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Identification of a coactivator that increases activation of transcription by serum response factor and GAL4–VP16 *in vitro*

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ABSTRACT **We have identified an activity in HeLa cell nuclear extracts that increases activation of transcription *in vitro* by serum response factor (SRF). This coactivator activity, termed CoS, did not affect basal (TATA only) transcription. CoS initially fractionated with transcription factors TFIIE and -F but was further purified to separate it from both TFIIE and -F as well as any other general transcription factor activity. We found that CoS was not specific for SRF activation, since it also increased transcriptional activation by the chimeric GAL4–VP16 activator. Differences among CoS and recently identified coactivators are discussed.**

Three classes of transcription factors are involved in eukaryotic mRNA gene regulation: general transcription factors, sequence-specific factors, and coactivators. At least five general transcription factors—TFIIA, TFIIB, TFIID, TFIIE, and TFIIF—have been identified (reviewed in ref. 1). These factors along with RNA polymerase II form a transcriptional initiation complex at the core promoter region, which is sufficient for basal (TATA only) transcription *in vitro*. Another general transcription factor, TFIIG, has been identified recently (2).

Transcriptional regulation of specific genes requires sequence-specific factors. Generally, these contain DNA binding and transcriptional activation domains (reviewed in ref. 3). How these sequence-specific factors regulate gene expression is unclear. There is some evidence that they may directly affect TFIID binding and preinitiation complex formation (4–6). Recently, it has been shown that TFIIB and TFIID bind to the herpes viral transcriptional activator VP16 on a VP16 affinity column, suggesting that these two general transcription factors interact directly with VP16 (7–9). In addition, TFIID has been shown to bind to the adenovirus E1A protein (10, 11).

Other evidence, however, strongly suggests that additional factors, termed coactivators, are required for the sequence-specific factors to activate transcription. First, *in vivo* and *in vitro* studies have shown that high levels of transcriptional activators inhibited or "squelched" activated but not basal transcription, suggesting that the activators titrated out cofactor(s) from the initiation complex (12–18). Second, the cloning of TFIID revealed that while recombinant TFIID could substitute for native TFIID (purified from HeLa cells) for basal transcription, it failed to support transcription activated by any of the sequence-specific factors tested (19–21). One interpretation of this observation is that there is a coactivator that copurifies with native TFIID, whose activity is required for activated but not basal transcription (16, 19, 20). Recently, coactivators have been identified and partially purified from yeast, *Drosophila*, and human cell extracts (22–24). The *Drosophila* coactivator appears to be distinct from the yeast and human coactivators; while these latter factors separated from TFIID under native conditions (22, 24), the *Drosophila* coactivator required denaturing conditions (23).

Serum response factor (SRF) is a transcriptional activator that binds to the serum response element (SRE) in the c-*fos* protooncogene (25–27). The SRE is required for serum and growth factor induction of the c-*fos* gene (27–31). SRF activates transcription *in vitro* specifically from templates with a SRE site, demonstrating that SRF is a positively acting transcription factor (32, 33). We have used a HeLa cell-derived *in vitro* transcription system to study the mechanism by which SRF activates transcription. Our previous studies, in which preincubation experiments were used, showed that TFIID may be the target of SRF activation because activation only occurred when SRF was present when TFIID bound to the template (21). We have also found, however, that high amounts of SRF inhibited transcription activated by itself and several other activators, including GAL4–VP16, but did not inhibit basal transcription *in vitro*. These results suggested that SRF can titrate out a coactivator required for factor-activated transcription (18).

In this report, we have identified a coactivator for SRF-activated transcription, termed CoS, by fractionating HeLa nuclear extract and reconstituting the partially purified transcription factors. We found that SRF-dependent transcription, but not basal transcription, was stimulated by a factor present in a crude TFIIE/F fraction. We have purified this coactivator through several columns and separated it from both TFIIE and TFIIF.

MATERIALS AND METHODS

Plasmids. The fosCAT plasmid pFC53X, containing a SRF binding site, was as described (33). A similar plasmid, pFC53G5, containing five GAL4 binding sites, was constructed by taking an *Xba* I/*Xho* I fragment, containing the GAL4 binding sites, from pG5E1bTATACAT (14). A *Sal* I linker was placed on the blunted *Xba* I end of this fragment and the fragment was ligated into pFC53 (34) at an *Xho* I site such that the five GAL4 binding sites were upstream of −53 in the fos promoter.

Chromatography of Transcription Factors. The purification procedures are summarized in Fig. 1. RNA polymerase II was purified as described from HeLa cell nuclear pellets (35). All the following chromatographic purifications were performed at 4°C in BC100 [100 mM KCl/20% (wt/vol) glycerol/20 mM Tris·HCl, pH 7.9/0.2 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/0.5 mM dithiothreitol/0.05% Nonidet P-40] except where indicated. The general transcription factors TFIIA, -B, -D, -E, and -F were fractionated from HeLa cell nuclear extracts generally as described (36) with changes described below. HeLa nuclear extracts were first fractionated on a phosphocellulose (P11) column as described (37). The TFIID-containing fraction, the P11 0.85 M KCl

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Abbreviations: TFIIA, -B, -D, -E, and -F, transcription factors IIA, -B, -D, -E, and -F; SRF, serum response factor; TAF, TFIID-associating factor; rTFIID, recombinant TFIID.

eluate, was further purified through DEAE-5PW (8 × 80 mm; Waters) and heparin-5PW (5 × 50 mm; Toso-Haas, Philadelphia) columns with linear 0.1–0.5 M KCl gradients. TFIID activity eluted from these columns at about 0.23 M and 0.37 M KCl, respectively.

TFIIB, -E, -F, and CoS derived from the P11 0.5 M KCl step fraction. For TFIIB and crude TFIIE/F, the P11 0.5 M fraction was applied to a DEAE-cellulose (DE52) column. The 0.25 M KCl step fraction contained TFIIE/F activity. The flow-through fraction was used for purification of TFIIB through a single-stranded DNA-agarose column. The 0.3 M KCl step eluate (2 ml) from this column contained TFIIB activity (as well as some TFIIE/F activity) and was precipitated with ammonium sulfate (6 ml of 3.8 M ammonium sulfate, pH 7.9). The precipitate was suspended in BC700 (same as BC100 but with 0.7 M KCl) containing 10% glycerol and loaded on a 300SW column (8.0 mm × 30 cm; Waters) equilibrated in the same buffer. The fractions with peak TFIIB activity were dialyzed against BC100.

For purification of TFIIE, TFIIF, and CoS, the P11 0.5 M KCl fraction was dialyzed in BC100 and loaded on a double-stranded DNA-Sepharose column as described (38). The flow-through fraction (0.1 M KCl) was loaded onto a DE52 column. The DE52 0.25 M KCl step fraction was further fractionated on a DEAE-5PW column as described above and eluted with a linear 0.1–0.5 M KCl gradient. Peak TFIIE activity eluted at ≈0.27 M KCl. The TFIIF- and CoS-containing fractions (eluting at ≈0.15 M KCl) were loaded onto a heparin-5PW column as described above and eluted with a linear 0.1–0.5 M KCl gradient. The peak TFIIF activity was in a 0.44 M KCl fraction, while CoS activity peaked at 0.38 M KCl. CoS-containing fractions were pooled and brought to 0.9 M ammonium sulfate and loaded onto a phenyl-5PW column (5 × 50 mm; Toso-Haas) equilibrated with BC0 (same as BC100 with no KCl) containing 0.9 M ammonium sulfate. The column was eluted with a linear 0.9–0 M ammonium sulfate gradient in BC0. Column fractions were dialyzed against BC100 and tested for activity. CoS activity eluted at 0.82 M ammonium sulfate, ahead of the protein peak at 0.66 M ammonium sulfate. Starting with 600 ml of nuclear extract (10 mg/ml), we attained 1 ml of peak CoS fractions at 0.035 mg/ml. From the DE52 0.25 M KCl fraction, we purified CoS ≈1000-fold, with a yield of ≈20%. Silver-stained SDS/polyacrylamide gels of the final CoS fractions showed ≈10 bands but it is difficult at this time to estimate the purity (data not shown).

***In Vitro* Transcription Reactions.** *In vitro* transcription reactions and the S1 nuclease hybridization method were as described (21). Templates pFC53X and pFC53G5 were used at 1 μg/ml (final concentration) except where indicated. The reactions were carried out at 30°C for 50 min. To assay for CoS activity, the following amounts of each fraction were used except where indicated: 0.09 μg of RNA polymerase II, 0.58 μg of TFIIA, 1.3 μg of TFIIB (single-stranded DNA-agarose fraction), and 0.15 μg of TFIID (DEAE-5PW fraction) or 0.2 μg of TFIID (heparin-5PW fraction). The amounts of CoS fraction (phenyl-5PW column; 0.035 mg/ml) used in the reactions were as indicated. Crude TFIIE/F (DE52 0.25 M fraction) was used at 0.2 μg/μl in Fig. 2. To assay for general transcription factor activity, 0.17 μg of TFIIB (300SW fraction), 1.0 μg of TFIIE (DEAE-5PW fraction), and 1.3 μg of TFIIF (heparin-5PW fraction) were substituted for the TFIIB fraction described above. The factors being tested were then removed one at a time to test for their requirement or for their presence in column fractions. Recombinant TFIID and SRF were overexpressed in *Escherichia coli* and purified as described (21). GAL4–VP16 was a kind gift of Jerry Workman (Pennsylvania State University) and was purified as described (39). SRF (0.4–0.8 pmol) and GAL4–VP16 (0.8 pmol) were used to activate transcription. Since a small amount of HeLa SRF could be detected in the TFIIB single-stranded DNA-agarose fraction by gel mobility-shift assay, a SRF binding site oligonucleotide, XGL (32), was added to a final concentration of 0.2 μg/ml in order to inhibit HeLa SRF from activating in the basal transcription reactions. The levels of transcripts were quantitated by PhosphorImager and ImageQuant software data analysis (Molecular Dynamics).

RESULTS

Activation of Transcription by SRF *in Vitro*. To investigate the molecular mechanism of transcriptional activation by sequence-specific factors, we have used partially purified factors to reconstitute transcription *in vitro*. We fractionated TFIIA, TFIIB, TFIID, and TFIIE/F from HeLa cell nuclear extracts (Fig. 1). RNA polymerase II was isolated from HeLa cell nuclear pellets (35). As template, we used a fosCAT promoter construct, pFC53X, with a high-affinity SRF binding site positioned upstream of −53 to +42 of the human *FOS* gene followed by the chloramphenicol acetyltransferase (CAT) gene (33). Specifically initiated fosCAT transcripts were detected by S1 nuclease analysis. We have previously found that SRF specifically activates transcription from this

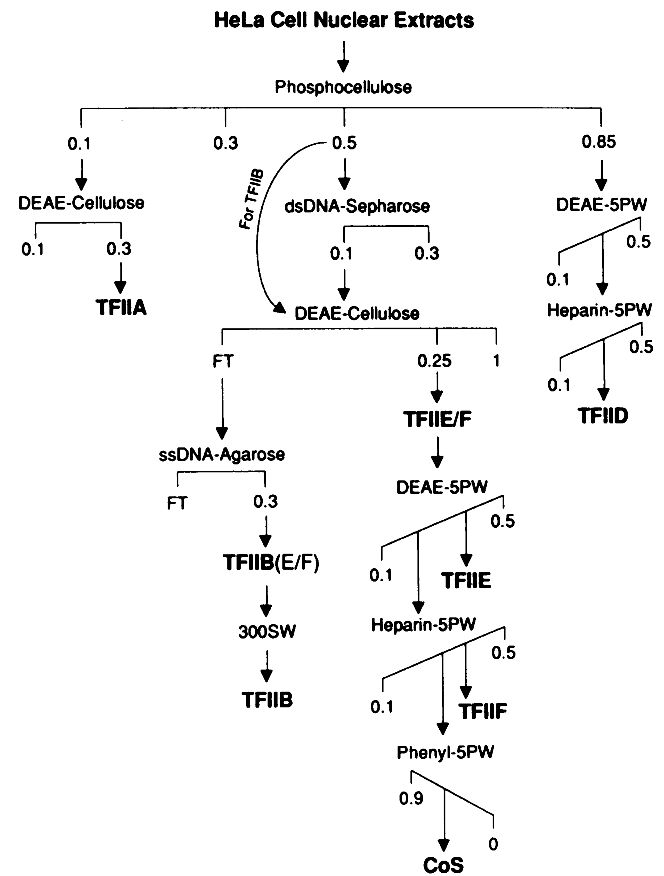

FIG. 1. Chromatography of transcription factors. Scheme for purification from HeLa cell nuclear extracts of the general transcription factors TFIIA, -B, -D, -E, -F, and CoS is diagramed. Elutions by steps or gradients of KCl (M) are indicated for all columns except the phenyl-5PW column, which was eluted with a decreasing gradient of ammonium sulfate. RNA polymerase II was purified separately from HeLa nuclear pellets. The crude TFIIB fraction (from the single-stranded DNA-agarose column) contained significant amounts of TFIIE/F activity, while the more pure fraction (from the 300SW column) was free of this contamination. dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; FT, flow-through.

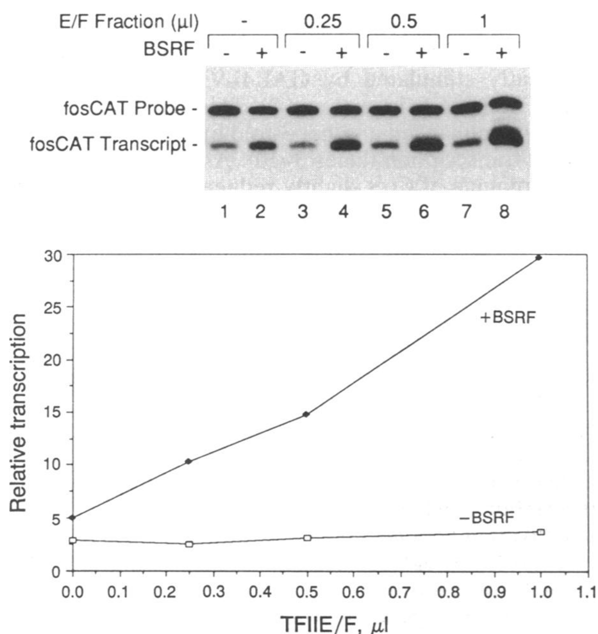

FIG. 2. The TFIIE/F fraction is required for SRF-activated but not basal transcription. Increasing amounts of the TFIIE/F fraction (from the DEAE-cellulose column) were added with or without SRF to transcription reaction mixtures containing TFIIB (single-stranded DNA-agarose fraction), TFIIA, TFIID (heparin-5PW fraction), and RNA polymerase II. The template used was pFC53X, which contains a SRF binding site, and the transcripts were detected by S1 nuclease analysis. The positions of migration of undigested fosCAT probe and specifically initiated transcripts are indicated. Quantitation of transcription levels is shown. BSRF, bacterially made SRF.

template (21, 33). This activation by SRF was typically 5- to 10-fold (Fig. 2, lanes 7 and 8).

Identification of Coactivator Activity. Surprisingly, when the TFIIE/F fraction was removed from the transcription reaction mixture, basal transcription (without SRF) was not reduced (Fig. 2, compare lanes 1 and 7). We found that this was because there was TFIIE/F activity contaminating our TFIIB preparation; when TFIIB was further purified, the TFIIE/F fraction was then required for basal transcription (data not shown). This contamination of the TFIIB fraction allowed us to detect coactivator activity in the TFIIE/F fraction. Without this fraction, activation of transcription by SRF was very poor (lanes 1 and 2). Increasing amounts of the TFIIE/F fraction greatly increased activation without having an effect on basal transcription (lanes 3–8). We have termed this activity required for SRF-activated transcription, CoS.

Separation of CoS from TFIIE and TFIIF. To further purify CoS and separate it from TFIIE and -F, we used several additional columns. The chromatographic procedure to fractionate CoS is diagramed in Fig. 1. CoS was separated from TFIIE on a DEAE-5PW column on which TFIIE and -F are known to separate (40). Partial separation of CoS from TFIIF was achieved on a heparin-5PW column. Finally, a phenyl-5PW column was used to separate the remaining TFIIF activity from the coactivator fraction. CoS eluted from this column, while no detectable TFIIF activity was recovered (see below). This CoS fraction increased transcription levels activated by SRF 5-fold, although higher amounts reproducibly resulted in a small decrease in transcription. Basal transcription (TATA only; without SRF) was not significantly affected (Fig. 3; see graph quantitating results). This fractionation procedure resulted in an ≈1000-fold purification of CoS relative to the crude TFIIE/F fraction (DEAE-cellulose 0.25 M KCl step; see Fig. 1).

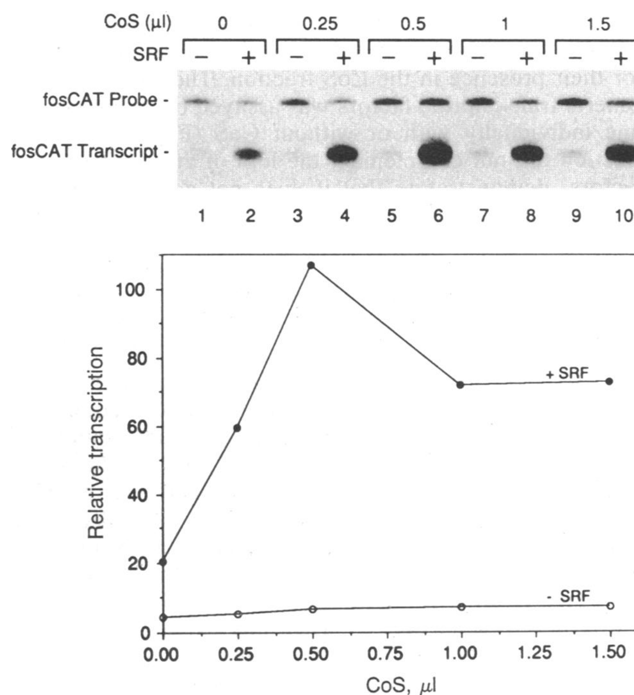

FIG. 3. CoS increases SRF-activated transcription. Increasing amounts of the phenyl-5PW CoS fraction were added to transcription reaction mixtures with or without SRF. Quantitation of transcription levels is shown.

The induction of transcription in the absence of CoS may be due to low level contamination of CoS in the general transcription factor preparations or because CoS is not absolutely required for activation. In some experiments, we have observed greater induction of transcription without CoS (e.g., compare Fig. 2, lanes 1 and 2, with Fig. 3, lanes 1 and 2). Even in cases in which greater induction occurred, CoS had a strong effect on activation (Fig. 3). It is not clear why the levels vary, but it may be due to differing effects of a general repressor, since the basal levels seem to vary more than the activated levels.

Absence of General Factor Activity from the CoS Fraction. The lack of effect on basal transcription suggests that CoS is not a general transcription factor (Figs. 2 and 3). In contrast, when additional TFIIB, -D, -E, or -F was added, basal levels of transcription increased (data not shown). We increased the level of basal transcription by increasing the template concentration and found that CoS had no effect (Fig. 4, lanes 1 and 2). This suggests that CoS is not simply a limiting factor for high-level transcription but that it is more specific to activated transcription. Similarly, we found that CoS had no effect on basal transcription from the strong adenovirus major late promoter (data not shown).

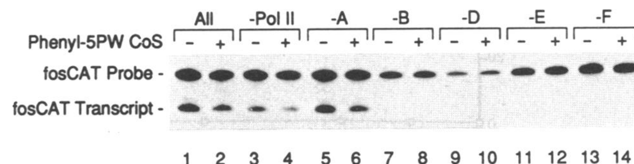

FIG. 4. Assay for general transcription factor activity in the CoS phenyl-5PW fraction. Activity of each of the general transcription factors was assayed with TFIIA, -B (300SW column fraction), -D, -E, -F, and RNA polymerase II by removing one at a time as indicated. CoS (phenyl-5PW fraction; 2 µl) was added, as indicated, to determine whether it could substitute for one of these general factors. The template pFC53X was used at 3 µg/ml.

To further show that CoS activity was not due to adding greater amounts of a general transcription factor, we tested for their presence in the CoS fraction. The presence of the general transcription factors was assayed by removing each one individually with or without CoS (Fig. 4). The CoS fraction did not complement the loss of any of the general factors, demonstrating that it does not contain significant amounts of these proteins. In fact, using these preparations of the general factors, CoS caused an ≈50% decrease in transcription levels (Fig. 4, lanes 1 and 2). Removal of RNA polymerase II and TFIIA did not abolish transcription because of contamination of RNA polymerase II in the TFIIE preparation (data not shown) and because TFIIA is either not absolutely required or is also cross-contaminating another fraction. In these two cases, as in the complete fractions case, the coactivator fraction did not increase transcription, but rather slightly inhibited it (Fig. 4, lanes 3-6). In addition, no SRF was detected in the CoS fraction when a gel mobility-shift assay was used (data not shown). We also tested transcription from the adenovirus major late promoter to determine whether CoS could complement the loss of any of the general transcription factors. Again, CoS had no general transcription factor activity when this strong promoter was used (data not shown). We further found that if we raised the transcription levels from pFC53X by adding SRF, CoS could not replace the requirement for any of the general transcription factors (data not shown). Together, the experiments described above suggest that CoS is not a general transcription factor.

Activation of Transcription by GAL4-VP16 Is Increased by CoS. To determine whether the CoS effect is specific for SRF activation or is more general for other transcriptional activators, we tested its function with the GAL4-VP16 activator. The herpes viral VP16 protein is one of the strongest transcriptional activators and contains an acidic activation domain (41, 42). GAL4-VP16 is a chimeric protein containing the GAL4 DNA binding and VP16 activation domains and stimulates transcription from promoters containing GAL4 binding sites (41). We constructed such a plasmid, pFC53G5, by inserting five GAL4 binding sites upstream of -53 of a fosCAT promoter construct. Transcription from this plasmid was greatly stimulated by GAL4-VP16 (20-fold) without adding CoS (Fig. 5, lanes 1 and 2). This activation, however, was further enhanced another 3-fold by CoS with little effect on basal transcription (lanes 3-10). As with SRF activation, higher amounts of CoS slightly reduced the activated levels (lanes 8 and 10; see graph quantitating results).

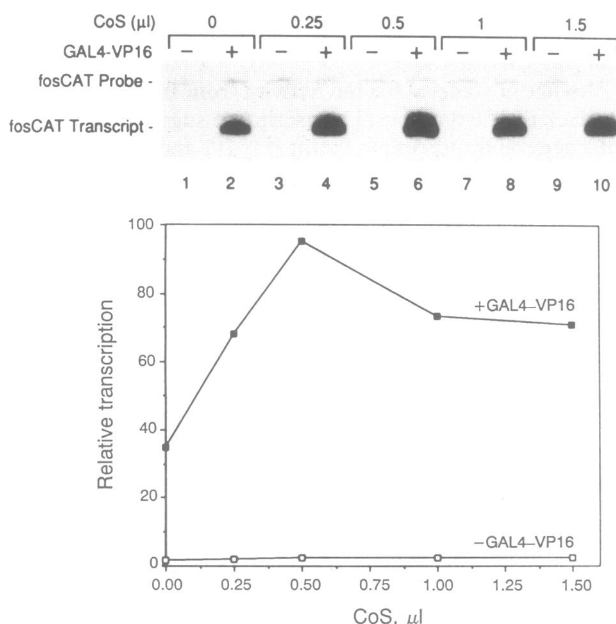

FIG. 5. Activation by GAL4-VP16 is stimulated by CoS. Increasing amounts of CoS were added with or without GAL4-VP16 (0.8 pmol) as indicated. Transcription conditions were identical to those of Fig. 3 except that pFC53G5 (containing five GAL4 binding sites) was used as template. Basal transcription levels were clearly visible upon longer exposure. Quantitation is shown.

DISCUSSION

We have identified and partially purified a coactivator for SRF-dependent transcriptional activation termed CoS. We define a coactivator as a factor that stimulates activated but not basal (TATA only) transcription. CoS was separated from the general transcription factors and did not affect the level of basal transcription from core (TATA only) promoters. It is not clear yet whether CoS is absolutely required for activated transcription or whether it only stimulates the activated level. The activation we observed without CoS, however, may be due to low levels of CoS contaminating the general transcription factor preparations.

GAL4-VP16-dependent transcription was also enhanced by CoS such that CoS is not specific for SRF and may be a general coactivator. Since SRF and VP16 do not contain similar activation domains, CoS appears to function with members of distinct classes of activators. While VP16 contains an acidic activation domain (42), we have mapped the transcriptional activation domain of SRF to the C-terminal one-third of the protein both *in vitro* and *in vivo* (ref. 18; F. Johansen and R.P., unpublished results), and this domain does not contain a recognizable activation motif.

The greater induction of transcription seen with GAL4-VP16, with and without CoS, may be because VP16 is a more potent activator than SRF. In fact, we have found that upon transfection to HeLa cells, a GAL4-VP16 construct activated transcription 10 times more than GAL4-SRF constructs (F. Johansen and R.P., unpublished results). Thus, without the CoS fraction, GAL4-VP16 may activate more effectively because it can function better with the low levels of CoS that may be contaminating the general transcription factors.

Recently, several other coactivators have been identified and partially purified from yeast, *Drosophila*, and human cells (22-24). The yeast coactivator, termed mediator, is difficult to compare to CoS because of the differences of the human and yeast systems; however, this mediator also stimulated activation by GAL4-VP16 and was purified under native conditions (22). In contrast, Dynlacht *et al.* (23) isolated coactivators from the *Drosophila* TFIID complex under denaturing conditions. These coactivators tightly associate with the DNA binding component of TFIID. Our TFIID preparation was partially purified under native conditions and is likely to contain these associating factors. In addition, since CoS separated from TFIID under native conditions, it is unlikely that CoS is similar to one of these TFIID-associating factors (TAFs). Meisterernst *et al.* (24) identified a coactivator, USA, in HeLa nuclear extracts. While USA separated from TFIID under native conditions, it does not appear to be similar to CoS because of differences in their chromatographic properties (e.g., CoS initially copurifies with TFIIE/F rather than with TFIID).

While CoS increased activation by SRF and GAL4-VP16, higher levels of CoS reproducibly resulted in a slight decrease in activated transcription levels. Under certain conditions, basal transcription levels were also reduced (Fig. 4). It is possible that the CoS fraction contains an inhibitory component. While the importance of this inhibitory activity is unclear, it is interesting that the USA coactivator also contains an inhibitory factor (24).

One of the original observations suggesting coactivators was that recombinant TFIID (rTFIID) could support basal but not activated transcription. This may be due to the absence of the TAFs from the rTFIID preparation; however, the TAFs alone were not sufficient to reconstitute activated transcription using rTFIID, but required additional factor(s) (23). The USA coactivator only poorly reconstituted activated transcription with rTFIID (24), and we observed no activation with CoS and rTFIID (data not shown). These results are consistent with the possibility that the TAFs are one class of coactivators and that a second class of coactivators is required that is not tightly associated with TFIID.

Thus, there appear to be multiple coactivators involved in the process of transcriptional activation. This may be for several reasons. First, there may be several factors involved in transcriptional activation such that all of these coactivators are required. These factors may all function in a complex with a transcriptional activator to affect the general transcription machinery. An alternative reason for multiple coactivators is that an activator may use any one of multiple coactivators. This could account for the activation we observed by GAL4–VP16 and SRF without CoS. Our preliminary results suggest that there may, in fact, be more coactivators, because when we tried to reconstitute our transcription system with more purified TFIIB, -E, and -F we observed only poor transcriptional activation by SRF even in the presence of CoS (unpublished data). This has made it difficult to determine whether the activation we observe without CoS is due to low levels of CoS contaminating these fractions or whether it is because CoS is not absolutely required for activation.

It is also possible that coactivators are specific to particular activators or classes of activators. This is supported by *in vivo* squelching experiments in which high amounts of an activator inhibited activation of its own target gene but not activation of genes by certain other factors (14, 15). Nevertheless, the coactivators identified to date, including CoS, have had an effect on all activators tested (22–24).

It will be important in further work to determine the specificity of the coactivators and the mechanism by which they act. The coactivators may directly bind the transcriptional activators and/or a general transcription factor(s). We have not detected CoS binding to a SRF affinity column; however, this may be because CoS and SRF do not form stable complexes (unpublished results). Purification and cloning of coactivators will greatly aid in determining with which proteins they directly interact. These interactions may be critical since gene regulation may be mediated by the enhancement or disruption of activator–coactivator binding.

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