Purified Cofactors and Histone H1 Mediate Transcriptional Regulation by CTF/NF-I

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The initiation of RNA polymerase II transcription is controlled by DNA sequence-specific activator proteins, in combination with cofactor polypeptides whose function is poorly understood. Transcriptional cofactors of the CTF-1 activator were purified on the basis of their affinity for the regulatory protein. These purified cofactors were found to be required for CTF-1-regulated transcription, and they counteracted squelching by an excess of activator in *in vitro* reconstitution experiments. Interestingly, the cofactors possessed an inhibitory activity for basal transcription, which was relieved by the further addition of the activator. Histone H1 also contributes to the regulation of transcription by CTF-1, whereby the activator prevents repression of the basal transcription machinery by the histone. However, histone H1 could not replace the cofactors for CTF-1-regulated transcription, indicating that they possess distinct transcriptional properties. Furthermore, the purified cofactors were found to be required, together with the activator, in order to antagonize the histone-mediated repression of transcription. These results suggest that CTF-1 and its cofactors function by regulating the assembly of the basal transcription machinery onto the promoter when the latter is in competition with DNA-binding inhibitory proteins such as histone H1.

Basal-level transcription by RNA polymerase II requires the ordered assembly of at least seven general factors on the core promoter, including transcription factor (TF) IIA, TFIIB, TFIID, TFIIE, TFIIIF, and TFIIIG (36). Transcription from the initiation complex can be regulated by activator or repressor proteins that bind promoter-specific distal sequences. Activators are thought to facilitate the assembly of the initiation complex onto the promoter (18, 37, 44) or favor the release of poised RNA polymerase II after transcription initiation (33). These activators have been found to regulate transcription by displaying transcriptional activation determinants composed of various primary structures, including acidic or glutamine- or proline-rich domains (29). However, the precise functioning of these activating sequences remains poorly understood. Transcriptional activation domains have been proposed to interact directly with components of the basal transcription machinery, such as TFIIB or the TATA box-binding protein (TBP), a component of TFIID (15, 21, 23). However, fractionation studies have revealed the existence of another class of transcription factors, termed cofactors (also called coactivators, mediators, or transcriptional intermediary factors), which are dispensable for basal initiation but are nevertheless necessary for transcriptional activation (13, 38, 42). For instance, TBP has been found to interact with a collection of cofactor polypeptides which mediate transcriptional regulation as a complex (10, 26, 27, 41). It has been proposed that these cofactors may bridge activators and the basal transcription machinery, thus facilitating the assembly of the initiation complex. However, all of these cofactors need not necessarily interact directly with activators. Alternatively, cofactors could modulate the assembly of the preinitiation complex, as in the association between TBP and TFIIA (26, 27), which could in turn be regulated by a direct interaction of activators with the basal transcription factors.

In addition to sequence-specific regulatory proteins and cofactors, chromatin structure also plays a fundamental role in RNA polymerase II transcription *in vitro* as well as *in vivo*. Nucleosome reconstitution generally represses *in vitro* transcriptional initiation, but this inhibition can be relieved in the presence of activators and/or components of the basal transcription machinery (12, 19). Recent studies have also implicated histone H1 in the repression of transcription *in vitro*, in both the presence and the absence of core histones. The inhibitory effect of histone H1 can be alleviated by activators, and this has been termed antirepression (7, 20). At present, the existence of a functional relationship between antirepression and the direct activation of the preinitiation complex has not been demonstrated. Although transcriptional activation domains have also been implicated in the antirepression of nucleosome-reconstituted promoters (43), suggesting similar mechanisms, antirepression does not appear to require TBP-associated cofactors, in contrast to transcriptional activation (25, 43). Therefore, a role for cofactors in the antirepression of transcription remains to be determined.

CTF-1, a member of the human CTF/NF-I family of sequence-specific DNA-binding proteins (16, 35), regulates transcription via a proline-rich activation domain (28). Previous competition (squelching) studies suggested that this activation domain recognizes other components of the transcription machinery in transfected cells and that this interaction mediates CTF-1 transcriptional activity (24). To further address this possibility, we utilized purified CTF-1 derivatives as a column ligand in affinity chromatography. This resulted in the purification of a set of polypeptides that associate specifically with the activator. *In vitro* reconstitution studies indicated that both these cofactor polypeptides and histone H1 are necessary to mediate high levels of transcriptional regulation by CTF-1. These results suggest a role for the purified cofactors in the antirepression activity of CTF-1.

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MATERIALS AND METHODS

Expression and purification of CTF-1 derivatives. HeLa cells were infected with recombinant vaccinia virus expression vectors (a kind gift from N. Tanese and R. Tjian) for full-length CTF-1 and for the CTF-1ΔC399 mutant, which lacks part of the proline-rich activation domain. HeLa cell infection and preparation of nuclear extracts were as described previously (9). Recombinant vaccinia virus-expressed vCTF-1 and vCTF-1ΔC399 proteins were purified from crude nuclear extracts of infected cells by DNA affinity chromatography and analyzed by denaturing polyacrylamide gel electrophoresis and silver staining (16). A total of 30 to 160 μg of purified proteins was obtained from approximately 10⁹ infected cells.

Transcription extract fractionation. A total of 300 to 400 ng of purified vCTF-1, vCTF-1ΔC399, or bovine serum albumin (BSA) was covalently coupled to 1 ml of CNBr-activated chromatography resin (Affi-Gel 10; Bio-Rad) as described previously (39). A total of 150 μl (1.5 mg of total proteins) of HeLa cell nucleus transcription extracts was applied to 1-ml columns in buffer D* (100 mM KCl) (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-KOH [pH 7.8], 2 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% [vol/vol] glycerol, 100 mM KCl). These and subsequent steps were performed at 4°C. The columns were incubated with the extract for 15 min and washed with 30 ml of D* (100 mM KCl). Bound proteins were eluted with D* (200 mM KCl), and columns were washed with 30 ml of the same buffer. This step was repeated with buffer D* (600 mM KCl). Occasionally, the cofactor-containing fractions were further concentrated by chromatography on a 100-μl DEAE resin column in D* (100 mM KCl) and were eluted in D* at 200 mM KCl. Flowthrough and dialyzed eluate fractions were stored in aliquots at -70°C, and the affinity columns were kept at 4°C for several weeks and remained functional for three to four passages of nuclear extracts.

In vitro transcription. In vitro transcription was performed with HeLa cell nuclear extracts depleted of CTF/NF-I with a DNA affinity resin (16), as described previously (3). In vitro transcription reactions were essentially as have been described (3), except that the final concentrations of Nonidet P-40 and MgCl₂ were 0.01% (vol/vol) and 1 mM, respectively. Reporter constructs used were either the pCAT-87Δ-3XAd or the pCAT-55Δ plasmid, containing three or no high-affinity CTF/NF-I binding sites, respectively (28), or pG5BCAT, consisting of five GAL4 recognition sequences upstream of a minimal synthetic promoter (22). The internal control template was the minimal vitellogenin B1 control promoter of pB1(-41/+8) CAT8+ (6). A total of 100 ng of each indicated template, in supercoiled form, was incubated for 20 min on ice in the presence or absence of 700 ng of carrier DNA (pBluescript; Stratagene), with or without purified vCTF-1 or vCTF-1ΔC399, bacterially expressed purified human TBP (TFIID; Promega), bacterially expressed purified GAL4-VP16 (a kind gift from J. Kadonaga [7]), and/or affinity column eluates, as indicated in the figure legends. This was followed by the addition of histone H1 purified from HeLa cells (a kind gift from J. Kadonaga [8]) when indicated, CTF/NF-I-depleted nuclear extract, and nucleotides (see Fig. 1, 3, 4A and B, 5, and 6). In one experiment, histone H1 was first preincubated with 100 ng of each template and 700 ng of carrier DNA for 20 min on ice and vCTF-1 was then added for a further 20-min of incubation, which was followed by the addition of CTF/NF-I-depleted nuclear extract and nucleotides (see Fig. 4C). In

FIG. 1. Purified vCTF-1 and vCTF-1ΔC399 activate in vitro transcription. (A) Purification of vCTF-1 and vCTF-1ΔC399 proteins. Purified vCTF-1 and vCTF-1ΔC399 proteins, expressed from recombinant vaccinia virus expression vectors, were analyzed by denaturing polyacrylamide gel electrophoresis and silver staining. The indicated amounts (in microliters) of vCTF-1 (lane 1) and vCTF-1ΔC399 (lane 2) were resolved in parallel with molecular mass standards (lane M; sizes in kilodaltons). (B) In vitro transcriptional activity of purified vCTF-1 and vCTF-1ΔC399. Reporter promoter template DNA, containing no (pCAT-55Δ [lanes 1 to 3]) or multiple (pCAT-87Δ-3XAd [lanes 4 to 6]) CTF/NF-I binding sites, was transcribed in vitro together with a minimal control promoter, with a CTF/NF-I-depleted HeLa cell nuclear extract (see Materials and Methods). Two microliters of vCTF-1 or vCTF-1ΔC399 (60 ng), which corresponds to equivalent DNA binding activities, was added as indicated. Primer extension signals of RNA transcribed from the reporter and control promoters are indicated by arrows.

another experiment, the CTF/NF-I-depleted nuclear extract was preincubated with 100 ng of each template and 700 ng of carrier DNA for 60 min at 30°C (see Fig. 4D). vCTF-1 was then added and incubated for a further 20 min, and then histone H1, nucleotides, and, within 10 s, Sarkosyl (final concentration, 0.2% [wt/vol]) were added as indicated. All transcription reaction mixtures were further incubated for 60 min at 30°C, and RNA was analyzed by primer extension as described previously (16). Transcription from the control and reporter promoters was inhibited by α -amanitin at a concentration of 2 μg/ml, indicating that these promoters are recognized by RNA polymerase II. Reproducibility was ensured by several independent experiments. Transcripts were quantified by densitometric scanning of autoradiographic exposures, and inductions of less than twofold were scored as nonsignificant.

RESULTS

In order to identify proteins that interact with CTF-1 in vitro and which may contribute to its transcriptional activity, we utilized the activator as a ligand in affinity chromatography. Full-length CTF-1 and a derivative lacking part of the activation domain (CTF-1ΔC399) (28) were overexpressed in HeLa cells with recombinant vaccinia virus vectors and purified essentially to homogeneity by DNA affinity chromatography (Fig. 1A). The microheterogeneity of the purified polypeptides is consistent with their known posttranslational modifications (41a). Transcriptional activities of the purified proteins were assessed by the complementation of a HeLa cell nuclear extract depleted of endogenous CTF/NF-I with a DNA affinity resin. Reporter templates consisted of

FIG. 2. Affinity purification of polypeptides that associate with vCTF-1 in vitro. vCTF-1, vCTF-1ΔC399, or BSA was covalently coupled to a chromatography resin, which was loaded with either a HeLa cell nuclear transcription extract depleted of CTF/NF-I and associated proteins (lanes 1 to 4) or a nondepleted extract (lanes 5 to 10). Bound proteins were successively eluted in the presence of 200 mM (0.2) and 600 mM (0.6) KCl from the BSA (lanes 9 and 10), vCTF-1ΔC399 (lanes 1, 2, 5, and 6), and vCTF-1 (lanes 3, 4, 7, and 8) columns and resolved on a denaturing polyacrylamide gel. Numbers and arrows indicate the sizes (in kilodaltons) of polypeptides that were reproducibly eluted from the vCTF-1ΔC399 and vCTF-1 columns at 200 and 600 mM KCl, respectively, with the nondepleted extract. The bracket labeled C indicates buffer contaminants revealed by silver staining. Lane M, molecular mass standards (see legend to Fig. 1).

α -globin-based promoters containing no (pCAT-55Δ) or multiple (pCAT-87Δ-3XAd) DNA recognition sites (28), while the strong minimal promoter of the vitellogenin B1 gene, with all regulatory sequences deleted (6), was used as an internal control. Full-length vCTF-1 stimulated pCAT-87Δ-3XAd transcription 3- to 11-fold as normalized to the internal control signal, depending on nuclear extract preparations (Fig. 1B). As expected, vCTF-1 was two- to threefold more active than the vCTF-1ΔC399 derivative, which is lacking part of the activation domain, and demonstrated inductions ranging from one- to fivefold. In both cases, activation was DNA binding site dependent.

In order to detect vCTF-1 interactions with other transcription factors, purified vCTF-1, vCTF-1ΔC399, or BSA proteins were covalently cross-linked to a chromatography resin, which was loaded with HeLa cell nuclear extracts under low-salt conditions (100 mM KCl). Bound proteins were then successively eluted in the presence of 200 and 600 mM KCl and analyzed by denaturing gel electrophoresis (Fig. 2). When a complete nuclear extract was loaded on the vCTF-1 affinity column, the 600 mM KCl eluate fractions consistently contained several polypeptides with apparent mobilities of >200, 120, 80, 70, 46, 34, 32, and 29 kDa (Fig. 2, lane 8), representing less than 0.04% of input proteins. Other polypeptides were occasionally obtained in independent experiments but did not affect the activity of the preparations (see below). In contrast, these polypeptides were not recovered from columns containing covalently

bound BSA (Fig. 2, lanes 9 and 10) or ethanolamine (data not shown). Interestingly, similar polypeptides were eluted from the vCTF-1ΔC399 affinity column (Fig. 2, lane 5), although at a lower salt concentration (200 mM KCl). This suggests weaker interactions of these polypeptides with the protein deprived of part of the activation domain. Eluted polypeptides did not migrate as vCTF-1 species, nor did they recognize a CTF/NF-I DNA binding site in gel mobility shift experiments (data not shown). In addition, the loading of a control extract, which had been previously subjected to multiple cycles of depletion of CTF/NF-I and associated proteins under low-salt conditions, did not allow the detection of such polypeptides in the eluate fractions (Fig. 2, lanes 1 to 4). This suggests that these polypeptides were depleted in part from this extract, through their interaction with CTF/NF-I. These experiments indicate that these polypeptides interact specifically with vCTF-1 and suggest that the proline-rich regulatory domain of the activator is at least in part responsible for the stabilization of this interaction.

To assess the role of the eluted proteins, we next depleted column flowthrough fractions of endogenous CTF/NF-I activity and assayed whether they could support vCTF-1 transcriptional regulation (Fig. 3A). The BSA column flowthrough was competent for a two- to threefold vCTF-1 transcriptional activation, as was the mock-treated and diluted load extract (Fig. 3A, lanes 1 to 4). The addition of the eluate fractions to the BSA column flowthrough did not markedly alter inducibility (Fig. 3A, lanes 3 to 8). In contrast, flowthrough fractions from the vCTF-1 and vCTF-1ΔC399 columns did not allow significant vCTF-1 regulation but displayed already elevated basal transcription levels (Fig. 3A, lanes 9, 10, 15, and 16). The addition of vCTF-1 occasionally resulted in a slight, twofold inhibition of transcription from the reporter template, with independent flowthrough preparations from the vCTF-1 species columns (data not shown; see also below). This indicates that these fractions are depleted of an activity necessary for vCTF-1 function but that they are not depleted of limiting components of the basal transcription machinery. Complementation of the vCTF-1 column flowthrough with its cognate 600 mM KCl eluate fraction consistently restored a three- to fivefold apparent induction by inhibiting reporter promoter transcription in the absence but not in the presence of the activator (Fig. 3A, lanes 19 and 20). Thus, the vCTF-1 column eluate fraction contains an inhibitory activity that can be alleviated by the activator. In independent preparations, the best inductions correlated with the highest levels of basal transcription repression activity of the eluate fractions. In contrast, the 200 mM, but not the 600 mM, KCl eluate fraction from the vCTF-1ΔC399 column restored a similar vCTF-1 response (Fig. 3A, lanes 11 to 14). The activity of these fractions correlates with the presence of the eluted proteins shown in Fig. 2 (lanes 5 and 8), which implies that they act collectively as cofactors for vCTF-1 transcriptional regulation.

That the vCTF-1 cofactors consist of a number of polypeptides is reminiscent of recently identified components of the human TFIID general transcription factor, which have also been proposed to act as cofactors for CTF-1 and other activators (10, 41). These cofactors have been shown to form stable complexes with TBP, the TATA box-binding component of TFIID. To address the possibility that the HeLa cell cofactors described here may also contain TBP activity, the TBP component of the general transcription machinery was inactivated by a mild heat treatment of a complete nuclear extract (30). As expected, this treatment abolished transcrip-

FIG. 3. In vitro transcriptional activity of vCTF-1 cofactors. (A) The pCAT-87Δ-3XAd reporter construct and control template DNA were incubated with 2 μ l of purified vCTF-1 and 6 μ l of 200 mM (0.2) or 600 mM (0.6) KCl eluate fractions from the BSA, vCTF-1ΔC399, or vCTF-1 columns, as indicated. This was followed by the addition of column load samples or cognate column flowthrough fractions, which had been depleted of endogenous CTF/NF-I activity, in subsequent in vitro transcription reactions (see also the legend to Fig. 1B). The bracket labeled D indicates downstream initiation signals from the reporter promoter. (B) vCTF-1 cofactors do not possess TBP activity. The indicated amounts (in microliters) of vCTF-1, TBP (40 ng), and 4 μ l of the 600 mM KCl eluate fraction from the vCTF-1 column were incubated with the pCAT-87Δ-3XAd reporter construct and control template DNA in a transcription reaction as described for panel A, and then either a heat-treated or a nontreated HeLa cell nuclear extract (lanes 3 to 8 and 1 and 2, respectively) was added. (C) vCTF-1 cofactor-depleted extract mediates GAL4-VP16 transcriptional activation. The GAL4-responsive pG5BCAT reporter construct and control promoter plasmid were incubated with or without GAL4-VP16 (2 μ l, 10 ng/ μ l), and then either column load samples or the BSA (FT BSA) or the vCTF-1 (FT vCTF-1) column flowthrough fraction was added in an in vitro transcription reaction.

tion from the reporter and control promoters, in both the absence and the presence of vCTF-1 (Fig. 3B, lanes 3 and 4). Addition of purified human TBP fully restored basal expression of the control template and partly restored that of the relatively weaker reporter promoter. In contrast, the addition of purified cofactors did not restore transcription from

either promoter (Fig. 3B, lanes 5 to 8), indicating that they do not possess significant TBP activity. Consistently, but unlike the bacterially expressed TBP, the cofactor polypeptides did not recognize a TATA box element in gel mobility shift experiments, nor did they comigrate with TBP in denaturing gel electrophoresis (data not shown). Moderate

but significant levels of vCTF-1-mediated induction were observed when TBP was added to the heat-treated extract, as reported previously (41). However, the subsequent addition of the cofactors did not restore higher induction levels (data not shown), suggesting either that additional heat-sensitive activities are needed for maximal transcriptional regulation or that the bacterially expressed TBP cannot fully replace its HeLa cell counterpart.

We next assessed whether the vCTF-1 cofactor-depleted extract may still support activation mediated by another type of regulatory protein, the negatively charged GAL4-VP16 chimeric activator (34). GAL4-VP16 was found to mediate a similar 7- to 10-fold transcriptional activation with either the cofactor-depleted vCTF-1 column flowthrough fraction or the nondepleted BSA column flowthrough fraction (Fig. 3C). Therefore, this indicates that the vCTF-1 cofactor-depleted fraction is not intrinsically refractory to transcriptional activation but that it has specifically lost the capacity to mediate the regulatory effect of the proline-rich activator. Induction with the flowthrough fractions was, however, not as strong as the 20-fold activation observed with the load extract, suggesting that a GAL4-VP16-specific cofactor activity was either nonspecifically lost or inactivated during chromatography.

The observation that vCTF-1-associated cofactors have an inhibitory effect on basal-level expression is reminiscent of the reported effect of histone H1, which is present in nuclear extracts such as the ones used in this study and has been reported to repress basal but not activated transcription in the presence of glutamine-rich or acidic activators (7). It is therefore possible that the repression effect of the cofactors resulted simply from the presence of histone H1. To address this possibility, we first assessed whether the proline-rich vCTF-1 activator may also counteract histone H1-mediated repression of transcription. It has been shown that the effect of transcriptional inhibitors such as histone H1 can be relieved by the addition of nonspecific competitor DNA (1, 7, 17). The addition of competitor DNA to the transcription reaction shown in Fig. 4A had little effect on initiation from the control and reporter templates, indicating that it does not prevent the association of the basal transcription machinery with these promoters (Fig. 4A, lanes 1 to 5). However, the addition of the carrier DNA abolished vCTF-1 transcriptional regulation. Gel electrophoresis mobility assays performed under conditions similar to those employed in the *in vitro* transcription reactions indicated that the lack of vCTF-1 response did not result from the inhibition of vCTF-1 promoter binding by the competitor DNA, in either the presence or the absence of histone H1 (Fig. 4E). To confirm the possibility that the loss of vCTF-1 regulation results from the removal of transcriptional inhibitors, we next complemented the transcription reaction with increasing amounts of purified HeLa cell histone H1. This process restored vCTF-1-regulated transcription by an antirepression mechanism: while the addition of histone H1 nearly abolished basal-level expression of both the reporter and the minimal control promoters, it did not inhibit reporter template transcription in the presence of the activator (Fig. 4A). This resulted in an apparent induction of up to 20-fold (Fig. 4A, lanes 8 and 9). Interestingly, histone H1 addition resulted in higher levels of activated transcription from the reporter promoter (Fig. 4A, lanes 5, 7, and 9; see also Fig. 4C). This could result from histone H1 shielding nonspecific DNA sequences from assembling alternative initiation complexes or from a more efficient initiation at the histone-bound promoter. With respect to the latter possibility, we note that

one previous study indicated higher transcriptional levels from a nucleosome-assembled template compared with naked DNA, an effect that might involve CTF/NF-I (6). However, higher amounts of histone H1 also showed an inhibitory effect in the presence of vCTF-1, as has been noted previously with other activators (7). The same antirepression effect was observed with threefold-lower amounts of vCTF-1 (data not shown), indicating that a precise stoichiometry of the histone relative to vCTF-1 is not involved in this process. In addition, the antirepression effect was dependent on the presence of sites vCTF-1 DNA binding site on the reporter promoter (Fig. 4B), which further excludes a nonspecific inhibition of histone H1 by vCTF-1, as could occur, for instance, via a direct interaction of the two proteins.

In the latter experiments, vCTF-1 was first bound to the template DNA and then histone H1 and the general transcription factors were added. To address the possibility that histone H1 may repress basal transcription by decreasing the accessibility of protein(s) to the promoters, we altered the order of addition of the different transcription reaction components. When histone H1 was first incubated with the reporter promoter and carrier DNA in conditions that allow reversible binding to DNA (7) and then vCTF-1 and the general transcription factors were added, similar antirepression effects were observed (Fig. 4C). This indicates that histone H1 does not prevent promoter recognition by vCTF-1. Therefore, histone H1 does not irreversibly sequester template promoters, as would occur, for instance, by the nonspecific precipitation of DNA. In contrast, when the preinitiation complex was first allowed to assemble and then vCTF-1 and histone H1 were added, the repression effect of the latter was decreased, resulting in a lower (two- to threefold) apparent induction (compare Fig. 4C and D, lanes 3 to 6). The addition of Sarkosyl, which inhibits initiation but not elongation by RNA polymerase II (14), revealed that multiple rounds of initiation occur in the absence of exogenous histone H1 (Fig. 4D; compare lanes 1 and 2 with 7 and 8). By contrast, in the presence of large amounts of histone, and in the absence of vCTF-1, reinitiation was essentially abolished (Fig. 4D, lanes 5 and 11). This implies that histone H1 acts by preventing the formation of an active initiation complex, as proposed earlier (7). However, the subsequent addition of vCTF-1 to the reaction mixture restored multiple rounds of transcription (Fig. 4D, lanes 6 and 12), indicating that this activator favors the formation of a functional transcription initiation complex. Distinctly, in the presence of both Sarkosyl and histone H1, no induction was detected (Fig. 4D, lanes 11 and 12), indicating that vCTF-1 does not activate previously assembled initiation complexes. In toto, these experiments indicate that vCTF-1 functions at least in part by counteracting the inhibitory effect of histone H1 on the assembly of a functional initiation complex *in vitro*.

These findings raised the possibility that an inhibitory protein such as histone H1 may be present in the cofactor preparation and may mediate its basal transcription repression effect. Consistently, the nonspecific association of histone H1 with numerous proteins has been reported (11), and cofactor preparations contain two polypeptides with similar, although not identical, mobilities compared with that of the purified histone (32 and 34 kDa [Fig. 2 and data not shown]). Therefore, we assessed whether purified histone H1 may complement the cofactor-depleted flowthrough from the vCTF-1 column. We found that vCTF-1 is no longer capable of preventing histone H1-dependent repression of transcription in conjunction with the cofactor-depleted extract (Fig. 5, lanes 1 to 6), in contrast with cofactor-

FIG. 4. Role of histone H1 in vCTF-1 transcriptional regulation in vitro. (A) Antirepression activity of vCTF-1. The pCAT-87Δ-3XΔ reporter and control templates were preincubated in the absence (lanes 1 and 2) or presence (lanes 3 to 9) of carrier DNA with the indicated amounts (in microliters) of vCTF-1. This was followed by the addition of histone H1 (amounts indicated in nanograms), CTF/NF-1-depleted nuclear extract, and nucleotides, in an in vitro transcription reaction (see also the legend to Fig. 1B). (B) DNA binding site dependence of vCTF-1 antirepression effect. In vitro transcription reactions were performed in the presence of carrier DNA as described for panel A, except that the pCAT-55Δ reporter template which contains no vCTF-1 binding site was used. (C) Addition of histone H1 prior to the activator does not prevent antirepression by vCTF-1. In vitro transcription reactions were performed as described for panel A, except that the reporter, control, and carrier plasmids were first incubated with histone H1 and then vCTF-1 and other components of the transcription reaction were added. (D) In vitro transcription reactions were performed as described for panel A, in the presence of carrier DNA, except that the CTF/NF-1-depleted nuclear extract was first incubated with DNA and then vCTF-1, histone H1, nucleotides, and Sarkosyl were added as indicated. (E) Addition of carrier DNA or histone H1 does not affect vCTF-1 sequence-specific DNA recognition. Gel mobility shift assays were performed under conditions similar to those used for in vitro transcription reactions as described for panel A, in the presence or absence of 250 ng of histone H1 and/or 2 μl of vCTF-1, with the exceptions that the reporter promoter plasmid was replaced by the same molar amount of a 27-bp gel shift probe containing the CTF/NF-1 binding site of the adenovirus replication origin (16) and that the nuclear extract was replaced by dialysis buffer. Reactions were resolved on a native 5% polyacrylamide gel (16) and revealed by autoradiography of the dried gel.

containing extracts (Fig. 4A). This indicates that histone H1 cannot substitute for, and is at least in part distinct from, the cofactors. Furthermore, this also suggests that the cofactors might be necessary for the vCTF-1 antirepression effect. Indeed, further addition of the purified cofactors to the vCTF-1 column flowthrough fraction restored vCTF-1-mediated antirepression, in the presence of the histone (Fig. 5, lanes 13 to 16). Cofactor addition resulted both in the inhibition of basal transcription and in an increase of the transcription level in the presence of the activator, which resulted in high overall levels of antirepression by vCTF-1 (14- to 30-fold), compared with the fourfold induction obtained in the absence of exogenous histone (Fig. 5, lanes 7 and 8). Furthermore, the addition of carrier DNA essentially abolished vCTF-1 regulation, even in the presence of the cofactors (Fig. 5, lanes 9 to 12), indicating that both the

cofactors and promoter-bound histone are required for transcriptional regulation by the activator. Together, these findings indicate that the cofactors participate in preventing histone H1-mediated repression of transcription, in concert with the activator.

CTF-1 cofactors were previously detected in competition (squenching) studies performed with transfected cells, in which overexpression of the activator was found to prevent transcriptional activation, most probably by titrating a limiting cofactor activity (24). To assess a possible relationship of the in vivo detected cofactor activity with the purified polypeptides, we attempted to reproduce the squenching effect in vitro. The use of a high concentration of a cofactor-containing transcription extract resulted in a similar sevenfold induction with either saturating (Fig. 6, lane 2) or excess (lane 3) amounts of vCTF-1. This suggested that the cofac-

FIG. 5. vCTF-1 cofactors mediate the antirepression effect of the activator. The pCAT-87A-3XAd reporter and control templates were incubated in the presence or absence of carrier DNA, with or without 2 μ l of vCTF-1 and 4 μ l of the 600 mM vCTF-1 column eluate fraction concentrated by DEAE chromatography (see Materials and Methods). This was followed by the addition of the indicated amount of histone H1 (in nanograms), vCTF-1 column flowthrough fraction, and nucleotides, in an *in vitro* transcription reaction (see also the legend to Fig. 1B).

tors present in the extract were not limiting, which would not allow the detection of a squelching effect. Consistently, further addition of the purified cofactors did not increase induction (data not shown). We therefore used lower amounts of the transcription extract, which resulted in a moderate induction by the small amount of the activator (threefold [Fig. 6, lanes 4 and 5]). This suggested that a cofactor activity was limiting under these assay conditions. Indeed, the addition of an excess of the activator essentially abolished induction (Fig. 6, lanes 4 and 6), while it did not inhibit a reporter promoter devoid of CTF-1 binding sites (lanes 10 to 12). Therefore, an excess of vCTF-1 titrates a

FIG. 6. vCTF-1 cofactors relieve squelching by an excess of activator. The pCAT-87A-3XAd or pCAT-55A reporter and control templates were incubated with or without 2 or 8 μ l of vCTF-1 and 2.6 μ l of the 600 mM vCTF-1 column eluate fraction concentrated by DEAE chromatography (see Materials and Methods). This was followed by the addition of 36 μ g (lanes 1 to 3) or 12 μ g (lanes 4 to 15) of total proteins of a CTF/NF-I-depleted extract and nucleotides, in an *in vitro* transcription reaction (see also the legend to Fig. 1B).

limiting cofactor activity involved in transcriptional induction, in a squelching mechanism, but does not inhibit the basal transcription machinery. Further addition of the purified cofactors increased transcriptional induction in the presence of a small amount of the activator (eightfold [Fig. 6, lanes 7 and 8]) and restored activation in the presence of an excess of vCTF-1 (threefold [lanes 7 and 9]). This indicates that these polypeptides counteract squelching and therefore can alleviate in part the titration of a positively acting cofactor by an excess of activator. This further supports the conclusion that at least one of the cofactor polypeptides interacts with vCTF-1. Interestingly, under these assay conditions the cofactors did not display an inhibitory effect on basal transcription, probably because the negatively acting cofactor component is not limiting in the diluted extract. In any case, these results indicate that the purified cofactors can act positively on transcriptional activation and that vCTF-1 does not simply prevent transcriptional repression by an inhibitory protein present in the cofactor preparation, such as by a direct competition for promoter binding.

DISCUSSION

Affinity purification of CTF-1 cofactors. In this study, we sought to purify protein cofactors that might participate in the regulation of transcription by interacting with the vCTF-1 activator. We found that a collection of approximately eight polypeptides can be isolated by virtue of their specific association with vCTF-1. These polypeptides could also associate with the vCTF-1ΔC399 derivative lacking part of the proline-rich activation domain but were eluted at a lower ionic strength compared with their interaction with full-length vCTF-1. Thus, the weaker activating properties of the truncated vCTF-1ΔC399 derivative correlate well with its lower apparent affinity for the cofactor polypeptides. In addition, this suggests that the proline-rich activation domain is involved in the interaction of vCTF-1 with these cofactors.

Depletion of the cofactors increased basal-level initiation and abolished the vCTF-1 regulatory effect, while their subsequent addition restored regulation by decreasing basal initiation transcription. This implies that one or more of these polypeptides function as cofactors for vCTF-1 regulation by inhibiting transcription in the absence but not in the presence of the activator. However, vCTF-1 affinity chromatography did not deplete limiting components of the basal machinery, since it did not abolish basal transcription. Consistently, the purified cofactors displayed no TBP activity. This contrasts with previous studies in which affinity chromatography with the negatively charged VP16 activator abolished basal transcription, which resulted from direct interactions with the TFIID and TFIIB general transcription factors (23, 40). Therefore, different classes of activation domains, e.g., proline-rich and acidic, appear to mediate distinct protein-protein interactions *in vitro*, a result which is in keeping with previous squelching evidence obtained from transfected tissue culture cells (24). In addition, the depletion of vCTF-1 cofactors did not inhibit GAL4-VP16-mediated activation, which is consistent with the proposal that the two types of activators may function by distinct mechanisms. Proline-rich CTF/NF-I species could regulate transcription, at least in part, by associating with specific cofactors, while negatively charged GAL4-VP16 may directly bind components of the initiation complex (15, 23) and/or interact with a different set of cofactors (13, 41, 42).

Interplay of cofactors with activators and the basal tran-

scription machinery. Recent studies have shown that TBP-binding polypeptides are involved in transcriptional regulation by several types of activators, including CTF-1 chimeric proteins (10, 26, 27, 41). However, the possible interaction of these TBP-associated factors (TAFs) with activators remains unknown. Our results indicate that a class of vCTF-1 cofactors can be purified by virtue of their association with the activator. This raises the possibility that the two sets of cofactors might be functionally related or even identical. For instance, both sets of cofactors act in conjunction with the proline-rich activator but appear not to be involved in transcriptional activation mediated by the negatively charged GAL4-VP16 protein (Fig. 3C) (41). However, several lines of evidence argue against the identity of the two classes of cofactors. Firstly, the vCTF-1-associated cofactors repress basal transcription, an effect antagonized by the activator, while highly purified TAFs increase activated transcription but have no effect on basal expression. Secondly, interaction between the TAFs and TBP is disrupted by chaotropic agents, while cofactors dissociated from vCTF-1 under mild chromatographic conditions do not possess detectable TBP activity. This indicates that if TBP was present in our cofactor preparation, it should be in a repressed or inactive form, unlike when it is present in the transcription-competent TAF-TBP complex. Thirdly, the polypeptide pattern of the vCTF-1-binding cofactors is distinct from that of the TAFs. However, we cannot exclude the possibility that some polypeptides might be common to both cofactor preparations and that functional differences may reflect the particular constellation of proteins present in each subset of cofactors. Particular cofactors may contribute to vCTF-1 regulation by lowering basal initiation levels, while other activities may increase activated transcription. That a vCTF-1-associated cofactor(s) may in turn interact with the TAF-TBP complex, therefore bridging the activator and the basal transcription machinery, would be an interesting possibility. Thus, vCTF-1 could stabilize or facilitate the assembly of the preinitiation complex onto the core promoter. This would be in keeping with the demonstration that vCTF-1 cofactors facilitate the assembly of the initiation complex, when the latter is in competition with histone H1 for promoter recognition (see below).

Surprisingly, we find that the cofactors which bind vCTF-1 inhibit basal but not activated transcription when assayed together with their cognate-depleted extract. This is reminiscent of the effect of the NC2 fraction, which displays cofactor activity for the Sp1 activator and which can be isolated in a TBP-free form under mild chromatographic conditions (26). NC2 has also been shown to repress basal but not activated transcription, probably by displacing TFIIA from TBP. This raises the question of why an activator like vCTF-1 would interact with a negatively acting cofactor. This apparent paradox could be resolved if the activator's interaction with the negatively acting cofactor might simply alleviate the repression effect of the latter. However, this would be difficult to reconcile with the fact that the cofactors increase activated transcription in squelching studies. Furthermore, this possibility would also be inconsistent with the finding that vCTF-1 cofactors facilitate the assembly of the preinitiation complex in the presence of histone H1. It is therefore more likely that vCTF-1-regulated transcription involves both negatively and positively acting cofactor protein(s), as suggested above. Thus, the negatively acting component would repress basal transcription, while the addition of the activator would restore favorable cofactor interactions with the initiation

complex. The positively acting component of vCTF-1 cofactors could nevertheless be part of the general transcription machinery, since our results do not exclude a direct interaction of vCTF-1 with an abundant basal factor, which may in turn associate with limiting cofactors. The development of simple procedures for the purification of vCTF-1 cofactors provides a means for the further separation and characterization of individual polypeptides, in order to evaluate such possibilities.

Histone H1 also participates in vCTF-1-regulated transcription. The finding that vCTF-1 cofactors repress basal but not activated transcription suggests that this activator in fact functions in part by preventing the repression of transcription. Histone H1 has been shown to be a general repressor of basal transcription *in vitro*, in either the presence or the absence of reconstituted nucleosomes. Here we report that histone H1-mediated repression of transcription can be counteracted by vCTF-1, a proline-rich activator, as was found previously for the glutamine-rich Sp1 and acidic GAL4-VP16 proteins (7, 20). Histone H1 most probably inhibits basal transcription by competing for the association of basal factors with DNA. In this context, vCTF-1 acts by favoring the assembly of the basal transcription machinery onto the promoter but does not detectably activate previously formed preinitiation complexes. Thus, vCTF-1 acts during preinitiation complex assembly but not at later stages such as the initiation of transcription by RNA polymerase II. vCTF-1 antirepression activity is lost from the cofactor-depleted extract, while it is restored by complementation with the purified cofactors. Therefore, transcriptional activity of the cofactors cannot merely be explained by the repression effect of histone H1 but must result from at least one distinct activity. These results also demonstrate that both the cofactors and histone H1 are necessary for vCTF-1 regulatory activity in the reconstituted reaction and further indicate that the cofactors participate in vCTF-1 antirepression effect. We therefore propose that vCTF-1 and its cofactors regulate at least one event during preinitiation complex assembly and that this event is rate limiting for transcription initiation in the presence of histone H1 (Fig. 7). In the absence of promoter-bound histone, however, the final rate of transcription would be determined by distinct vCTF-1-independent events along the pathway leading to initiation.

In this study, histone H1 was used as a nonspecific inhibitor of transcription, in order to probe preinitiation complex assembly. Whether purified histone H1 interacts with naked DNA *in vitro* in a fashion similar to its association with chromatin remains to be determined. Nevertheless, previous evidence indicated that histones such as H1 may also contribute to CTF/NF-I-regulated transcription *in vivo*, in the context of native chromatin. *In vitro* and *in vivo* studies have suggested that CTF/NF-I recognition of the mouse mammary tumor virus promoter is favored by the previous binding of another activator, the glucocorticoid receptor (5, 31, 32). Promoter binding by the glucocorticoid receptor results in alterations of the chromatin structure, including the removal of histone H1 from stably replicated template DNA *in vivo*, which is accompanied by the concomitant binding of CTF/NF-I and formation of the initiation complex (2, 4). These analyses indicate that a cascade of events occurs in the transition from an extinct promoter to one undergoing maximal gene expression. Our results suggest that CTF/NF-I cofactors may participate in regulating one of the later events along this pathway, i.e., the assembly of the transcription initiation complex.

FIG. 7. Model for the role of CTF-1 cofactors in in vitro transcription antirepression. (A) In the absence of promoter-bound inhibitors of transcription such as histone H1, transcription initiation by RNA polymerase II (POL II) and basal factors is determined by CTF-1-independent rate-limiting events and is constitutively high (+). (B) In the presence of histone H1, but without cofactors, promoter recognition by one or several basal transcription factors, but not by CTF-1, is inhibited. As a result, transcription initiation is constitutively low (-). (C) In the presence of CTF-1 cofactors (COFs), however, transcription initiation is restored in an activator-dependent fashion (+). Therefore, histone H1 and the cofactors mediate CTF-1 transcriptional regulation synergistically. As suggested here, the cofactors could interact directly or indirectly with basal transcription factors such as TBP (labeled D) and/or TFIIB (labeled B) or with other basal factors (TFIIA, TFIIE, and TFIIF are labeled A, E, and F, respectively). An alternative possibility not ruled out by our results would be that the cofactors act directly on histone H1 by displacing it from the promoter.

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