

**HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN R ENHANCES
TRANSCRIPTION FROM THE NATURALLY-CONFIGURED *C-FOS* PROMOTER
*IN VITRO***

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Transcription of a proto-oncogene *c-fos* is induced rapidly to high levels by various extracellular stimuli. To explore the molecular mechanism of *c-fos* gene induction, we established a defined *in vitro* transcription system for the *c-fos* promoter that consists of purified activators (SRF, Elk-1, CREB and ATF1), general transcription factors (GTFs) and RNAP II. In this reconstituted transcription system, activation of *c-fos* transcription was highly dependent upon coactivators such as PC4 and Mediator, indicating a very weak activation potential of the activators in the context of an unaltered promoter structure. This heightened coactivator-dependency, however, allowed us to identify from HeLa nuclear extract a coactivator-like activity termed transcriptional regulator of *c-fos* (TREF) that enhanced *c-fos* transcription but not GAL4-VP16-dependent transcription. TREF cooperated with Mediator to enhance *c-fos* transcription by ~60 fold over its basal level, and like Mediator, stimulated activator-independent (basal) transcription as well. Further purification of TREF revealed that it consists of at least three distinct components, one of which was purified to near homogeneity and identified as heterogeneous nuclear ribonucleoprotein R (hnRNP R). Recombinant hnRNP R enhanced transcription from the *c-fos* promoter and displayed cooperativity with PC4 and Mediator, thus demonstrating its direct transcriptional activity.

studied gene promoters and responds in a rapid and transient manner to a myriad of extracellular signals such as growth factors, cytokines and cellular stress (1). These signals are transmitted via cascades of kinases including mitogen-activated protein (MAP) kinases to the nucleus, wherein the transcription factors bound on the inducible cis-elements are activated upon phosphorylation (2). Among the important cis-elements within the *c-fos* promoter are the serum response element (SRE) upon which a dimer of SRF and one molecule of Elk-1 form a ternary complex (3) and the cAMP response elements (CREs) which are bound by a heterodimer of CREB and ATF1 (4). Although the signal transduction and the subsequent regulation of the activators are well characterized, much less is known about the mechanism by which the activators elicit dramatic *c-fos* induction.

Transcription of a typical protein-coding gene such as *c-fos* requires general transcription factors (GTFs), including TFIIA, TFIIB, TFIID, TFIIE, TFIIIF and TFIIH, which form the preinitiation complex (PIC) with RNA polymerase II (RNAP II) on the promoter (5). PIC formation and the following steps of transcription are ultimate targets of activators, whose activating signals are transduced to GTFs and RNAP II in a process that is poorly understood. This process, nonetheless, has been shown to require another class of cofactors termed coactivators or coregulators that serve as a physical and functional bridge between activators and GTFs (6,7). In the case of the *c-fos* gene, well-characterized p300/CBP serves as a coactivator for SRF (8), Elk-1 (9) and CREB (10,11), and coactivates

The *c-fos* promoter is one of the very well

transcription not only as a bridging factor but also as a histone acetyltransferase (12). More recent studies indicate that another type of coactivator termed Mediator is also involved in *c-fos* transcription. The Med23 subunit of Mediator, first identified as an E1A-interacting protein (13), has been demonstrated to interact with Elk-1 and plays a role for SRE-mediated transcriptional activation (14). Indeed, Med23 (-/-) knockout cells show reduced transcription of SRE-containing genes such as *egr-1* and *c-fos* (14,15).

Mediator was initially discovered by the genetic and biochemical analyses in yeast (16-18). Subsequent biochemical dissection of mammalian nuclear extracts by several independent groups lead to the purification of mammalian Mediator complexes (6), variously named as negative regulator of activated transcription (NAT), the mouse Mediator complex, thyroid hormone receptor-associated proteins (TRAP), SRB-MED-containing cofactor (SMCC), vitamin D receptor-interacting proteins (DRIP), activator-recruited cofactor (ARC), cofactor required for Sp1 activation (CRSP), positive cofactor 2 (PC2). The identified complexes turned out to be highly related to each other and probably constitute the identical Mediator or its various subcomplexes (6).

Given the diversity of the employed biochemical systems, it is somewhat surprising that only the requirement of Mediator was eventually revealed. The apparent unity, however, could be due to the use of artificially constructed activators and templates that may alter, or even obviate, the requirement of some cofactors. Indeed, for producing sufficiently strong signals of transcript, *in vitro* transcription systems typically utilize a strong activation domain fused to a DNA-binding domain (often the DNA binding domain of yeast GAL4) and an artificially constructed model promoter with tandem repeats of activator-binding sites. Because of the activator's intrinsically strong activation potential and the optimized geometry of activator-binding sites, it is possible that these systems obscure the requirement for some class of cofactors.

To identify a novel cofactor requirement in an unbiased manner, we chose the *c-fos*

promoter as a model and initiated to recapitulate transcription from the naturally-configured promoter with only purified components. We employed a defined *in vitro* transcription system using purified GTFs (19), together with SRF, Elk-1, CREB and ATF1 to analyze transcriptional activation from the *c-fos* promoter that retains cis-elements in its original configuration.

Unlike the GAL4-VP16-based model promoter, activation of the *c-fos* promoter by SRF, Elk-1, CREB and ATF1 was much more dependent on coactivators such as PC4 and Mediator.

This heightened coactivator-dependency allowed us to identify from HeLa nuclear extract a novel coactivator-like activity termed transcriptional regulator for *c-fos* (TREF), which augments activated transcription from the *c-fos* promoter but not the GAL4-VP16-dependent transcription. Upon further purification, TREF was separated into three activities, one of which was identified as hnRNP R. Recombinant hnRNP R stimulated *c-fos* transcription and cooperated with PC4 and Mediator, evoking a potent activation (~100-fold) of the *c-fos* promoter over its basal level. Since hnRNP R is a presumptive RNA binding protein and is possibly involved in regulating mRNA stability, our results suggest a potential link between transcription and the subsequent posttranscriptional processes for rapid and transient *c-fos* induction.

EXPERIMENTAL PROCEDURES

Construction of baculoviruses for expressing FLAG-tagged proteins— Complementary DNAs (cDNAs) of human SRF, Elk-1, CREB, ATF1 and hnRNP R were isolated from HeLa 1st-strand cDNA by a PCR-based method. Since the 5' region of SRF cDNA could not be amplified by PCR due to a high GC content, ~300 bp of the 5' region was constructed by annealing synthetic oligonucleotides based on the published SRF DNA sequence. PCR was used to add *NdeI* and *BamHI* sites to the N- and C-terminal ends of each cDNA, and when necessary, internal *NdeI* and *BamHI* sites were mutated without altering the amino acid sequences. Each cDNA was sequenced in its entirety and

then subcloned into a modified baculovirus transfer vector pTOF, which was constructed from its parental plasmid, pVL1392, by mutating the unique *NdeI* site within the plasmid and adding FLAG-encoding DNA sequence as well as *NdeI* and *BamHI* sites. Recombinant baculoviruses were prepared by transfection of *Sf9* cells with each transfer vector (1.0 μ g), linearized baculoviral DNA (0.25 μ g) and cellfectin (Invitrogen), and the obtained viruses were plaque purified and amplified.

Preparation of High Five cell extract— High Five cells were infected with each amplified baculovirus and cultured at 27°C for 50 hours. The collected cells were washed once with PBS, resuspended in one packed cell volume (PCV) of hypotonic buffer (10 mM Tris-HCl, pH 7.3 at 4°C, 1.5 mM MgCl₂, 10 mM KCl) containing 0.5 mM PMSF, 1 mM DTT, 100 nM MG132, 10 mM β -glycerophosphate and protease inhibitor cocktail (Sigma) and then homogenized mildly in a dounce homogenizer. After centrifugation, the supernatant was saved as the cytoplasmic (S100) fraction and the pellet (crude nuclei) was suspended in one PCV of low salt buffer (10 mM Tris-HCl, pH 7.3 at 4°C, 25% glycerol, 0.2 mM EDTA, 1.5 mM MgCl₂, 10 mM KCl) and extracted by addition of one PCV of high salt buffer (10 mM Tris-HCl, pH 7.3 at 4°C, 25% glycerol, 0.2 mM EDTA, 1.5 mM MgCl₂, 1.2 M KCl). The nuclear suspension was fractionated by centrifugation into the nuclear extract and nuclear pellet fractions.

Purification of F:SRF, F:Elk-1, F:CREB, F:ATF1 and F:hnRNP R— FLAG-tagged SRF (F:SRF)-containing S100 fraction was loaded onto HiTrap heparin (GE Healthcare) equilibrated with BC(100) containing 0.5 mM PMSF and 1 mM DTT. BC buffer consists of 20 mM Hepes-KOH, pH 7.9, 1 mM EDTA and 10% glycerol, and the number in a parenthesis shows its KCl concentration (mM). The bound proteins were eluted with a 10 CV (column volume) linear gradient from 100 to 1000 mM KCl. F:SRF bound to HiTrap heparin was eluted at 300~500 mM KCl. The fractions containing F:SRF were adjusted to 100 mM with BC(0) and loaded onto HiTrap Q (GE Healthcare), and the bound proteins were eluted with a 10 CV linear gradient from 100

to 1000 mM KCl. F:SRF was eluted at 200~300 mM KCl, and the fractions containing F:SRF were loaded onto Mono S. F:SRF bound to Mono S was eluted at 150~250 mM KCl. F:Elk-1 was purified with the same columns as those used for F:SRF (HiTrap heparin, HiTrap Q and Mono S). F:Elk-1 was eluted at 200~300 mM KCl from HiTrap heparin and was in the flow-through fractions from both HiTrap Q and Mono S. The High Five S100 fraction containing recombinant F:CREB (CREB α isoform) was loaded onto HiTrap SP (GE Healthcare) equilibrated with BC(100) containing 0.5 mM PMSF. F:CREB bound to HiTrap SP was eluted stepwise with BC(700). The eluate was dialyzed against BC(100) containing 0.5 mM PMSF and 0.1% Triton X-100, and F:CREB was further purified with anti-FLAG M2 agarose. The same method was also used for purifying F:ATF1. F:hnRNP R was purified similarly by SP Sepharose and Q Sepharose columns, followed by affinity purification using anti-FLAG M2 agarose.

GST pull-down assays— GST pull-down assays were performed essentially as described (19) using a FLAG-tagged PC4 expressed in *E. coli*.

In vitro transcription— The promoter region of the *c-fos* gene (from -380 to -10) was isolated from human genomic DNA by PCR using the following primers; 5'-GGCCGAATTCGCACTGCACCCTCGG TGTTG-3' and 5'-GGCCGTCGACGGCCGCGCCGCAGC CACTGCTTTTATAAC-3'.

After disruption of the *EagI* site at -121, the *c-fos* promoter fragment was subcloned between *EcoRI* and *SalI* sites of pUC19, and the G-less cassette from pG5HMC2AT (20) was fused to the *c-fos* promoter by using an *EagI* site introduced at -8. *In vitro* transcription assays were performed as described (21).

Purification of Mediator— To produce a 3xFLAG-Nut2-expressing cell line, pLNCX2-3xFLAG-Nut2 was introduced into AmphoPack-293 cells by Lipofectin (Invitrogen) and the produced viruses were used to infect HeLa S3 cells, which were then selected in 0.8 mg/ml G418 medium containing 10% FBS and 1% penicillin-streptomycin solution (GIBCO).

The G418-resistant colony that expressed the highest level of 3xFLAG-Nut2 was expanded for nuclear extract preparation. The nuclear extract was fractionated on a P11 column, and the resulting 0.85 M KCl eluate was adjusted to BC(300) containing 0.1% Triton X-100 and mixed with M2-agarose (Sigma) at 4°C for 1hr. After extensive washes with the same buffer, bound proteins were eluted with the buffer containing 0.1 mg/ml 3xFLAG peptide.

HeLa NE fractionation— Fifty ml of HeLa nuclear extract (~10 mg/ml protein) was adjusted to BC(100) and loaded onto a 50-ml P11 column, and the flow-through fraction was collected as fraction A. The bound proteins were then eluted stepwise in BC(300), BC(500) and BC(850), and the eluates were collected as fractions B, C and D, respectively. Each fraction was then adjusted to BC(100) by dialysis and loaded onto an appropriate volume (~10 ml per 100 mg protein) of DE52 column, and the flow-through fraction and the BC(300) eluate were collected.

RESULTS

Expression and purification of natural activators for the c-fos gene— The *c-fos* promoter contains binding sites for several transcription factors, including serum response factor (SRF), Ets-related transcription factor (Elk-1), cAMP-response element binding protein (CREB) and activating transcription factor 1 (ATF1). SRF binds the serum response element (SRE) as a dimer and forms a ternary complex with Elk-1, and CREB binds the *c-fos* AP-1 site (FAP-1) and cAMP-response element (CRE) either as a homodimer or as a heterodimer with ATF1 (Fig. 1A). These transcription factors play important roles for *c-fos* transcription, with each transcription factor differentially regulated by extracellular signals (22). The activities of SRF, Elk-1, CREB and ATF1 are enhanced upon phosphorylation by MAP kinases or their downstream effector kinases such as the mitogen- and stress-activated kinases (MSKs) and the ribosomal S6 kinases (RSKs) (23).

As a first step to better understand the regulatory mechanisms of *c-fos* transcription

in vitro, it was essential to obtain intact SRF, Elk-1, CREB and ATF1. For this purpose, we chose the baculovirus expression system, which can provide posttranslational modifications as opposed to the *E. coli* system, because these transcription factors are subject to multiple posttranslational modifications, including phosphorylation, glycosylation and sumoylation that regulate their activity and stability (24,25). Moreover, to prevent rapid degradation of the expressed proteins, we found that the inclusion of a proteasome inhibitor MG132 during purification was crucial, presumably because of the ubiquitination and proteasome-mediated protein degradation (26). As shown in Fig. 1B, the expressed activators were purified through combinations of conventional and affinity columns. Specifically, SRF and Elk-1 were purified by HiTrap heparin, HiTrap Q and Mono S columns, while CREB and ATF1 were purified by a HiTrap SP column and then by anti-FLAG M2 affinity purification. The SDS-PAGE analysis showed that the obtained activators were essentially homogenous with no apparent degradation (Fig. 1C).

The purified transcription factors were also tested for their DNA-binding using gel-shift (supplemental Fig. S1) and DNase I footprinting assays (supplemental Fig. S2). As expected, SRF and Elk-1 bound specifically to the SRE, and CREB and ATF1 bound to both the FAP-1 and CRE. Elk-1 binding to the SRE was completely dependent upon the presence of SRF. Both CREB and ATF1 bound to the FAP-1 and CRE as either homodimer or heterodimer; however, a heterodimer of CREB and ATF1 appeared to show the strongest binding. Together, the purified recombinant SRF, Elk-1, CREB and ATF1 are intact and retain the binding activity to their cognate cis-elements.

SRF, Elk-1, CREB and ATF1 fail to activate transcription in the reconstituted in vitro transcription system— Having confirmed the DNA binding activities of recombinant SRF, Elk-1, CREB and ATF1, we then tested their transcriptional activities. First, to reveal the intrinsic activation potential of these cellular activators, we employed an *in vitro*

transcription system reconstituted only with RNAP II and six GTFs (TFIIA, TFIIB, TFIID, TFIIIE, TFIIF and TFIIH). The reconstituted *in vitro* transcription system was devoid of any known coactivators but capable of supporting activated transcription by GAL4-VP16 (19). A *c-fos* template, pfMC2AT, was constructed by replacing the upstream GAL4-binding sites and the TATA box of pG5HMC2AT with the *c-fos* promoter region (Fig. 2A). Thus, the pfMC2AT template encompasses from -380 to -10 of the human *c-fos* gene, including the SRE, FAP-1, CRE and TATA box, which allows simultaneous binding of seven activators; namely, one Elk-1 and two SRFs to the SRE, and each CREB/ATF1 heterodimer to the FAP-1 and CRE (Fig. 1A).

Consistent with the previous results, the reconstituted transcription system allowed GAL4-VP16 to activate transcription from its cognate template with five GAL4-binding sites (pG5HMC2AT) in the absence of any coactivator, achieving ~2 to 3-fold activation at its saturating amount (Fig. 2D) (19). The same transcription system, however, failed to activate transcription when one of the *c-fos* gene activators (SRF, Elk-1, CREB and ATF1) was added individually (Fig. 2B). Moreover, even when various combinations of the activators (SRF/Elk-1, CREB/ATF1 or SRF/Elk-1/CREB/ATF1) were tested, minimal levels of activation was observed (Fig. 2C). At the highest amounts of the four activators, where *c-fos* transcription increased apparently, basal (i.e. activator-independent) transcription also increased to similar extents, indicating no net effect on activator-dependent transcription under these conditions (Fig. 2C, lanes 9 and 10). Given that a single *c-fos* promoter binds seven activators (Fig. 1A), with each activator possessing at least one activation domain, it is unlikely that the number of activators or activation domains is insufficient for *c-fos* promoter activation. Thus, unlike viral activators (e.g. VP16), which can activate transcription in the absence of any coactivator, the tested activators (SRF, Elk-1, CREB and ATF1) alone are insufficient to evoke activated transcription, suggesting a higher degree of dependence on coactivators.

A coactivator PC4 permits transcriptional activation of the c-fos promoter by SRF, Elk-1, CREB and ATF1— As an initial step to explore their coactivator requirement for activating the *c-fos* promoter, we tested the effect of PC4, a well-characterized coactivator that augments *in vitro* transcription for a wide variety of activators (27,28). We added PC4 to *in vitro* transcription reactions, in which various combinations of the *c-fos* activators were examined. Consistent with our earlier study (19), PC4 enhanced GAL4-VP16-dependent transcription from 2.5-fold to 38 fold, giving rise to additional increase (over 10 fold) in activation (Fig. 3C). Similarly, PC4 enabled the *c-fos* activators individually (Fig. 3A) or as combinations of SRF/Elk-1, CREB/ATF1 or SRF/Elk-1/CREB/ATF1 (Fig. 3B), to activate transcription by ~5-9 fold. Thus, SRF, Elk-1, CREB and ATF1 are indeed capable of activating transcription, though in a markedly PC4-dependent manner.

PC4 exerts its coactivator activity by mediating interactions between activators and GTFs such as TFIIA (27), TFIIB (29) and TFIIH (19). Given the ability of PC4 to enhance the transcriptional activities of SRF, Elk-1, CREB and ATF1 (Fig. 3, A, B and C), we wished to know if PC4 could interact with these activators. To determine this, we performed GST pull-down assays using GST-fused activators and FLAG-tagged PC4. Figure 3D shows that PC4 interacted strongly with CREB and Elk-1, moderately with ATF1, and very weakly with SRF. The results indicate that these interactions may, at least in part, provide a mechanistic basis for PC4-enhanced transcriptional activities of SRF, Elk-1, CREB and ATF1.

A novel activity enhances in vitro transcription from the c-fos promoter— The numerous *in vivo* studies have shown that various extracellular stimuli elicit *c-fos* gene activation that typically reaches well over a hundred fold. Thus, even in the presence of PC4, the observed activation of the *c-fos* promoter *in vitro* (Fig. 2, A and B) was unremarkable as compared to that generally observed within cells. To further explore the coactivator requirement for the *c-fos* promoter in an unbiased manner, we fractionated the nuclear extract from rapidly

growing HeLa cells into eight fractions (Fig. 4A) and examined their effects on *c-fos* transcription *in vitro*.

As expected, the D0.3 fraction, which contains TFIID (supplemental Fig. S3), increased both basal and activated transcription (Fig. 4B, lanes 17 and 18), as the amount of TFIID in the current transcription system was set below the saturating amount to reveal the effect of activators (Fig. 4C, lanes 7-10). Furthermore, the D0.1 fraction showed an activity reminiscent of Mediator (Fig. 4B, lanes 15 and 16) (30), indicating that the D0.1 activity is, at least in part, attributable to Mediator, though the presence of additional coactivators cannot be excluded. Intriguingly, the A0.3 fraction, in which no biochemically defined coactivator had been reported, stimulated transcription from the *c-fos* promoter (Fig. 4B, lanes 5 and 6). Although this fraction includes TFIIA (31), which is important for activated transcription *in vitro* (19), further addition of TFIIA did not affect the level of *c-fos* transcription at all (Fig. 4C, lanes 3-6), thus excluding the possibility that the observed stimulatory effect was due to TFIIA. Collectively, our biochemical analyses identified a novel activity, which we termed TREF (Transcriptional REgulator of *c-Fos*), that can stimulate *in vitro* transcription from the *c-fos* promoter.

TREF enhances transcription from the c-fos, but not the 5xG promoter and displays cooperativity with Mediator— We then addressed whether or not TREF was specific to the *c-fos* promoter. As shown in Fig. 5A, TREF increased both basal transcription (lane 3) and activator-dependent transcription (lane 4) from the *c-fos* promoter, eliciting a ~17-fold net increase of transcription from the basal level (compare lanes 1 and 4). However, while TREF increased basal transcription from the 5xG promoter, it somewhat diminished GAL4-VP16-dependent transcription from the same promoter (Fig. 5A, lanes 9 and 10). Thus, although TREF enhances transcription through the core promoter as well as the upstream regulatory elements, the stimulatory effect of TREF through the upstream elements appears to have some

activator specificity. By contrast, the D0.1 fraction, which principally reflects the activity of Mediator, stimulated both basal and activator-dependent transcription from the *c-fos* (Fig. 5A, lanes 5 and 6) and 5xG promoters (Fig. 5A, lanes 11 and 12) in a similar manner. Finally, the fully phosphorylated activators and its unphosphorylated (i.e. phosphorylation-defective) mutants displayed essentially indistinguishable response to TREF, indicating that phosphorylation of the activators do not affect the TREF activity (data not shown).

Next, we tested the functional cooperativity of TREF and Mediator, a major coactivator component of the D0.1 fraction, in stimulating *c-fos* transcription. As shown in Fig. 5C, both TREF and Mediator, which was isolated from HeLa cell line stably expressing FLAG-Nut2 (Fig. 5B), enhanced activator-dependent transcription from the *c-fos* promoter (lanes 4, 6, 12 and 14). When TREF and Mediator were added simultaneously to the transcription reactions, they displayed further enhancement of transcription from the *c-fos* promoter, resulting in ~60 fold net activation (Fig. 5C, lanes 8 and 16) over the basal levels (Fig. 5C, lanes 1 and 9). Importantly, this cooperativity between TREF and Mediator was observed in the range of amounts where 2-fold increase of TREF (Fig. 5C, compare lanes 3 and 4 versus 11 and 12) or 3-fold increase of Mediator (Fig. 5C, compare lanes 5 and 6 versus 13 and 14) had little additional effect on transcription individually. Thus, TREF not only stimulates *c-fos* transcription through the core promoter and the upstream regulatory elements, but also displays cooperativity with Mediator for enhancing *c-fos* transcription, indicating that the *c-fos* gene may require multiple coactivators for its dramatic level of transcriptional activation observed *in vivo*.

hnRNP R is an active component of TREF—

To further characterize the TREF activity, we purified the A0.3 fraction using *c-fos* transcription assays with SRF, Elk-1, CREB and ATF1 as activators and PC4 as a coactivator. Our initial attempt to purify TREF resulted in a dramatic reduction in the TREF activity; however, we realized that the

reduced activity was due to the separation of the original TREF activity into different fractions. Indeed, as shown in Fig. 6A, purification with a larger amount of the A0.3 fraction by Q Sepharose and Heparin Sepharose showed that the TREF activity could be separated into at least three activities that were tentatively designated as TREF α , TREF β and TREF γ . TREF α was purified further to near homogeneity by a Mono S column, and SDS-PAGE analysis revealed that active fractions contained a ~80-kDa protein as a major polypeptide that correlated with the TREF α activity (Fig. 6B). Mass spectrometric analysis identified this 80-kDa polypeptide as hnRNP R, a putative RNA-binding protein, whose function remains ill defined. hnRNP R is a 633-aa protein with three RRM and one arginine-glycine-glycine rich (RGG) box, and belongs to a group of proteins termed hnRNPs (32) (Fig. 6C).

To demonstrate that hnRNP R possesses the TREF α activity, we expressed recombinant hnRNP R by baculovirus expression system and purified it to homogeneity (Fig. 6D). As shown in Fig. 6D, recombinant hnRNP R displayed a coactivator activity in a dose-dependent manner in the presence of PC4. The effect of hnRNP R on GAL4-VP16-dependent transcription, however, was minuscule (supplemental Fig. S4), indicating that its coactivator activity varies depending upon the used activators. When PC4, Mediator and hnRNP R were tested individually or in combination, each coactivator stimulated *c-fos* transcription by 4 to 12 fold. hnRNP R and Mediator also stimulated activator-independent transcription to similar extents, indicating that both of them act directly to the core promoter in the absence of activators. Similar to the activity of a more crude TREF fraction (i.e. A0.3), hnRNP R also showed cooperativity with PC4 and Mediator, (Fig. 6D), and the combined effect of the three coactivators on activator-dependent *c-fos* transcription reached well over 100 fold (Fig. 6E). Together, our results indicate that hnRNP R serves as a transcriptional coactivator for *c-fos* transcription.

DISCUSSION

In this study, we have established an *in vitro* transcription system that utilizes unaltered cellular activators (SRF, Elk-1, CREB and ATF1) and the natural *c-fos* promoter to explore its coactivator requirement in an unbiased manner. We have identified from HeLa nuclear extract a novel coactivator activity, termed TREF, that specifically augments transcription from the *c-fos* promoter and cooperates with Mediator. TREF appears to be composed of at least three activities, one of which was identified as hnRNP R. Recombinant hnRNP R stimulated transcription from the *c-fos* promoter and cooperated with PC4 and Mediator to elicit a high level of *c-fos* activation.

Functional distinction between viral and cellular transcription factors— Our transcription assays showed that the tested cellular activators fail to activate transcription in the absence of any coactivator, a condition under which the archetypical transcriptional activator GAL4-VP16 can still activate transcription. Viral activators such as VP16, E1A, Tax and pX typically do not bind DNA directly but piggyback on DNA-bound cellular activators to enhance gene expression (33). For example, a prototypical viral protein VP16 becomes tethered to DNA by forming a complex with Oct-1 and HCF, and then interacts with GTFs (TBP, TAFs, TFIIB and TFIID) (34). Similarly, another viral protein pX is recruited to DNA via its interactions with transcriptional activators and then activate transcription through interactions with TBP, TFIIB and TFIID (35,36). Given these properties, viral activators resemble a coactivator rather than a mere transcriptional activator.

Besides acting as a coactivator, viral activators may have usurped part of the TFIID function as well. For example, the SV40 large T antigen (37), the hepatitis B viral protein pX (38) and the human cytomegalovirus IE proteins (39) can functionally obviate the requirement of TAF1, a TFIID subunit that functions as a coactivator. Thus, viral activators in general may depend less on coactivators than the

cellular activators. This would allow viral activators to maximize their transcriptional potency in a manner detached from cellular regulatory networks. Cellular activators, by contrast, may retain their regulatory potential at the cost of activation potency, possibly necessitating a greater dependency on coactivators.

hnRNP R possesses a coactivator activity for the c-fos promoter— The current study has identified a novel type of coactivator, termed TREF, that stimulates transcription from the *c-fos* promoter *in vitro* and is apparently distinct from the more generally required Mediator (40). The TREF activity increases activator-dependent as well as basal transcription from the *c-fos* promoter in a manner similar to that of Mediator (18,41,42). Unlike Mediator, however, TREF does not stimulate GAL4-VP16 dependent transcription *in vitro*. Together with its cooperativity with Mediator in stimulating transcription from the *c-fos* promoter and its differing chromatographic behavior from Mediator (30,43), TREF appears to be a novel activity distinct from the well-characterized Mediator. Indeed, several steps of chromatography revealed that TREF is separated into three distinct components, and one of which, TREF α , turned out to be hnRNP R. As it is, TREF α is also distinct from the reported coactivators for SRF or CREB, including CoS (coactivator for SRF-activated transcription) (44), MRTFs (myocardin-related transcription factors, (45,46), CBP (CREB binding protein)/p300 (47) and TORC (transducer of regulated CREB) (48,49).

hnRNP R belongs to a group of diverse RNA-binding proteins termed heterogeneous nuclear ribonucleoproteins (hnRNPs), which are characterized by RNA binding motifs such as the RGG box, RRM and KH domains (50). hnRNPs are involved in a wide array of RNA metabolism including transcription, splicing, RNA export, RNA stability and translation (50). No specific function, however, has been assigned to hnRNP R except for a recent report that suggests its role in ARE-mediated RNA degradation (51). Most of hnRNPs that have been analyzed so far do not appear to affect transcription (50); however, several lines of evidence indicate

that hnRNP K serves as a transcription factor. Indeed, hnRNP K binds to a C-rich DNA sequence termed the CT-element, which is located upstream of the *c-myc* gene, and interacts directly with TBP (52). Moreover, hnRNP K has been demonstrated to activate transcription *in vitro* and *in vivo* (52). Although hnRNAP R is structurally unrelated to hnRNP K, which has three KH domains but no RRM or RGG box (53), our purification and functional analyses of TREF α indicate that hnRNP R is another member of hnRNPs that directly stimulate transcription in addition to its possible role in regulating mRNA stability (51).

Recent studies have revealed that in addition to stimulating transcription, some coactivators participate actively in posttranscriptional processes. For example, the thermogenic coactivator PGC-1 regulates alternative splicing of the transcript from the gene promoter on which it is loaded (54). This coupling of transcription and splicing requires the RRM and serine- and arginine-rich (SR) domain within PGC-1 (54), indicating the importance of RNA-binding domains for the coupling. A coactivator for nuclear receptors, CoAA, also influences alternative splicing of the transcript in a steroid hormone-specific manner (55). CoAA contains two RRMs and is structurally an hnRNP-like protein (56). Thus, a subset of diverse proteins with RNA-binding domains such as the RRM, KH domain, SR domain and/or RGG box may be actively involved in both transcription and posttranscriptional processes.

The mechanism by which hnRNP R stimulates *c-fos* transcription awaits future studies. However, given that hnRNP R is found among the proteins associated with phosphorylated CTD (57) and thereby presumed to be complexed with elongating RNAP II, one attractive hypothesis is that hnRNP R stimulates RNAP II elongation, perhaps through its interactions with phosphorylated CTD and with transcribed RNA. If so, CTD phosphorylation by TFIIH and/or p-TEFb might regulate the binding of hnRNP R to the CTD and alter its coactivator activity. All in all, our results support the emerging view that transcription is tightly coupled to the subsequent RNA metabolism.

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FOOTNOTES

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The abbreviations used are: GTF, general transcription factor; TREF, transcriptional regulator of *c-fos*; RNAP II, RNA polymerase II; hnRNP R, heterogeneous nuclear ribonucleoprotein R; RRM, RNA recognition motif; RGG, arginine-glycine-glycine rich

LEGENDS TO FIGURES

FIGURE 1. Expression and purification of the full-length activators for the *c-fos* gene. *A*. Schematic diagram of the *c-fos* gene. The regulatory cis-elements (SRE, FAP-1 and CRE) in

the *c-fos* promoter and their cognate activators (SRF, Elk-1, CREB and ATF1) are depicted in the diagram. *B.* Purification schemes of SRF, Elk-1, CREB and ATF1. Recombinant FLAG-tagged proteins were expressed in insect (High Five) cells, and their extracts were used for the following purification. Three columns (HiTrap heparin, HiTrap Q and Mono S) were used for purification of SRF and Elk-1, while HiTrap SP and anti-FLAG M2 affinity resin were used for purification of CREB and ATF1. *C.* SDS-PAGE analysis of purified activators. Purified activators (~1.0 mg) were resolved on a 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue.

FIGURE 2. Transcriptional activities of SRF, Elk-1, CREB and ATF1 in the reconstituted *in vitro* transcription system. *A.* Schematic diagram of the templates used for *in vitro* transcription assays. The *c-fos* template, pMC2AT, contains the SRE, FAP-1, CRE and the TATA box, which are derived from the human *c-fos* promoter, together with a 390-nt G-less cassette. The 5xG template, pG5HMC2AT, contains five GAL4-binding sites, the HIV-1 TATA box and a 390-nt G-less cassette. The control plasmid, pMLΔ53C2AT, containing the Ad2MLP-derived TATA box and a 290-nt G-less cassette, was used for measuring basal transcription as a control. *B.* *In vitro* transcription assays in the absence of coactivators. Transcription reactions were performed in a reconstituted system consisting of GTFs and RNAP II with the indicated recombinant activator. The amounts of activators were 5, 20 or 80 ng for SRF, CREB and ATF1, and 2.5, 10 or 40 ng for Elk-1. The arrow and arrowhead indicate the positions of the transcripts from activator-dependent (pMC2AT) and basal transcription (pMLΔ53C2AT), respectively, and the relative levels of activator-dependent transcription are shown below each lane. *C.* *In vitro* transcription assays were done with various combinations of the activators. The relative levels of activator-dependent and basal transcription are shown below each lane. *D.* GAL4-VP16-dependent transcription using pG5HMC2AT. The reactions contained 1.6, 6.3 or 25 ng of GAL4-VP16. The arrow indicates the transcripts from activator-dependent transcription (pG5HMC2AT).

FIGURE 3. Effect of coactivator PC4 on transcriptional activation by SRF, Elk-1, CREB and ATF1. *A.* PC4 (200 ng) was added to the *in vitro* transcription reactions containing one of the activators (SRF, Elk-1, CREB and ATF1). The positions of the transcripts from activator-dependent (arrow) and basal (arrowhead) transcription are indicated on the right. *B.* PC4 (200 ng) was added to the *in vitro* transcription reactions with various combinations of SRF, Elk-1, CREB and ATF1 as activators. *C.* PC4 (200 ng) was added to *in vitro* transcription reactions from pG5HMC2AT with GAL4-VP16 as an activator. The arrow indicates activator-dependent transcription from pG5HMC2AT. *D.* Interactions of PC4 with SRF, Elk-1, CREB, ATF1 and VP16. GST-fused activators were immobilized on Glutathione Sepharose 4B and incubated with FLAG-PC4. The bound proteins were eluted in high salt buffer and analyzed by immunoblot analysis using anti-FLAG M2 antibody. The position of FLAG-PC4 is indicated by an arrow on the right.

FIGURE 4. A novel activity enhances transcription from the *c-fos* promoter. *A.* Fractionation scheme of HeLa nuclear extract. HeLa nuclear extract was fractionated stepwise by P11 (0.1, 0.3, 0.5, 0.85M KCl) and the derived fraction was further fractionated by DE52 (0.1, 0.3M KCl) into 8 fractions. The names of the DE52-derived fractions are as indicated at the bottom. *B.* *In vitro* transcription assays. Each fraction was added to the *in vitro* transcription reactions using the *c-fos* template, pMC2AT, in the presence or absence of the activators (SRF, Elk-1, CREB and ATF1). PC4 was also added with the activators. *C.* Effect of varying amounts of purified TFIIA or TFIID on *c-fos* transcription.

FIGURE 5. Promoter specificity of TREF and its cooperativity with Mediator. *A.* A novel coactivator activity, TREF, is specific to the *c-fos* promoter. *In vitro* transcription was performed using the *c-fos* template (pMC2AT) or 5xG template (pG5HMC2AT) in the presence or absence of the activators (SRF, Elk-1, CREB, ATF1 and PC4 for pMC2AT; GAL4-VP16 and

PC4 for pG5HMC2AT). Either A0.3 or D0.1 fraction was added to the indicated reactions. The arrow indicates the transcripts from pfMC2AT (*lanes 1-6*) or pG5HMC2AT (*lanes 7-12*). *B.* Purification of Mediator. The nuclear extract from the HeLa cell line that expresses FLAG-tagged Nut2 (F:Nut2) was fractionated by P11, and Mediator was purified from the 0.85 M KCl fraction using anti-FLAG M2 affinity gel. *C.* TREF cooperates with Mediator in activating transcription from the *c-fos* promoter. TREF and Mediator were added to the transcription reactions and the positions of transcripts from pfMC2AT and pMLΔ53C2AT are indicated by arrow and arrowhead, respectively.

FIGURE 6. hnRNP R stimulates transcription from the *c-fos* promoter. *A.* Purification scheme of TREF activities. The TREF activity (A0.3) was further purified by Q Sepharose, Heparin Sepharose and Mono S columns, and separated into three chromatographically distinct activities designated TREF α , TREF β and TREF γ . *B.* Transcriptional activities of the Mono S fractions of TREF α were assayed using pfMC2AT in the presence of SRF, Elk-1, CREB, ATF1 and PC4. The corresponding fractions were analyzed by SDS-PAGE and silver staining. The position of a ~80 kDa protein that correlated with the transcriptional activity is indicated on the right. *C.* Structural domains of hnRNP R. RRM, NLS and RGG indicate the RNA recognition motif, nuclear localization signal and arginine-glycine-glycine-rich domain, respectively. The regions rich in acidic amino acids or in glutamine and asparagine is indicated by acidic or QN, respectively. *D.* Purification of recombinant hnRNP R. FLAG-tagged hnRNP R was expressed in insect cells and purified by SP Sepharose, Q Sepharose and anti-FLAG M2 agarose. The purified hnRNP R was analyzed by SDS-PAGE. Transcriptional activity of hnRNP R was tested in the transcription assays using pfMC2AT, in which the presence of SRF, Elk-1, CREB, ATF1 and PC4 is indicated by +. Relative levels of activator-dependent transcription from pfMC2AT are indicated below the panel. *E.* hnRNP R, PC4 and Mediator were added in various combinations to the transcription reactions containing SRF, Elk-1, CREB and ATF1 as activators. Relative levels of activator-dependent transcription from pfMC2AT are indicated below the panel.

Figure 1

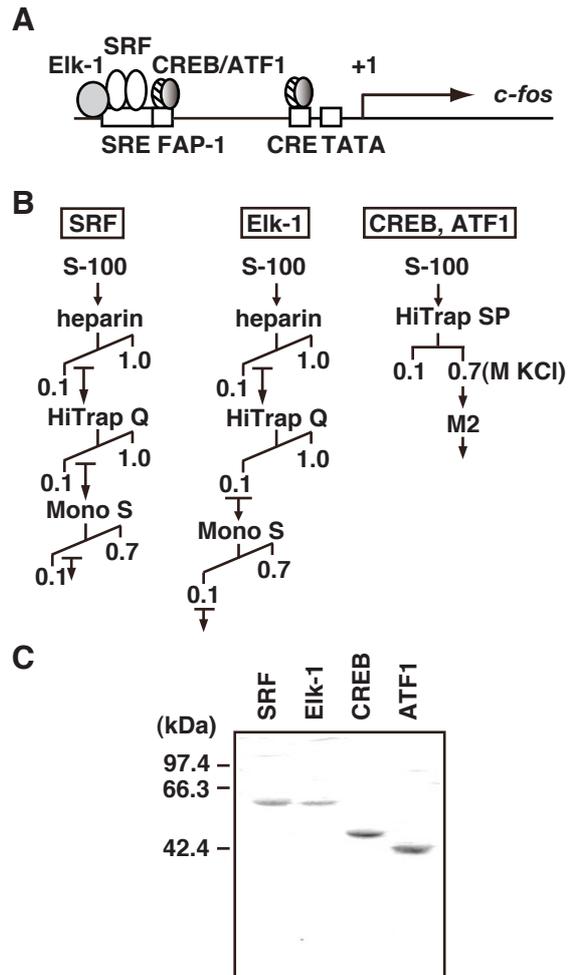
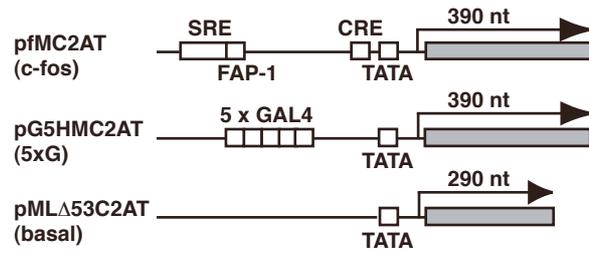
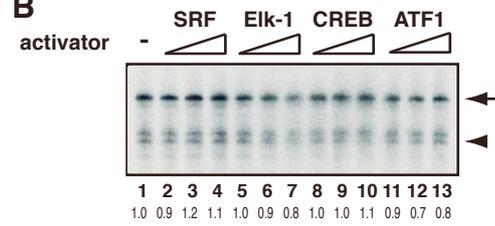


Figure 2

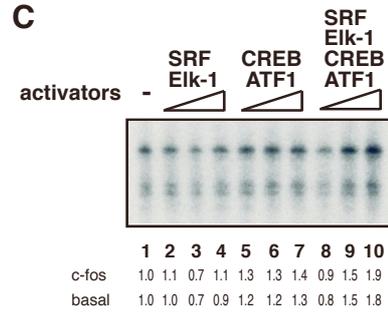
A



B



C



D

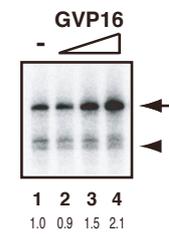


Figure 3

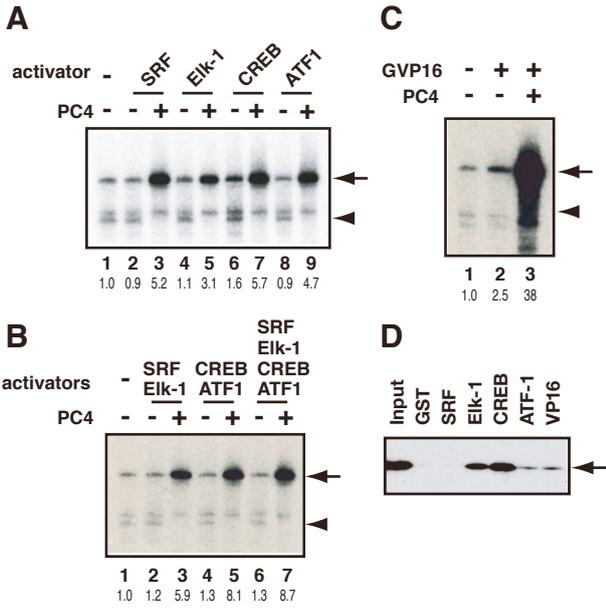


Figure 4

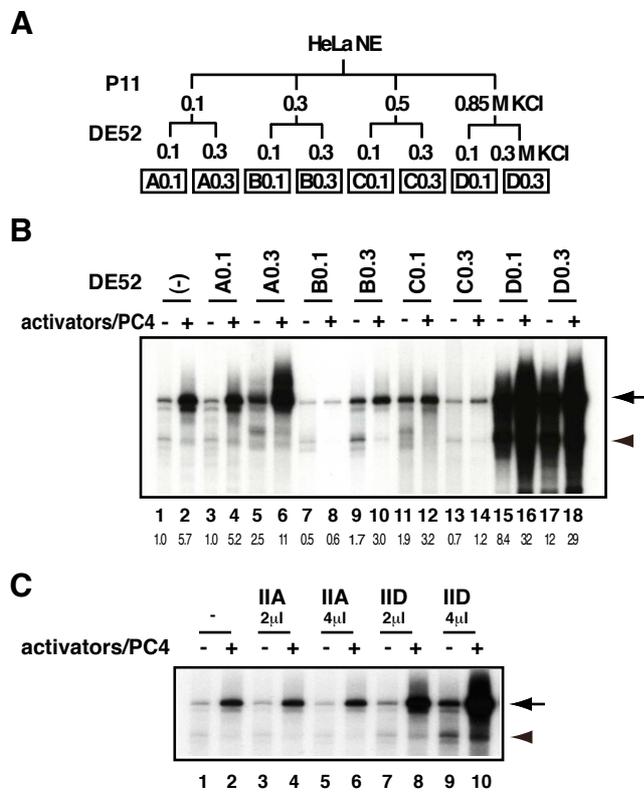
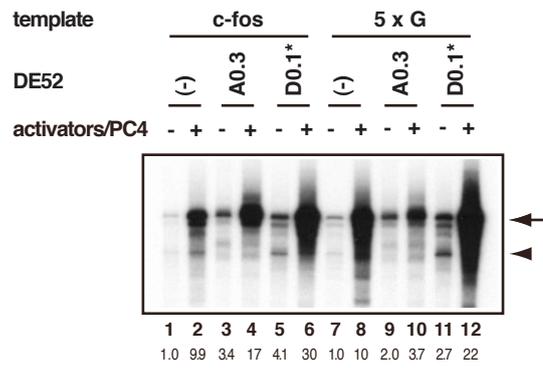
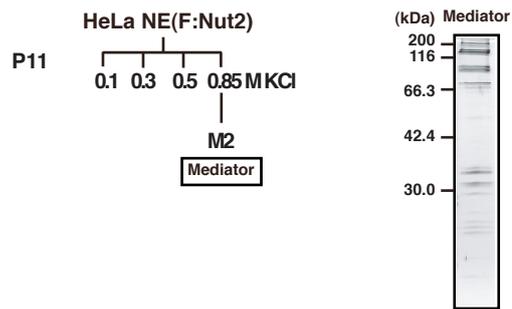


Figure 5

A



B



C

