAT (Δ905), 5′-gatcagtctagaTTCGGGGTCTCGGAGTTTCC-3′; and TF170CAT (Δ930), 5′-gatcagtctagaGAGGGGAGTGGGGCGGGGGCAG-3′. A TF basal promoter construct (TF60CAT or Δ1040), which is a deletion of all promoter sequences upstream of −60 and thus contains only the tissue factor TATA box, was created from TF264CAT by PCR as above using the following forward primer: 5′-gacatcggatccGGGTGGAGAGGAGCCGGTGT-3′ and TFCATBglIIrev. The lower case letters denote the *Bam*HI restriction site and extra nucleotides to facilitate cloning. The PCR product was gel-purified, digested with *Bam*HI and *Eco*RV, and ligated into the similarly digested and purified TF264CAT vector. For creation of LPSTF60CAT and AP1TF60CAT, synthetic LPS oligonucleotides 5′-GATCGTTGAATCACGGTTGAGTCACCCCTTTCGGGGTCTCGGAGTTTCCTAC-3′ and 5′-GATCGTAGGAAACTCCGAGACCCCCG-AAAGGGGTGACTCAACCGTGATTCAAC-3′ were annealed and cloned into the *Bam*HI site of TF60CAT. Similarly, the AP1 oligonucleotides 5′-agtcagtctagaTTGAATCACGGTTGAGTCACggatccagtcag-3′ and 5′-ctgactggatccGTGACTCAACCGTGATTCAAtctagactgact-3′ were annealed, digested with *Xba*I and *Bam*HI, and ligated into the similarly digested TF60CAT or pBLCAT2.

All plasmid DNAs were purified by double cesium gradient centrifugation. Plasmid sequences were confirmed by DNA sequencing.

The mammalian expression vectors RSV-c-Fos, -JunB, and -JunD contain cDNAs encoding the respective proteins under transcriptional control of the Rous sarcoma virus long-terminal repeat and were obtained from H. Iba (Suzuki *et al.*, 1991).

Nuclear Extracts and Gel Mobility Shift Assays. Nuclear extracts were prepared from quiescent or stimulated AKR-2B cells according to the mini-extraction procedure as modified by deGroot *et al.* (1992). Briefly, 1×10^6 cells were plated in 5 mL of growth medium for 16−24 h. The cells were then rendered quiescent in MCDB402 medium for 36−48 h. Extracts were prepared from individual plates using 25−30 μL of buffer C for each plate. Extracts from similarly treated cultures (usually 3−4 plates) were pooled, frozen on dry ice, and stored at −80 °C. The protein concentration of each extract was determined by the Bio-Rad protein assay. An oligonucleotide containing both TF AP-1 elements, 5′-TTGAATCACGGTTGAGTCA-3′, or the TF NFκB element, 5′-CTCGGAGTTTCCTAC-3′, was cloned into the *Sma*I site of pSP72. The approximately 70 bp *Eco*RI/*Hin*dIII fragments were liberated and purified on a 2.5% GTG LMP agarose (FMC) followed by extraction with hot phenol and ethanol precipitation. The purified fragments were end-labeled with [α-^{32}P] dATP and Klenow (New England Biolabs) as recommended by the manufacturer. For gel shift analysis, approximately 3 μg of nuclear extract was added to a reaction cocktail containing 20 000 cpm of labeled probe (0.2 ng of DNA), 0.8 μg of poly(dIdC)−poly(dIdC) (Pharmacia), and buffer to make the final reaction conditions 20 mM HEPES, pH 8.0, 60 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol. The reactions were incubated at room temperature for 20 min and resolved on a 6% (29:1) native polyacrylamide gel in TGE buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA, pH 8.5). For antibody "supershifts", large-scale gel shift reactions were incubated as described and then aliquoted into tubes, each containing 1 μL of specific Fos- or Jun-family antibody (Santa Cruz). The reactions were incubated an additional 60 min at 4 °C prior to loading onto the gel.

RESULTS

Identification of Tissue Factor Promoter Elements Required for Serum Induction. Mouse TF was independently cloned in our laboratory (Ranganathan *et al.*, 1991) and that of Nathans (Hartzell *et al.*, 1989) as a cellular immediate early gene, which was rapidly induced upon serum or growth factor stimulation of G_0-arrested fibroblasts. TF gene transcription was shown to be maximally induced by 30 min following stimulation with serum (Ranganathan *et al.*, 1991; Blatti *et al.*, 1988; Lau & Nathans, 1987). The induction of transcription was transient and attenuated to near basal levels by 3 h following stimulation. Serum stimulation in the presence of cycloheximide resulted in TF mRNA superinduction owing in part to a prolonged transcriptional response, a characteristic common to many cellular immediate early genes (Hartzell *et al.*, 1989; Ranganathan *et al.*, 1991; Lau & Nathans, 1987).

Mackman and co-workers recently reported the sequence of the murine TF gene from NIH3T3 cells (Mackman *et al.*, 1992). To further study TF regulation, we used synthetic oligonucleotides corresponding to this clone to amplify approximately 900 bp of TF promoter sequence from AKR-2B mouse fibroblasts. This DNA was cloned upstream of the chloramphenicol acetyltransferase (CAT) gene for transient transfection into AKR-2B fibroblasts. The proximal 300 bp of the mouse promoter is highly conserved with the promoter for human TF (Mackman *et al.*, 1992). We, therefore, truncated our TF promoter clone to a *Bam*HI site at −264 with respect to the start site of transcription. We found the expression of the initial promoter clone and the truncated clone (TF264CAT) to be identical and therefore chose to use TF264CAT for further study. This construct is illustrated schematically in Figure 1.

Because of the high degree of conservation of the previously described LPS-response element and its importance in regulating TF transcription in monocytes (Mackman *et al.*, 1991; Oeth *et al.*, 1994) and endothelial cells (Moll *et al.*, 1995), we focused our studies on the possible role of this element in mediating serum inducibility in fibroblasts. As shown in Figure 1, specific mutations were introduced in this element within the context of the wild-type TF264 promoter. The AP-1 sites were mutated individually or in combination to *Sal*1 restriction sites while the NFκB site was mutated as described by Mackman *et al.* (1991). Deletion mutants were also created that specifically removed the AP-1 elements (TF195CAT) or the entire LPS-response element (TF170CAT). The ability of the various constructs to

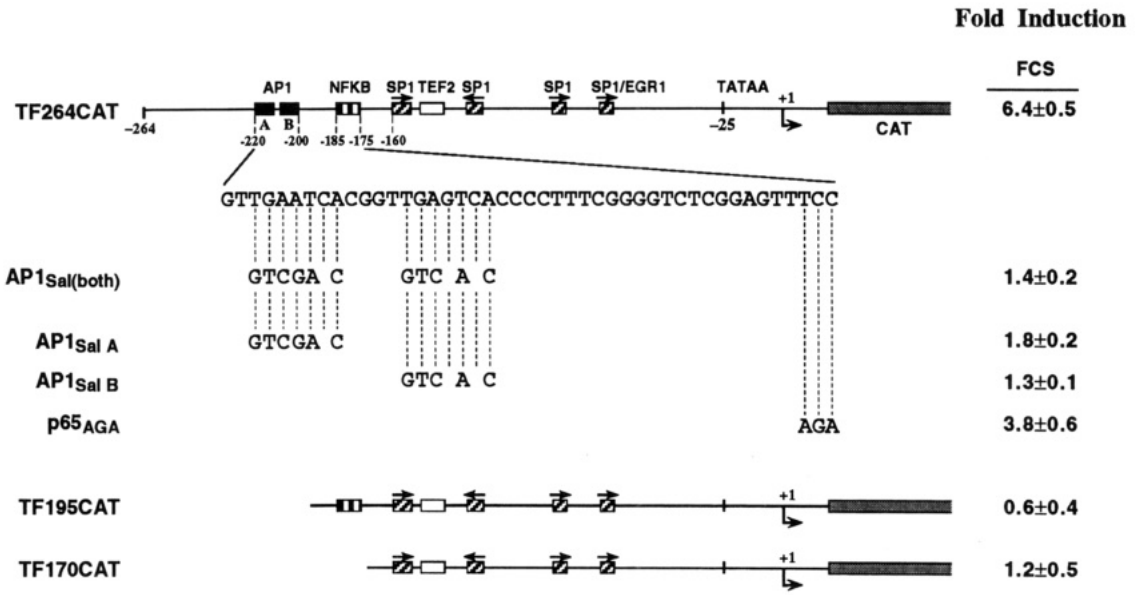

FIGURE 1: Dissection of TF promoter elements required for serum induction in fibroblasts. Site-directed mutations and specific deletions of the AP-1 and NFκB elements were created within the context of the intact TF promoter construct (TF264CAT) as indicated. AKR-2B fibroblasts were transfected and rendered quiescent as described under Materials and Methods. The cells were then stimulated with serum (FCS, 20%) for 5 h and harvested, and extracts were assayed for CAT enzymatic activity. The fold inductions were calculated from parallel quiescent and stimulated transfectants, after correcting for transfection efficiency using TF264luc and are expressed as the average (plus or minus the standard deviation) of duplicate plates.

respond to stimulation by serum was then tested by transient transfections into AKR-2B cells.

As shown in Figure 1, the TF264CAT promoter responded to serum stimulation approximately 6-fold. Mutation of one or both AP-1 elements essentially abolished serum inducibility. Deletion of both AP-1 elements in the TF195CAT construct also eliminated serum inducibility despite retention of the NFκB element. Mutation of the NFκB element within the context of the TF264CAT construct ($p65_{AGA}$) decreased the promoter response to serum by approximately 40%, suggesting that while the NFκB binding site alone does not confer detectable serum inducibility (TF170CAT), it may augment the ability of the AP-1 elements to do so.

To further examine the relative importance of the AP-1 and NFκB elements in TF transcriptional regulation, the AP-1 elements or the entire LPS-response element were cloned upstream of a basal TF promoter containing only the TATA box (TF60CAT). The data in Figure 2 show that the AP-1 elements alone were able to confer a level of serum inducibility to the TF basal promoter indistinguishable from that displayed by the wild-type TF264 promoter. Inclusion of the NFκB site had no additional effect in this context. Similar results were obtained in transiently transfected NIH3T3 fibroblasts (data not shown). Furthermore, the AP-1 elements alone were able to confer serum inducibilty onto the heterologous tk promoter from the herpes simplex virus thymidine kinase gene (tkCAT), although this construct displayed considerably higher basal transcription. Although these experiments do not rule out a contribution from other regulatory elements in the complete TF promoter, they strongly indicate that the AP-1 elements are the primary mediators of serum inducibility in fibroblasts.

Effect of Serum Stimulation on AP-1 Binding and Subunit Composition. We next tested the effect of serum stimulation on AP-1 DNA-binding activity in AKR-2B fibroblasts. Nuclear extracts were prepared from quiescent or stimulated cells and assayed for AP-1 DNA-binding activity by gel mobility shift. Figure 3A illustrates that AP-1 DNA binding was readily detected in unstimulated cells but dramatically increased within 30 min following serum stimulation (compare lanes 1 and 9). The specificity of this complex was determined by its ability to be competed with unlabeled probe and its failure to be competed by a mutant DNA fragment in which the TF AP-1 sites were changed to *Sal*1 restriction sites as shown in Figure 1 (data not shown). Because AP-1 dimers composed of different members of the Fos and Jun families also differ in their ability to activate or repress transcription (Suzuki *et al.,* 1991; Schutte *et al.,* 1989), we examined the composition of the various AP-1 complexes using antibodies specific for individual family members. An antibody directed against a highly conserved region of c-Fos (amino acids 128−156) that recognizes all Fos family members gave a quantitative supershift of the AP-1 complexes in extracts from both quiescent and serum-stimulated cells (lanes 5 and 13, panel A; lanes 6 and 15, panel B). Because AP-1 complexes are known to be composed of either Fos/Jun heterodimers or Jun/Jun homodimers (Montesano & Orci, 1988), this result provided strong evidence that the observed complexes consisted exclusively of Fos/Jun heterodimers. Irrespective, no strong supershifts were detectable with antibodies specific for individual Fos family members using extracts from quiescent cells. Although not shown in panel A, these also included FosB. Within the Jun family, a strong supershift was observed using anti-JunD while anti-JunB gave a weaker supershift. No supershifts were observed using anti-c-Jun. These results suggest that the TF AP-1 DNA-binding complexes in quiescent cells consist mainly of an unidentified Fos-related protein heterodimerized with JunD and perhaps JunB.

When AP-1 DNA-binding complexes from serum-stimulated cells were analyzed, a dramatically different picture emerged. Most notably, strong c-Fos reactivity was detected within 30 min of stimulation (lane 10, Figure 3A). By 4 h, however, c-Fos levels decreased and were replaced by high

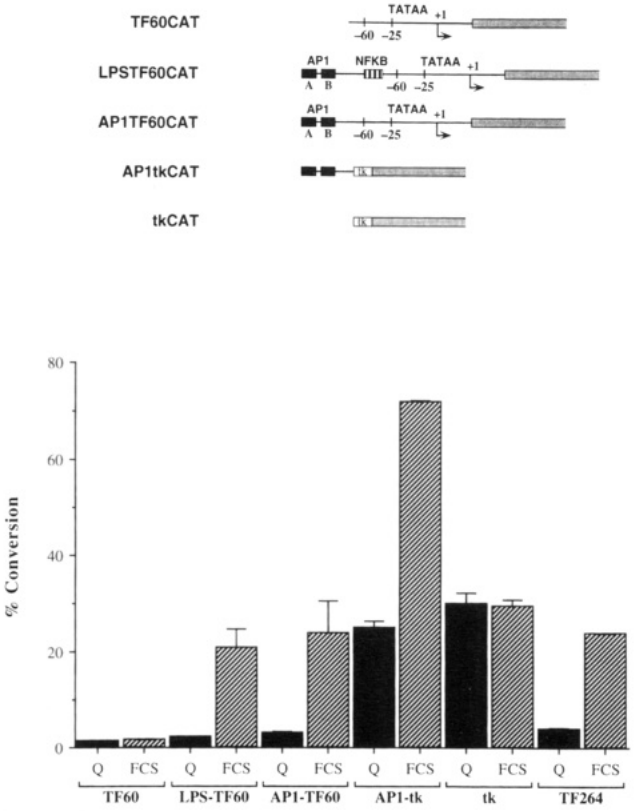

FIGURE 2: AP-1 DNA-binding elements are sufficient for serum induction of tissue factor. The TF LPS element, containing the AP-1 and NFκB elements from the TF promoter, or the AP-1 elements were linked to the homologous minimal TATA box-containing promoter (TF60CAT). The AP-1 elements were also linked to the heterologous tk promoter in pBLCAT2. These DNAs were transiently transfected into fibroblasts. Equal amounts of cellular protein from duplicate quiescent or serum (FCS)-stimulated transfectants were assayed for CAT activity, expressed as percent conversion of [^{14}C]chloramphenicol to its acetylated derivatives. Error bars represent the range of the duplicate samples.

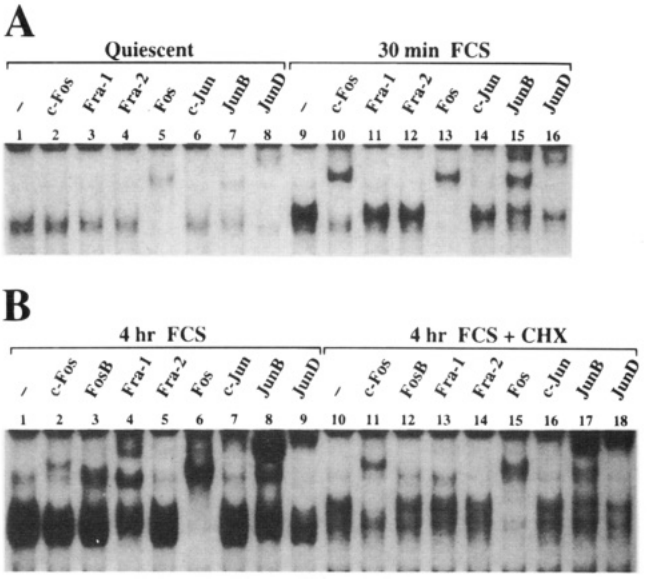

FIGURE 3: Serum induction of TF AP-1 DNA-binding activity. Nuclear extracts from quiescent (panel A, lanes 1−8), 30-min serum-stimulated (panel A, lanes 9−16), 4-h serum-stimulated (panel B, lanes 1−9), or 4-h serum plus cycloheximide-stimulated (panel B, lanes 10−18) AKR-2B fibroblasts were assayed for AP-1 DNA-binding activity by gel mobility shift using a DNA fragment containing the two TF AP-1 elements. Large-scale reactions were incubated for 20 min at room temperature. Aliquots of the reaction were then incubated for 1 h at 4 °C with antibodies to various AP-1-binding proteins as indicated prior to loading onto the gel. Lanes indicated with "−" are parallel reactions in the absence of antibody.

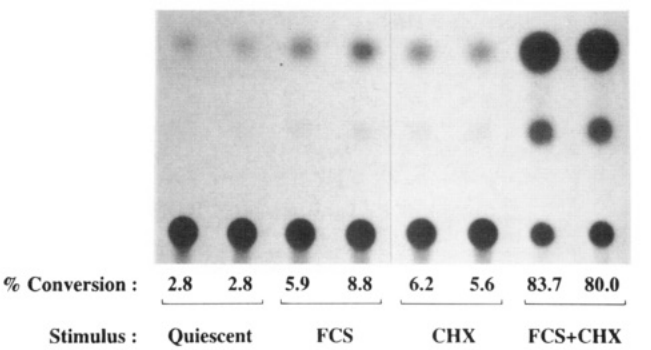

FIGURE 4: Effect of cycloheximide on TF promoter activity. TF264CAT was transfected into AKR-2B cells. After being rendered quiescent, duplicate transfectants were stimulated for 5 h with serum (FCS, 20%), cycloheximide (CHX, 10 μg/mL), or both. Cells stimulated with cycloheximide were then washed and incubated in serum-free medium for an additional 2 h to allow recovery of CAT protein synthesis. Cellular extracts were prepared and assayed for CAT enzymatic activity, expressed as percent conversion of [^{14}C]chloramphenicol to its acetylated derivatives for each sample.

levels of both Fra-1 and FosB (lanes 2−4, Figure 3B). Changes in the composition of the Jun components were less striking although JunB appeared to become predominate over JunD by 4 h. When considering the possible significance of these changes in AP-1 composition, it is important to recall that serum stimulation of TF transcription in AKR-2B cells is transient, peaking at 30 min following stimulation, but attenuating to a near basal level by 3 h (Blatti *et al.*, 1988). Thus, while the appearance of c-Fos at 30-min post-stimulation correlates with transcriptional activation, the subsequent increase in FosB, Fra-1, and JunB is actually correlated with transcriptional attenuation. Because of this, we also examined AP-1 DNA-binding complexes in cells stimulated for 4 h with serum in the presence of cycloheximide, a combination which superinduces TF transcription by inhibiting transcriptional attenuation for at least 4 h (Ranganathan *et al.*, 1991; Lau & Nathans, 1987). Under these conditions, the increases in Fra-1, FosB, and JunB were largely inhibited while c-Fos reappeared as a major component of AP-1 complexes (lanes 10−18, Figure 3B).

Cycloheximide Synergizes with Serum To Superinduce TF Promoter Activity. The observation that maintenance of c-Fos DNA-binding activity and inhibition of Fra-1, FosB, and JunB synthesis by cycloheximide (Figure 3B) correlated with maintenance of TF gene transcription (Ranganathan *et al.*, 1991; Blatti *et al.*, 1988) was further investigated by testing the effect of cycloheximide on TF promoter activation. The results shown in Figure 4 show that TF264CAT was induced 2−3-fold by either serum or cycloheximide. When these and other samples were reassayed at higher amounts of cellular protein, the fold-induction by CHX averaged over 3-fold (data not shown). When cells were co-stimulated with serum and CHX, a synergistic induction of TF promoter activity was obtained that averaged nearly 50-fold. Thus, maintenance of a c-Fos-containing AP-1 complex correlates with increased TF promoter activity in fibroblasts.

TF NFκB DNA-Binding Activity in AKR-2B Cells Is Unaffected by Serum Stimulation. Because mutation of the

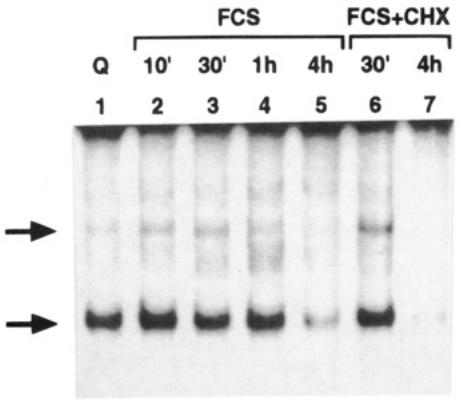

FIGURE 5: TF NFκB DNA-binding activity in AKR-2B cells. Nuclear extracts from quiescent (Q) or stimulated AKR-2B fibroblasts (FCS, serum; FCS + CHX, serum plus cycloheximide) were assayed for DNA-binding activity using a DNA fragment containing the TF NFκB element. Arrows indicate mobility shift bands which comigrate with those obtained using the same DNA probe and recombinant p50 (Promega).

NFκB site within the context of the TF264 promoter reduced serum-stimulated transcription by 40% (Figure 1), we examined NFκB binding activity in AKR-2B nuclear extracts using a DNA fragment containing the TF NFκB site. The data in Figure 5 show that, while specific NFκB binding complexes were detected, they were quantitatively indistinguishable in quiescent cells and cells stimulated with serum for periods up to 1 h (lanes 1−4). Some reduction in NFκB DNA-binding activity was observed by 4 h post-stimulation (lane 5). However, this reduction was not inhibited by co-stimulation with CHX (lane 7). Thus, increased TF promoter activity in fibroblasts does not correlate with increased TF NFκB DNA-binding activity.

Specific Combinations of Growth Factors Mimic Serum-Induction of TF Promoter Activity and Recruitment of c-Fos into AP-1. To gain insight into serum components that are active in regulating the TF gene in fibroblasts, we tested the ability of various recombinant growth factors to induce TF promoter activity. As shown in Figure 6A, transforming growth factor type β1 (TGF-β1), PDGF, and acidic FGF produced little stimulation of promoter activity. However, when tested in combination with TGF-β1, both PDGF and acidic FGF exhibited significant activity. In particular, the combination of TGF-β1 and acidic FGF resulted in a cooperative stimulation of activity which approached that of serum. As shown in Figure 6B, acidic and basic FGF and PDGF, but not TGF-β1, also stimulated the rapid entry of c-Fos into AP-1 DNA-binding complexes as determined by antibody supershifts. Since these factors alone did not significantly stimulate TF promoter activity, this could indicate that post-translational modifications of c-Fos by cooperating growth factors may play an important role in regulating TF gene expression in serum-stimulated cells.

Overexpression of JunD and c-Fos Stimulates TF Promoter Activity in Fibroblasts. To further test the hypothesis that AP-1 heterodimers containing c-Fos are involved in TF activation in fibroblasts, AKR-2B cells were cotransfected with AP1TF60CAT and expression vectors encoding particular AP-1 proteins. As shown in Figure 7, expression of JunD alone elevated reporter gene activity approximately 2-fold in both quiescent and serum-stimulated cells when compared to control transfectants. In contrast, expression of c-Fos alone had little effect on TF promoter activity (data

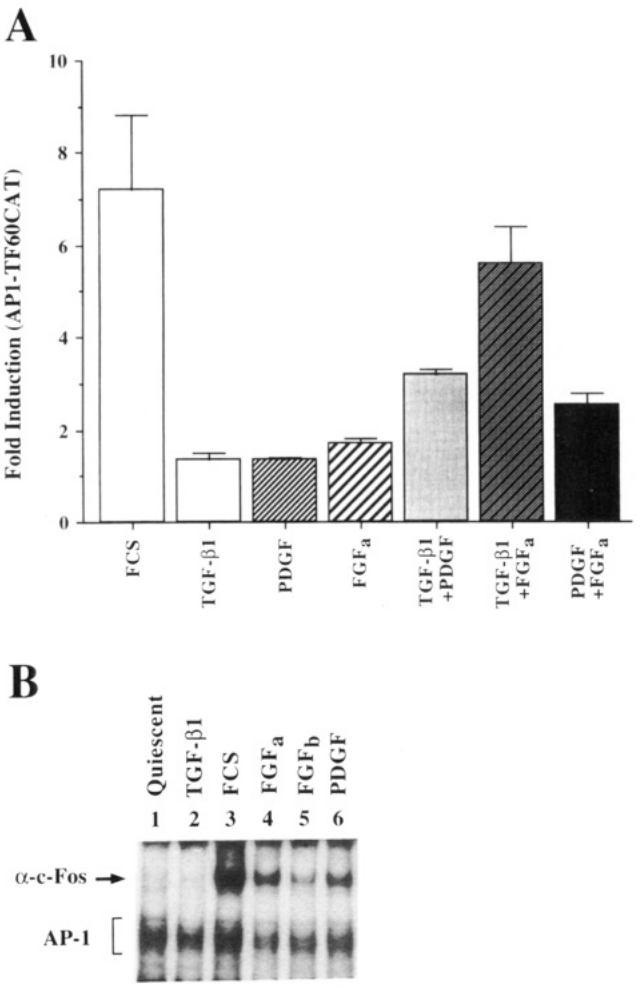

FIGURE 6: Effect of certain growth factors on TF promoter activity and c-Fos DNA binding. (Panel A) AP1TF60CAT was transfected into AKR-2B fibroblasts and assayed for its induction upon stimulation of the cells with serum (FCS) or purified growth factors, TGF-β1 (5 ng/mL), PDGF (10 ng/mL), and/or acidic FGF (5 ng/mL). Fold inductions were calculated from CAT assays of duplicate quiescent and stimulated transfectants. (Panel B) Nuclear extracts from cells stimulated for 30 min with the indicated growth factor were assayed for c-Fos-containing AP-1 DNA-binding activity as in Figure 3.

not shown). However, when JunD was co-expressed with c-Fos, TF promoter activity was increased 12-fold in quiescent cells. A small additional increase was evident upon serum treatment. Thus, co-expression of c-Fos with JunD virtually abrogated the requirement for serum factors in activating the TF reporter gene. In contrast, co-expression of JunD with JunB had little, if any, stimulatory effect. Indeed, JunB was actually inhibitory to the ability of JunD alone to stimulate TF reporter gene activity, particularly in serum-treated cells. JunB also had a modest inhibitory effect on its own (data not shown).

DISCUSSION

The results presented in this study support a model in which the rapid entry of c-Fos into heterodimeric AP-1 DNA-binding complexes is a key event underlying the activation of TF gene transcription in serum-stimulated fibroblasts. First, the TF AP-1 elements were found to be both necessary and sufficient to confer a high degree of serum inducibility to both a minimal TF promoter and to a heterologous tk promoter. Second, an analysis of the composition of TF

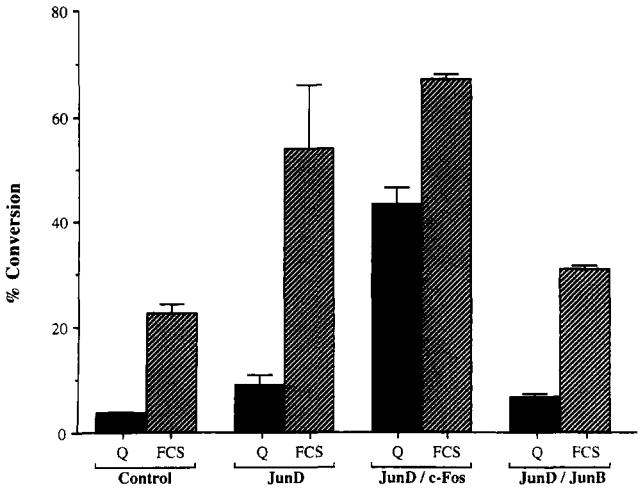

FIGURE 7: TF AP-1 elements are activated by cotransfection with c-Fos. AP1TF60CAT (7 μg) was co-transfected into AKR-2B fibroblasts with RSV-neo (control, up to 8 μg) or RSV-JunD (3 μg) and RSV-c-Fos or RSV-JunB (5 μg). Duplicate quiescent and serum-stimulated transfectants were assayed for CAT activity as described previously.

AP-1 DNA-binding complexes revealed a strong induction of c-Fos binding activity by 30 min following serum stimulation. Earlier nuclear run-on transcription assays (Blatti *et al.*, 1988) demonstrated that TF transcription (then termed c70) was activated within 20 min following serum stimulation of AKR-2B cells and peaked by 30 min. Thus, the appearance of c-Fos in AP-1 complexes correlates well with the timing of TF transcriptional activation. In addition, maintenance of c-Fos in the AP-1 complex by co-stimulation with serum and cycloheximide correlates with promoter superinduction. Lastly, co-expression of exogenous c-Fos with JunD, a prominent Jun family component in quiescent cells, largely eliminated the need for serum in fully activating the expression of a TF reporter gene. While this last observation appears somewhat at odds with the inability of recombinant factors such as PDGF and FGF to stimulate TF promoter activity despite their ability to activate c-Fos binding, expression of abnormally high levels of c-Fos in transfected cells may well compensate for a lack of post-translational modifications that may otherwise be required. In this regard, TGF-β1 is an attractive candidate for stimulating modifications that potentiate DNA binding and/or the transactivation potential of c-Fos because it functionally cooperated with c-Fos-inducing factors in stimulating TF promoter activity while lacking any c-Fos-inducing ability of its own. Consistent with this possibility, TGF-β1 has been recently shown to stimulate phosphorylation of the CREB transcription factor in mink lung cells (Kramer *et al.*, 1991).

The central role of the AP-1 elements in mediating serum inducibility of the TF gene in fibroblasts (both AKR-2B and NIH3T3) contrasts sharply with the situation in epithelial cells. In both COS-7 (Mackman *et al.*, 1990) and HeLa cells (Cui *et al.*, 1994), serum inducibility of TF transcription appears to be mediated by elements lying downstream of both the AP-1 and NFκB DNA-binding sites. Thus, signaling pathways that operate to induce TF transcription in response to serum growth factors appear to be cell-type specific. Additional studies will be required to determine exactly how these pathways differ in epithelial cells, fibroblasts, and possibly other cell types.

An unresolved issue concerns the identity of the Fos-related AP-1 component in quiescent fibroblasts. In repeated experiments, we have been unable to obtain convincing supershifts using any of the Fos family member-specific antibodies, although weak supershifts have been occasionally observed with anti-Fra-1. Nevertheless, the broad spectrum Fos antibody consistently produced quantitative supershifts of AP-1 complexes, strongly suggesting that such complexes are composed exclusively of Fos/Jun heterodimers. These results may reflect a post-translational modification or conformational difference in quiescent cells that obstructs access to one of the Fos family antibodies, or alternatively, the existence of a new Fos-related protein. This is an important issue for future study.

The proposed role for c-Fos in transcriptionally activating the TF gene promoter is consistent with recent studies showing that c-Fos is a more potent transcriptional activator than other Fos-related proteins (Suzuki *et al.*, 1991) and that c-Fos, but not Fra-1, specifically interacts with the TATA binding protein (TBP) of the TFIID basal transcription factor complex (Metz *et al.*, 1994). Thus, the transient interaction of c-Fos with TBP or other basal factors may underlie the transient activation of TF promoter activity in fibroblasts. The reduction in c-Fos DNA-binding activity by 4 h post-stimulation (Figure 3B), a time when TF transcription is severely attenuated (Ranganathan *et al.*, 1991; Blatti *et al.*, 1988), provides further support for this interpretation. In addition, the ability of cycloheximide to superinduce serum-stimulated TFCAT activity correlates well with its ability to inhibit both transcriptional attenuation (Ranganathan *et al.*, 1991; Lau & Nathans, 1987) and the reduction in c-Fos DNA-binding activity. It is also possible that the increase in other AP-1 components such as Fra-1, FosB, and JunB that occurs by 4 h post-stimulation is similarly related to transcriptional attenuation. The increase in all these components is strongly inhibited by cycloheximide; and JunB has been previously implicated in the suppression of AP-1-dependent transcription in other systems (Schutte *et al.*, 1989; Chiu *et al.*, 1989; Deng & Karin, 1993). Moreover, co-expression of JunB inhibited JunD-dependent transcription in AKR-2B cells (Figure 7). Thus, both the activation and subsequent attenuation of TF gene transcription in fibroblasts appear to be mediated by changes in the subunit composition of AP-1 DNA-binding complexes.

In contrast to the increased AP-1 binding following serum stimulation, NFκB binding appeared unaltered by serum stimulation. Because we did not analyze the composition of NFκB complexes, changes in the NFκB dimerization partners cannot be ruled out. However, it seems unlikely that any qualitative changes are essential to serum-stimulated activity per se since mutation or deletion of the NFκB site did not eliminate serum inducibility of promoters retaining intact AP-1 binding sites. Mutation of the NFκB site within the context of the wild-type TF264 promoter did, however, reduce AP-1-dependent transcription, suggesting a potentiating role for NFκB in serum-stimulated transcription in fibroblasts. However, the addition of the NFκB element to the AP1-basal promoter construct (i.e., LPS-TF60CAT versus AP1-TF60CAT) had no effect. Thus, the abbreviated construct requires no potentiation by κB-like proteins, perhaps due to the closer spacing between the AP1 elements and the TATA box. In monocytes (Mackman *et al.*, 1991; Oeth *et al.*, 1994) and endothelial cells (Moll *et al.*, 1995),

the NFκB site was shown to be essential for LPS stimulation of TF transcription while the AP-1 sites appeared to play a supporting role by augmenting NFκB-dependent transcription. Thus, depending on the cell type and inducing agent, either the AP-1 sites or the NFκB site appears to play the primary role in the activation of TF transcription with the adjacent element functioning to modulate the overall level of expression. The interplay of functionally diverse combinations of AP-1 or NFκB transcription factors on these two closely positioned sites provides an elegant and highly versatile mechanism for cell-type-specific regulation of TF activity. Further delineation of the proteins that occupy these two sites in different cell types may provide additional insight into the mechanisms which regulate TF expression during normal physiological processes and in specific disease states.

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