The ternary complex factors (TCF) are a subfamily of ETS domain transcription factors that bind and activate serum response elements (SREs) in the promoters of target genes in a ternary complex with a second transcription factor, serum response factor (SRF). Here, we have identified the SRF gene as a target for the TCFs, thereby providing a positive feedback loop whereby TCF activation leads to the enhancement of the expression of its partner protein SRF. The binding of the TCF Elk-1 to the SRF promoter and subsequent regulation of SRF expression occurs in a ternary complex-dependent manner. Our data therefore reveal that SRF is an important target for the ERK and Rho signaling pathways that converge on a ternary TCF-SRF complex at the SRE on the SRF promoter.

Elk-1, SAP-1, and SAP-2/ERP/Net comprise the ternary complex factor (TCF) subfamily of ETS-domain transcription factors (reviewed in Refs. 1 and 2). These proteins form ternary complexes on target promoters together with the MADS-box protein, serum response factor (SRF). Both protein-DNA and protein-protein interactions with SRF are required to form ternary complexes at the promoters of target genes like c-fos. The conserved B-box region of the TCFs plays a pivotal role in mediating these protein-protein interactions (3–8). The TCFs can be phosphorylated within their transcription activation domains by members of all three of the major mitogen-activated protein kinase pathways present in mammals: ERK, JNK, and p38 (reviewed in Refs. 1, 2, and 9). In the case of Elk-1, this phosphorylation leads to the enhancement of its transactivation properties both by the recruitment and activation of the coactivator proteins Sur-2 and p300/CRP (10, 11) and also the loss of corepressor complexes containing histone deacetylase 2 (12). Thus, Elk-1 provides an adaptor protein for SRF that can link it to the mitogen-activated protein kinase signaling pathways.

In addition to binding to the TCFs, SRF has recently been shown to be able to bind to members of the MAL/myocardin family of coactivator proteins (13–18). In the case of MAL, this interaction permits linkage of the MAL-SRF complex to the Rho signaling pathway and subsequent activation of SRF-dependent gene expression (13, 16). The SRF gene itself is thought to be one target gene for the MAL/myocardin-SRF complex. The interaction of the TCFs and MAL/myocardin proteins with SRF is mutually exclusive, where the Elk-1 B-box inhibits MAL/myocardin recruitment by SRF (19, 20). This suggests either the existence of two different classes of SRF target genes (21) or the possibility of sequential (13) or mutually exclusive interactions of different coregulatory proteins with the same SRF target genes (20).

To date the number of TCF target genes identified is limited and comprises well studied immediate-early genes such as c-fos and egr-1 (reviewed in Refs. 1, 2, and 9). On these latter targets, SRF appears to aid TCF recruitment, but the reciprocal situation appears to operate on other genes such as nur77 where the TCFs recruit SRF to the promoter (22, 23). It is currently unclear if the TCFs act in an SRF-independent manner although evidence has been gathered to suggest such a role on genes encoding tumor necrosis factor-α (24) and 9E3/cCAF (25).

Because of the possibility of functional redundancy among the TCFs, suggested by the minimal phenotypes obtained in mouse knock-out studies (23, 26, 27), we developed a cell line encoding an inducible repressible form of Elk-1 (Elk-1 fused to the engrafted repression domain; Elk-En) to probe the potential role of TCFs in regulating gene expression (28). The induction of Elk-En caused apoptosis and one key target gene identified was the anti-apoptotic gene Mcl-1. Importantly, the activity of Elk-En was B-box dependent demonstrating that it functions in a SRF-dependent manner. Here, we have extended these studies and show that the gene encoding the TCF partner protein SRF is also a target for the TCF Elk-1. Elk-1 works through a ternary complex with SRF on the SRF promoter, thereby providing a link to the ERK signaling pathway in addition to the well-characterized link to the Rho pathway that acts through SRF on its own promoter.

**MATERIALS AND METHODS**

**Plasmid Constructs**—pSRF-Luc(WT) (pAS2159) and pSRF-Luc-(mETS-103) (pAS2160) contain a 322-bp fragment of the mouse SRF promoter (−322 to +1) upstream from the firefly luciferase gene, with the wild-type promoter or containing a mutated ets site at position −103, respectively (Ref. 29; kindly provided by Ravi Misra), pCH110 (Amersham Biosciences) contains an SV40 driven β-galactosidase (LucZ) gene and is used to monitor transfection efficiency. pRSV-Elk-1-VP16 (pAS348) is a Rous sarcoma virus promoter-driven vector encoding full-length wild-type Elk-1 fused to residues 410–490 of the VP16 C-terminal sequence (30). pAS1408 and pAS1411, encoding, respectively, wild-type (WT) and L158P mutant derivatives of Elk-1 (full-length Elk-1 fused to the engrafted repression domain) have been
described previously (28). pAS383 encodes full-length wild-type FLAG-tagged Elk-1 (31). pAS1801 encodes full-length mouse PEAK3, and was constructed by ligating a HindIII/SalI-cleaved PCR product (primers, A36537) into HindIII/Xhol cleaved pCDNA3. The plasmid pCOP (pAS2158) encodes full-length SRF (kindly provided by Ravi Misra). pEFp-link-MALAN encodes an N-terminal truncated, constitutively active version of MAL (kindly provided by Richard Treisman; Ref. 16). pAS278 (encoding full-length His-FLAG-tagged Elk-1) (31) and pAS58 (encoding glutathione S-transferase fused to amino acids 132–222 of SRF-core (28)) (6) for expressing proteins in bacteria have been described previously.

**RESULTS**

**Repressive Elk-1 Constructs Down-regulate the Expression of SRF—**To study the effects of inhibiting TCF-mediated gene expression, and thereby uncover novel TCF target genes, we created a 293-derived stable cell line that inducibly expresses a fusion of Elk-1 with the powerful Engrailed (En) repression domain (EcR293(Elk-En)#1.3) (28). Upon induction of its expression with PA, this fusion protein is expected to be recruited to all TCF-dependent promoters and hence ablate the expression of genes normally controlled by TCFs. To identify potential target genes, we used Affymetrix microarray analysis to compare the mRNA expression profiles of unstimulated EcR293-EcR293(Elk-En)#1.3 cells with the same cells stimulated with PA for 6 h to induce the expression of Elk-En. One of the downregulated genes identified was SRF (1.28-fold reduced). Northern analysis confirmed the microarray data, demonstrating a clear reduction in SRF mRNA following PA induction, which coincides with the induction of the expression of Elk-En (Fig. 1A). Similarly, SRF is also down-regulated at the protein level following loss of the SRF message (Fig. 1B). We also tested whether the prior induction of Elk-En could block the activation of SRF expression by serum (FCS) stimulation. In comparison to the uninduced cells, pretreatment of cells with PA to induce Elk-En expression led to a reduction in SRF induction by serum (Fig. 2A). Thus, Elk-1 appears to be able to regulate the transcription of the gene encoding its partner protein SRF.

**Elk-En Represses SRF Expression in a B-box-dependent Manner—**Elk-1 is thought to act primarily through ternary complexes in conjunction with SRF on SREs (reviewed in Refs. 1 and 2). However, it is possible that overexpression of Elk-En fusions could result in the repression of genes usually regulated by different ETS domain transcription factors in an SRF-independent manner by virtue of their overlapping DNA binding specificities. We therefore created a control cell line (EcR293(EcR293L158P)#8L) that contains an Elk-En derivative that has a point mutation in its B-box region that blocks its ability to bind to SRF and hence be recruited to DNA in an SRF-dependent manner (28). This fusion protein should still inhibit transcription through high affinity binding sites. Control experiments demonstrated that this was the case in reporter assays and that the expression levels of the wild-type and L158P version of Elk-En were similar (28).

The prior induction of Elk-En(L158P) expression had little effect on serum-stimulated SRF expression (Fig. 2B). This contrasts with the induction of WT Elk-En, which caused a clear reduction in serum-inducible SRF expression (Fig. 2A).
We also examined the protein levels of SRF in EcR293(Elk-En(L158P))#L8 cells following induction of Elk-En(L158P), in contrast to the reductions in SRF levels observed upon induction of wild-type Elk-En (Fig. 1B), no change in SRF expression was observed, even after 63 h of induction with PA (Fig. 2C). To demonstrate that Elk-En could act directly on the SRF promoter, we carried out transient transfection assays with increasing amounts of Elk-En and a SRF promoter-driven luciferase reporter construct (Fig. 3A). Wild-type Elk-En efficiently repressed the activity of the SRF promoter. In contrast, the mutant derivative, Elk-En(L158P), was unable to repress the activity of the SRF promoter. This differential effect was not because of differences in expression of the Elk-En fusions (Fig. 3B). Although Elk-En(L158P) is unable to affect the activity SRF promoter, it is possible that other ETS domain proteins might regulate this promoter. Indeed, overexpression of a different ETS domain protein, PEA3, also caused upregulation of this promoter (data not shown). To establish the specificity of ETS domain protein action, we determined whether PEA3 could compete with Elk-En fusions in regulating the SRF promoter. However, PEA3 was unable to compete with Elk-En (Fig. 3C), suggesting that Elk-1 is an important regulator of this promoter. Collectively, these data therefore demonstrate that Elk-1-mediated regulation of the SRF promoter takes place in a B-box-dependent manner, suggesting that it is recruited by SRF into a ternary, promoter-bound complex.

Elk-1 Recruitment to the SRF Promoter Is Mediated by an Ets Binding Site—The SRF promoter contains two CArG boxes that are bound by SRF and are important for signal-mediated activation of SRF expression (33, 34). In addition, there is a functionally important ets binding motif located upstream from these binding sites (29) (Fig. 4A). We therefore tested whether Elk-1 can bind to this module in vitro using a DNA fragment encompassing the upstream CArG box and the ets site (Fig. 4A). In the absence of SRF, Elk-1 was unable to bind to the SRF promoter (Fig. 4B, lane 3). However, in the presence of SRF, the binding of Elk-1 was strongly stimulated and a ternary complex was formed on the promoter (Fig. 4B, lane 4). This binding was dependent on the integrity of the B-box region of Elk-1 as Elk-1(L158P) was unable to bind to the promoter (Fig. 4B, lane 5).

**Fig. 2.** SRF down-regulation by Elk-En is B-box dependent. SRF expression was analyzed by Northern blotting in EcR293(Elk-En#1.3 (A) and EcR293(Elk-EnL158P)) cells (B). Cells were serum starved for 24 h, pretreated with 5 μM PA for 19 h, and stimulated with 20% serum for 2 or 3 h. The middle panels show the expression levels of Elk-En, whereas the bottom panels show the total RNA loading control. C, Western blot analysis of SRF expression (top panel) in EcR293(Elk-EnL158P) cells treated with 5 μM ponasterone A for the indicated times. The middle panel shows the expression levels of Elk-En, whereas the bottom panel shows glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading control.

**Fig. 3.** Elk-En down-regulates the SRF promoter. A, 293 cells were transiently transfected with a luciferase construct containing the SRF promoter and with increasing concentrations of Elk-En(WT) and Elk-En(L158P) (0, 1, 5, 10, 25, and 50 ng). Cells were starved for 48 h and then stimulated with 20% serum. Luciferase activity was measured 6 h after stimulation. The results were normalized to β-galactosidase activity. B, Western blot analysis of Elk-En(WT) and Elk-En(L158P) from samples derived from cells transfected with 10 (lanes 1 and 5), 25 (lanes 2 and 6), 50 (lanes 3 and 7), and 100 ng (lanes 4 and 8) of expression vector. C, reporter gene analysis of the WT SRF promoter (0.4 μg) in 293 cells grown as in A, in the absence and presence of 20 ng of Elk-En and increasing amounts of a construct encoding PEA3 (0, 2.5, 10, and 25 ng).
demonstrating the importance of interactions with SRF for its recruitment. Next, we tested the requirement for the ets motif for recruitment of Elk-1 to the SRF promoter. Mutation of the ets motif reduced the ability of Elk-1 to bind to the SRF promoter in a complex with SRF (Fig. 4B, lane 9). Thus, both protein-DNA contacts and protein-protein interactions with SRF are essential for the efficient recruitment of Elk-1 to the SRF promoter.

To extend these observations in vivo, we investigated the ability of Elk-1 derivatives to regulate an SRF promoter-reporter construct that contains a mutation in the ets motif (Fig. 5A). The wild-type SRF promoter was repressed by Elk-1 and activated by a constitutively active Elk-VP16 fusion protein (Fig. 5B). We also investigated the ability of wild-type Elk-1 to activate the SRF promoter in the presence of PMA, which stimulates the mitogen-activated protein kinase pathway (Fig. 5C). Elk-1 activated the wild-type SRF promoter in a dose-dependent manner. In contrast, its ability to activate the mutant SRF promoter was severely reduced. Collectively, these data demonstrate the importance of the ets motif within the SRF promoter for the binding of Elk-1 and the subsequent ability of Elk-1 to regulate its expression.

FIG. 4. Elk-1 binds the SRF promoter in a B-box-dependent manner. A, schematic diagram showing the sequence of the wild-type and mutated ets binding site in the SRF promoter. The region encompassed by the dashed box was used in the gel retardation assay. B, gel retardation assay with a fragment of SRF promoter (−49 to −165) containing the WT or mutated ets binding site (ets-mut) and one CArG box. The DNA was incubated with the indicated combination of coreSRF and C-terminal truncated Elk-1-(1–168)(WT) or Elk-1-(1–168)(L158P). The ternary complex containing Elk-1 and SRF and binary complex containing SRF alone are indicated by closed and open arrows, respectively.

FIG. 5. The ets motif in the SRF promoter plays an important role in its regulation by Elk-1. A, schematic diagram showing the sequence of wild-type and the mutated ets binding site in the SRF promoter used in luciferase assay. B and C, reporter gene analysis of the WT and ets mutated (ets-mut) SRF promoter. B, 293 cells were transiently transfected with a luciferase construct containing the WT or ets-mut SRF promoter and with 2.5 ng of Elk-En or 5 ng of Elk-VP16 fusion protein. Cells were starved for 48 h and then stimulated with 20% serum. Luciferase activity was measured 6 h after stimulation. The results were normalized to β-galactosidase activity. C, 293 cells were transiently transfected with SRF-luc reporter constructs as in B but with increasing concentrations of Elk-1 (2.5, 5, and 10 ng, respectively). Cells were starved for 48 h and then stimulated with 10 nM PMA. Relative luciferase activity was determined as in B.

Elk-1 Regulates SRF Expression
Elk-1 regulates SRF expression

Elk-1 binds to the SRF promoter in vivo. A, schematic diagram of the SRF gene showing the location of oligonucleotides used in the chromatin immunoprecipitation assay. B, chromatin immunoprecipitation of Elk-1 bound to the SRF promoter. HeLa cells were starved in serum-free DMEM for 48 h and then stimulated with 10 nM PMA for 5 min where indicated. Sonicated chromatin was immunoprecipitated with either an anti-Elk-1 antibody or nonspecific IgG. PCR analysis of eluted DNA was performed using oligonucleotides specific for the SRF promoter (top panel), SRF intronic sequence (middle panel), or egr-1 promoter (bottom panel). 2% of input DNA is shown in lanes 1 and 2. The panels shown are inverted images of ethidium bromide-stained gels.

Elk-1 regulates SRF expression by the ERK and Rho Pathways—Previously, SRF has been identified as a target gene of the Rho signaling pathway (21), and this regulation is mediated by coactivator proteins from the MAL family that act through SRF binding to the promoter (Fig. 7A). The binding of MAL is mutually exclusive with TCF binding to SRF (19), suggesting that regulation by TCFs and MAL might also be mutually exclusive. However, it is also possible that the ERK pathway, through the TCFs, can contribute to activation of the SRF promoter. This would be important physiologically, as under different conditions, signaling through either the Rho or ERK pathway alone or simultaneous activation of several pathways might occur.

To investigate the convergence of these two pathways on the SRF promoter, we first examined the expression of SRF in response to activating the ERK pathway with PMA or the ERK and Rho pathways by serum (FCS). The ERK and Rho pathway inhibitors, U0126 and LB, respectively, were used to identify the individual contributions of these pathways to SRF induction. SRF expression was increased by PMA stimulation, and this increase was selectively blocked with U0126, demonstrating that this activation was ERK pathway dependent (Fig. 7B, lanes 2–4). Similarly, FCS stimulated SRF expression, and this was inhibited by both LB and, to a lesser extent, U0126 (Fig. 7B, lanes 5–7). Similar observations have been made in NIH-3T3 cells (21). Thus, the ERK pathway can activate SRF expression and contribute to its expression following serum induction.

We also tested whether the ERK and Rho pathways contributed directly to the regulation of the SRF promoter using reporter gene assays. First, we overexpressed Elk-1 to sensitize the SRF promoter to ERK pathway-mediated stimulation. Under these conditions, both PMA and FCS treatment led to an increase in the activity of a SRF promoter–driven luciferase reporter (Fig. 7C). The inhibition of the ERK pathway reduced the activation of the SRF promoter by both treatments. However, LB was less effective in reducing the activation of the SRF promoter by FCS, presumably because of Elk-1 competing with MAL recruitment. To probe this possibility further, we tested the response of the SRF promoter to activation of the Rho pathway by jasplakinolide in the presence and absence of exogenous Elk-1 (Fig. 7D). The addition of Elk-1 inhibited the activation of the SRF promoter by jasplakinolide. This was not a general inhibitory effect as the promoter now became more responsive to activation by PMA. Thus, the level of Elk-1 in the cell can determine whether the ERK or Rho pathway can activate the SRF promoter.

Conversely, we asked whether overexpression of a constitutively active form of MAL, MALN (16), could interfere with Elk-1-mediated SRF promoter regulation. We transfected 293 cells with either the WT or mutant (ets-mut) versions of the SRF-luciferase reporter construct in the presence of a low amount of Elk-En to dampen down the activity of the promoter. A dose-dependent increase in the activity of the WT promoter was observed upon transfection of increasing amounts of MALN expression plasmid (Fig. 7E). However, in contrast, the ets-mut promoter was activated to maximal levels at the lowest concentration of MALN expressed, with no further increases seen at higher levels of MALN (Fig. 7F). Thus, the absence of the ets motif within the SRF promoter makes it more sensitive to activation by MALN, thereby demonstrating that occupancy of this ets motif in vivo can inhibit activation via MAL.

Finally, we compared the ability of wild-type Elk-1 and an alternative ETS domain transcription factor, PEA3, to affect the activity of MALN on the SRF promoter in serum-starved cells. Experiments were carried out under serum-starved conditions where the ERK pathway and hence the ETS domain protein targets are not activated. Elk-1 inhibited the action of MALN in a dose-dependent manner, but PEA3 did not, and instead weakly potentiated the activity of MALN on the SRF promoter (Fig. 7F). This is consistent with a model whereby Elk-1 binds through SRF interactions, thereby inhibiting MAL recruitment, but that other ETS domain proteins like PEA3 may in some circumstances function in an SRF-independent manner to activate the SRF promoter.

Collectively, these data corroborate previous observations that indicate that TCF and MAL/myocardin recruitment by SRF is mutually exclusive (19, 20). However, importantly, they demonstrate a clear role for the ERK pathway in regulating SRF expression and suggest how multiple signaling inputs might lead to up-regulation of the SRF promoter (see “Discussion”).

DISCUSSION

The TCF transcription factors play an important role in transducing extracellular signals into a nuclear response by acting as targets for the mitogen-activated protein kinase signaling pathways (reviewed in Refs. 1, 2, and 9). To fully understand the physiological role of the TCFs, it is important to gain a fuller insight into the spectra of target genes that they regulate. To date, the focus has been primarily on the immediate-early genes such as c-fos and egr-1 (reviewed in Refs. 1, 2, and 9). Here we have identified SRF as a direct TCF target gene. This provides an elegant positive feedback loop whereby the TCFs can regulate the expression of the TCF partner protein SRF.

We initially identified SRF as a TCF target gene by demonstrating down-regulation of SRF expression in cell lines expressing the repressive Elk-En fusion protein (Fig. 1). By using a combination of reporter gene and chromatin immunoprecipitation analyses, we have shown that the TCFs can directly
affect the SRF promoter and that endogenously expressed Elk-1 can be found on this promoter in vivo (Figs. 3, 5, and 6).

It is currently not known whether TCFs other than Elk-1 can participate in the regulation of SRF. Indeed, as overexpressed Elk-En can potentially block regulation by all the TCFs because of their structural similarity, it will be important to probe whether the other TCFs can work in the same way. It remains possible that other ETS domain proteins might act on the SRF promoter through the ets site. Indeed, overexpression of PEA3, a target of the ERK pathway (37), can activate the SRF promoter in an ets site-dependent manner (data not shown). However, PEA3 is unable to compete with Elk-1 for promoter occupancy (Fig. 3C). Indeed, we show that in cells containing Elk-1, the SRF promoter is occupied by Elk-1 (Fig. 6). It remains possible that in other cell types, where low levels of Elk-1 and high levels of ETS domain proteins such as PEA3 exist, that different ETS domain proteins contribute to SRF regulation. However, the interplay with the Rho/MAL pathway would likely differ, as interference with SRF-dependent MAL recruitment would not occur (Fig. 7F).

Elk-1 appears to act to couple signals generated by the ERK pathway to the activation of the SRF promoter (Fig. 7), whereas the coactivator MAL has previously been shown to couple Rho pathway signals to this promoter (21). Signaling through the ERK pathway is of importance where signaling does not activate the Rho pathway, here induced using the mitogen PMA, and as a potential contributing pathway to more complex signaling triggers such as serum. Importantly, we show that the ability of Elk-1 to regulate SRF expression is dependent on the formation of a ternary complex with DNA-bound SRF (Figs. 2, 4, and 5). Previous studies have shown that signaling via the Rho pathway also functions through SRF bound to its own promoter, although in this case, the MAL/myocardin family protein MAL represents the coregulatory
partner that links to this pathway (13, 16). We demonstrate that a functional antagonism can occur depending on the relative levels of MAL and the TCFs within the cell (Fig. 7), which is consistent with previous data demonstrating that Elk-1 can inhibit MAL recruitment in a B-box-dependent manner to SRF-regulated promoters (19, 20). Thus depending on the relative levels of the TCF and MAL/myocardin family proteins, the relative contributions of these two classes of coregulators might differ. Alternatively, there may be a role for both coregulators in permitting convergence of the ERK and Rho pathways through SRF bound to promoters. Our data suggest that the latter scenario may well exist at the SRF promoter, as inhibitor studies demonstrate a contribution of the ERK pathway to mitogenic stimulation of SRF expression (Fig. 7B) that is consistent with results from a previous study (21). In addition, it has been proposed that the TCFs might contribute to the early induction phase of other genes like c-fos in response to mitogens, whereas the MAL/myocardin family contributes to the later phases (13). Furthermore, recent studies demonstrate that promoters in several smooth muscle genes can be regulated through SRF by either the TCFs or MAL/myocardin family members depending on the signaling pathways that are active (20). Thus our data add further weight to an emerging model that SRF can form a platform that can differentially recruit coregulatory transcription factors and permit selective or combinatorial responses to different signaling pathways depending on the target promoter.

Finally, our data further suggest an important role for SRF expression in cell survival. Previously, SRF has been shown to be a target for degradation in response to apoptotic pathways (38, 39) and also to regulate the expression of anti-apoptotic proteins like Bcl-2 (40). Recently we showed that the induction of Elk-En causes apoptosis and that one key anti-apoptotic target gene was the antiapoptotic gene Mcl-1 (28). As SRF expression is also down-regulated upon Elk-En induction, then this too might play a role in triggering apoptosis. Indeed, the down-regulation of SRF transcript levels is a natural process observed upon apoptotic induction caused by etoposide treatment. However, although we could partially rescue apoptosis induced by Elk-En (data not shown), we have been unable to determine whether this was because of replacement of the down-regulated endogenous SRF, or merely titration of the Elk-En away from the promoters of antiapoptotic genes. Thus, the TCFs can potentially promote cell survival through the regulation of multiple genes and provide an important link to the prosurvival effects of the ERK pathway.

Acknowledgments—We thank Anne Clancy for excellent technical support. We also thank Ravi Misra and Richard Treisman for reagents, and Shen-Hai Yang and Alan Whitmarsh for comments on the manuscript.

2 A. Kasza and A. D. Sharrocks, unpublished data.