The transcription factor USF1 belongs to the family of basic helix-loop-helix proteins that are involved in the regulation of various important cellular processes. Here we characterized the factors involved in the activation of transcription by upstream stimulatory factor 1 (USF1) in a reconstituted class II gene transcription system. Activation of transcription by both wild type USF1 and a GAL-USF (amino acids 1-94 of the yeast activator protein GAL4 fused to amino acids 17-196 of USF) fusion protein required the presence of at least one positive cofactor. A novel positive cofactor (PC5) that functions specifically through the activation domain of USF1 was partially purified and biochemically distinguished from previously described positive cofactors. The mechanism by which PC5 mediates activation of transcription through USF1 was investigated in order-of-addition experiments. PC5 had to be present during binding of transcription factor (TF) II D to the TATA box to observe transcriptional activation. However, this event alone did not result in transcriptional activation, which also required the presence of the activator and of PC5 after binding of TFII D. Hence, PC5 may enter transcription during binding of TFII D to function in concert with the activator during subsequent steps in transcription.

Upstream stimulatory factor (USF) was originally described as an activity derived from HeLa nuclear extract binds to an element (E-box) upstream of the adenovirus major late promoter (1-3). Purification of USF (4) led to identification of two polypeptides of 43 (USF1) and 44 kDa (USF2). The corresponding cDNAs were cloned (5, 6), and the proteins encoded by the cDNAs were shown to form homo- and heterodimers. All three different USF dimers bind to the E-box element in vitro and in vivo (6).

Sequence analysis showed USF1 and USF2 to be members of the basic helix-loop-helix (bHLH)/leucin zipper family of transcription factors. This family of transcription factors also includes the mammalian proteins MYC, MAD, MAX, MXI1, TFEB, TFE3, and AP-4, which represents a subfamily of the large family of bHLH transcription factors (for review see Refs. 7 and 8). Homo- and heterodimers of bHLH proteins via binding to E-boxes regulate a variety of genes that are involved in a number of important cellular processes ranging from differentiation to cell cycle regulation and apoptosis. In addition to its important role in regulation of the adenoviral major late promoter, USF is involved in the regulation of cellular genes, including the murine metallothionein I gene (9), the rat gamma-fibrinogen gene (10), the human growth hormone gene (11), the p53 gene (12), and the cardiac ventricular myosin light chain 2 gene (13).

USF has been widely used as a model activator to study mechanisms of transcriptional activation by bHLH transcription factors. In nuclear extracts recombinant USF1, indistinguishable from purified USF, activates transcription of the major late promoter (14). The bHLH/leucin zipper region at the carboxyl terminus of USF1 is sufficient for DNA binding (5) but does not have an intrinsic activation potential (15). Transcriptional activation is dependent on the amino-terminal half of the polypeptide, and a fusion of this region to the GAL4 DNA-binding domain gives rise to an transcriptionally active protein (15).

Activation of transcription appears to require interactions between the regulatory sequence-specific DNA-binding proteins and the general transcription machinery. There is evidence for functional interaction of USF with TFII D (3, 16-19) with one of the TBP-associated factors (TAF) (20). USF also binds cooperatively with TFII D to both USF recognition sites and sequences close to the initiation sites of transcription (21, 22). Although recombinant USF1 is able to activate transcription in nuclear extracts indistinguishable from natural USF (14, 15), activation potential in reconstituted transcription systems is substantially reduced during purification of natural USF (16).

The activity of partially purified USF can be restored in vitro by including an upstream factor stimulatory activity (23, 24). Four different positive cofactors (PC1, PC2, PC3/Dr2/Topol, and p15/PC4) (Refs. 25-29; reviewed in Refs. 30 and 31) have been isolated from this fraction that are essential for stimulation of transcription by activators in highly purified class II gene transcription systems (32). At least in one example (p15/PC4), these coactivators interact with activators (29) and the general transcription factor TFIIA (29). More recently, functional analysis revealed that PC4, in conjunction with an acidic activator, stimulates transcription predominantly during TFII D-TFIIA promoter complex formation (33).

Here, we have analyzed transcriptional activation by recombinant USF1 and the USF1 activation domain fused to the minimal DNA-binding domain of GAL4 in a reconstituted class II gene transcription system. We have isolated and partially purified a novel cofactor termed PC5. PC5 displayed specificity for the USF1 activation domain and, in contrast to previously identified PCs, had no effect on transcription in the presence of
a GAL4 DNA binding domain. Mechanistic studies suggest that PC5 enters transcription during and function after binding of TFIIID to the promoter.

MATERIALS AND METHODS

Purification of Transcription Factors—TFIID, TFIIA, TFIIF, TFIIF, and RNA polymerase II were purified as described previously (34, 35). Recombinant transcription factors TFIIA, TFIIF, and GAL4, GAL4-AH (26, 27), USF1, and GAL-USF (amino acids 1–94 of GAL4 fused to amino acids 17–196 of USF) (15) were expressed and purified as described.

Purification of PC1 to PC4—PC1 was purified up to the heparin-Sepharose step as described previously (23). It was further purified to near homogeneity on MonoS and phenylsuperose columns, and 2.5 μl of the peak fraction (40 ng of PC1) were used in the transcription reactions.

HeLa nuclear extracts derived from epitope-tagged TBP-expressing HeLa cells (37) were fractionated on phosphocellulose (P11, Whatman) essentially as described (38). PC2 and PC3 were isolated from the P11 0.85 m KCl fraction, which was dialyzed to buffer HP (10 mM HEPES-KOH, pH 8.2, 10 mM NaPO4, pH 7.6, 200 mM KCl, 1 mg/ml phenylmethylsulfonyl fluoride, 0.01% Nonidet P-40, 20% glycerol) containing 5 mM imidazole and applied to a nickel-agarose column (QIAGEN Inc.). PC3, which is identical to topoisomerase I and contains a large number of clustered histidine residues, and also PC2 could be purified from this crude fraction on nickel columns. The column was washed with buffer HP containing 15 mM imidazole, and the activity was eluted using buffer HP containing 300 mM imidazole. PC2 and PC3 were separated according to their different native sizes of 500 and 300 kDa, respectively (25, 26), using Superose 12 (SMART) chromatography. The column (load 50 μl, 70 μg of protein) was developed in buffer P (200 mM NaPO4, pH 7.6, 0.2 mM EDTA, 10% glycerol). 1.5 and 3.0 μl of the peak fractions (fraction size, 50 μl) of PC3 and PC2, respectively, were used for transcription reactions.

PC4 (p15) was expressed and purified as described (27). 50 ng of purified protein was used in transcription reactions.

Purification of PC5—PC5 was isolated from nuclear extracts of HeLa cells fractionated on P11, on which it eluted in the 0.3 and the 0.5 m KCl steps. It was further purified as described earlier for NC2 (34). PC5 activity coeluted on DE52, A25, and S-Sepharose columns with NC2. The S-Sepharose peak fraction was concentrated 10-fold and further purified on Superose 12 columns (SMART) in buffer P, and NC2 and PC5 activities were separated on Superose 12, on which PC5 eluted with an approximate native size of 300 kDa. 2 μl of the peak fraction (160 ng/ml) was used for transcription reactions. Alternatively, the P11 0.5 m KCl fraction was dialyzed to buffer C (10% glycerol, 0.2 mM EDTA, 20 mM HEPES-KOH, pH 8.2, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing 0.5 M KCl. 200-μl aliquots were heat-treated at 55°C for 15 min. Precipitated proteins (40%–80%) were removed by centrifugation, and 1 μl (total protein, 0.45 μg/ml) of the heat-treated 0.5 m KCl P11 fraction yielded full levels of PC5 activity in transcription. Heat-treated PC5 was further purified using a MonoQ PC 1.5/5 column (SMART system). A linear gradient from 0.1 M to 0.5 M KCl in buffer C, PC5 eluted between 250 and 370 mM KCl. 1.5 μl of the peak fraction was used for transcription reactions. For size dermination, 0.5 ml of the crude heat-treated PC5 fraction was concentrated 10-fold by centrifugation in Centricon-10 vials (Amicon) and fractionated on a Superose 12 column (SMART System) in buffer C containing 0.2 M KCl.

Transcription Reactions—Templates were the adenovirus major late promoter including the USF binding site upstream of a G-less cassette (pMLC2AT) (39), a truncated adenovirus major late promoter lacking the USF binding site upstream of a shortened G-less cassette (pMLL53) (40), and the HIV-1 core promoter carrying five GAL4 recognition sites (23). Standard transcription reactions contained 50 ng of each template, 10 ng of recombinant TFIIF, 0.8 μl of TFIIID (DE52 fraction, 0.35 μg/μl protein), 10 ng each of recombinant TFIIIEα and TFIIIEβ, 1.5 μl of TFIIH- and TFIIIF-containing fraction (DE52, 0.5 μg/μl protein), and 0.2 μl of RNA polymerase II (DE52 fraction, 0.5 μg/μl protein). The TFIIID fraction used contained substantial amounts of TFIIA (34); however, in order-of-addition experiments (see Fig. 4), 0.5 μl of TFIIA (MonoQ fraction, 2 μg/ml) were also added. Transcription reactions were performed in a buffer containing 25 mM Hepes, pH 8.2, 10–15% glycerol, 4 mM MgCl2, 60–65 mM KCl, 5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 700 ng/ml bovine serum albumin, 0.002% Nonidet P-40, 100 μM each of UTP and ATP, 5 μM CTP, 20 μM 3′-O-methyl GTP, 0.5 μM [α-32P]CTP (3,000 Ci/mmol), and 20 units of RNase-Block (Stratagene). As described in the figures, activators (45 ng of GAL-USF, 15 ng of GAL4-AH, 35 ng of GAL4, 70 ng USF1) were added to the transcription buffer containing the templates followed by the PCs and the remaining GTFs. Reactions were carried out for 60 min at 28°C and processed and analyzed as described (34). In order-of-addition and restriction digest protocols (see Fig. 4), templates, GAL-USF and PC5 (as indicated in the figures), TFII1D, and TFIIA were mixed in transcription buffer lacking nucleotides. After 50 min, 1 μl of Sphi (40 units/μl; Boehringer Mannheim) was added, and the mixtures were further incubated for 20 min at 28°C. Thereafter, GAL-USF and PC5 (if indicated) and the remaining GTFs, and the nucleotides were added, and the reactions were carried out for 60 min at 28°C. Transcription levels were quantified densitometrically (see Figs. 1 and 2) or by counting on an InstantImager (Canberra-Packard; see Figs. 3 and 4).

RESULTS

Identification of a Novel Positive Cofactor Termed PC5—We had previously characterized four different PCs that were isolated from a fraction termed upstream factor stimulatory activity (23). These protein fractions (PC1 to PC4) were chromatographically separated, and the PCs were shown to be required and sufficient to facilitate activator-dependent transcription in a purified transcription system (reviewed in Ref. 27), which contained a complete set of GTFs, including a native TFIID complex consisting of TBP and TAFs (41). Upon fractionation of HeLa nuclear extracts on phosphocellulose columns (P11), all previously defined cofactors were isolated from the fraction that eluted between 0.5 and 0.85 m KCl. Here we have identified and partially purified (Fig. 1A) a novel coactivator, which eluted at lower salt from phosphocellulose columns (0.3–0.5 m KCl). This cofactor, termed PC5, coeluted with NC2 during four chromatographic steps (34) and was subsequently further enriched on gel filtration columns (Fig. 1A). PC5 eluted from Superose 12 according to a native size of 300 kDa (Fig. 1A) and was completely separated from NC2 as judged by electrophoretic mobility shift assay (performed as described in Ref. 34; data not shown). PC5 was assayed by its ability to stimulate activation of transcription by a GAL-USF protein (Fig. 1B), which is composed of the complete USF activation domain fused to the amino-terminal 94 amino acids of GAL4 (15). Whereas PC5 enhanced transcription in the presence of GAL-USF, a GAL4 control protein (GAL94) lacking the USF activation domain had no effect on transcription (Fig. 1B).

PC5 appears to be distinct from PC1 to PC4 by several criteria. On Superose 12 columns, PC5 eluted according to a native size of 300 kDa (Fig. 1A). This distinguishes PC5 from most of the PCs, specifically PC2 (500 kDa) (26), PC4 (30–100 kDa) (27), and PC1 (400 kDa).2 PC5, if purified as shown in Fig. 1C, did not contain topoisomerase I activity (PC3), which eluted according to a similar native size on gel filtration columns (25). PC5 could be further distinguished from PC1 and PC4 by its chromatographic behavior on strong anion exchanger columns. In contrast to PC4 and PC1, which pass through MonoQ-Sepharose, from which it elutes at 250–360 mM KCl (Fig. 1C). Furthermore, in contrast to most of the PCs, PC5 activity was found to be resistant to heat treatment for 15 min at 55°C. When the cofactors were heat-treated at identical salt (100 mM KCl) and protein concentrations (0.5 mg/ml) only PC5 and PC4 retained cofactor activity (data not shown). Thus, PC5 fractions appear to contain a novel coactivator distinct from previously defined PCs. We have subsequently used a heat-treated P11 0.5 m KCl fraction (see Fig. 1A) to study the mechanism of PC5 function. This PC5 fraction had similar specific activity as highly purified PC5 (Fig. 1B) and apparently

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was not contaminated by other PCs, because it eluted as a single peak from Superose 12 according to a native size of 300 kDa (Fig. 1D) and displayed functional properties indistinguishable from purified PC5.

Functional Comparison of PC5 and Previously Identified PCs—PC5 was compared with other PCs (PC1 to PC4) with respect to its capacity to mediate activation of transcription by GAL-USF. In addition to GAL-USF we also assayed GAL94, to assess the effects of the DNA-binding domain, and GAL4-AH, which had been shown to be active in conjunction with all previously identified PCs. Initially, activators and cofactors were titrated to maximum levels of transcription. At these concentrations 50–70% of the five GAL4 binding sites of the template were occupied by the activators (revealed by electrophoretic mobility shift assay; data not shown). The effects of GAL94, GAL-USF, and GAL4-AH were subsequently analyzed in combination with the different PCs (PC1 to PC5). Fig. 2A shows a representative analysis in the presence of either PC5 (lanes 1–4) or PC4 (lanes 6–9). Quantitative comparison of the effects of the different PCs is shown in Fig. 2B. Clearly, different PCs displayed preferences for either GAL-USF or GAL4-AH. Although PC1, PC2, and PC5 most efficiently facilitated activation of transcription by GAL-USF, all PCs acted through GAL4-AH. PC5, and less pronounced PC1, potentiated transcription most specifically through the USF activation domain, whereas in the presence of PC3 and PC4, GAL94 and GAL-USF exerted similar and weak effects on transcription (less than 3-fold). Fold stimulation by both GAL-USF and GAL4-AH above levels of basal transcription was maximal in the presence of PC2. However, as reported previously (26), PC2 also stimulated transcription in the presence of the DNA-binding domain of GAL4. Taken together, the specificity of PC2 for the USF activation domain was very low (approximately 2-fold). By contrast, PC5 followed by PC1 facilitated the strongest specific response of transcription to GAL-USF (if the ratio of GAL-USF versus GAL94 effects was taken as a measure). Exclusively, PC5 was completely inactive in the presence of GAL94 and functioned preferentially through GAL-USF (as compared with GAL4-AH). Hence, PC5 could play an important role during activation of transcription by USF1.

PCs that were active in combination with GAL-USF (PC1, PC2, and PC5) were also tested for their capacity to facilitate activation of transcription by the recombinant wild type USF1 protein, which was expressed in and purified from Escherichia coli. In these experiments the adenovirus major late promoter was used as a template that carries a single USF recognition site located between positions −54 and −60 (39). These experiments could not be performed with crude PC5 fractions because these fractions activated the major late promoter in the absence of exogenously added recombinant USF1 (Fig. 3A). Consistent with this observation, we detected substantial amounts of native USF in crude PC5 fractions by gel shift experiments (data not shown). However, more purified PCs, as well as PC2 and PC1, were depleted of USF and facilitated activation of transcription by USF1 (Fig. 3B, lanes 6 and 3, 3.5- and 3.2-fold activation, respectively, and data not shown). In the absence of PCs, USF1 had no effect on transcription in a highly purified transcription system. Thus, PCs that are active in conjunction with GAL-USF also facilitate activation of transcription by the wild type USF1 protein.
Mechanistic Aspects of Transcriptional Activation of GAL-USF Mediated by PC5—We analyzed the mechanism underlying the stimulation of transcription by PC5 and GAL-USF. Here we asked whether PC5 acts on the formation of active TFIID-TFIIA promoter (DA) complexes, as was shown previously for PC4 (33). To address this question we removed the activator from the transcription machinery after the formation of the DA complex (Fig. 4A). Templates were incubated with GAL-USF and PC5 together with TFIID and TFIIA and subsequently digested between the GAL4 recognition sites and the TATA box using SphI (Fig. 4A, lanes 3, 4, 7, and 8). Digestion of the templates led to loss of activation by PC5 and GAL-USF (Fig. 4A, compare lanes 5 and 6 and lanes 7 and 8). Lack of activation was not due to conformational change of the template because GAL-USF and PC5 could activate transcription on templates that were linearized downstream of the promoter (data not shown). These data suggest that GAL-USF and PC5 function after binding of TFIID. In line with this observation, effects of GAL-USF and PC5 were independent of TFIID concentrations and does not function if added after DA complex formation. As indicated, GAL-USF and PC5 were included in the preincubation together with TFIID and TFIIA (lanes 1-4) or added after the preincubation (lanes 5-6). Standard (lanes 1, 2, 5, and 6) or 4-fold higher concentrations of TFIID (lanes 3 and 4) were used. After a 50-min preincubation period, the remaining GTFs and NTPs were added, and transcription reactions were allowed to proceed for 60 min.

least one additional factor to activate transcription, which is apparently removed during purification of the protein (3, 4, 15, 16). This analysis suggests that this missing factor may be a member of the family of PCs. Although none of the PCs directly coelute with native USF, we could indeed detect PC5 activity in fractions that contain native USF. Furthermore, PC5 is heat stable and binds to DE52 columns, suggesting that it may have contributed to USF activity during the first purification steps (1, 3, 4, 16). It seems reasonable to assume that crude transcription systems contain PCs and that these factors contribute to transcriptional activation. Upon purification of general transcription factors, PCs are at least in part removed, leading to reduction of effects of activators on transcription (23). In agreement with this interpretation, PCs do not strongly affect activation of transcription by USF in nuclear extracts.3

For the first time, we have systematically compared effects of different PCs in conjunction with an activator, in this example the bHLH/leucin zipper protein USF1. Evidence is provided that PC5 displays specificity for the USF1 activation domain. Very little is known about specificities of PCs for domains of regulatory proteins that are essential for transcriptional activation in vivo. Weak preferences of the coactivator PC2 for the AH domain in GAL4-AH (26, 42) and of PC1 fractions for the acidic carboxyl-terminal activation domain of NFkB subunit p65 (TA-1 domain), and not vice versa, have been demonstrated previously (26, 43). Preferences of certain PCs for defined ac-

3 J.-P. Halle and M. Meisterernst, unpublished observation.
tivators are most easily explained if we assume that these two classes of transcription factors interact with each other. Indeed, at least in the case of PC4, direct interactions with VP16 and other activation domains have been reported (29, 44). However, in most examples functional interactions of activators with PCs remain to be demonstrated.

Other putative targets of activators include TFIIB (45) and the TAFs (Refs. 32, 39, 46, and 47; reviewed in Ref. 48). Analyses included USF, which was recently shown to interact with TAF 55, one of the integral components of the TFIID complex (20, 36). In previous investigations we and others provided evidence that PCs are distinct of TAFs (26, 32). This was subsequently confirmed by cloning of some members of the PCs (25, 27–29). Also in the case of PC5 we have no evidence for a relationship of this factor to members of the TBP-associated factors. PC5 did not coelute with TFIID, and our transcription system contained the complete native TFIID complex.

We have begun to study the mechanisms by which PC5 mediates activation of transcription by USF1. This analysis revealed clear differences to the mode of action of PC4. Effects of PC4 were found to be strictly dependent on TFIID concentrations leading to moderate (down from 10-fold to 3-fold) stimulation at saturating TFIID concentrations (33). In contrast, activation by GAL-USF through PC5 did not depend on TFIID concentrations. This observation is in agreement with the assumption that PC5 does not stimulate binding of TFIID, as does PC4, but acts on subsequent steps of preinitiation complex formation.

We had previously analyzed the activation mechanism of PC4 by introducing a combined order-of-addition/restriction digest protocol. In this experiment the activator could be removed in active transcription complexes through restriction digest from general factors after formation of stable DA complexes (33). These experiments confirmed that PC4 acted mostly on binding of TFIID. However, they also demonstrated moderate effects of PC4 on subsequent steps of preinitiation complex formation. Utilizing this protocol we found that PC5, independent of the TFIID concentration, stimulates transcription after DA complex formation. Basal levels of transcription were observed in reactions in which templates were preincubated with activator, PC5, TFIIB, and limiting concentrations of TFIID if templates were digested after DA complex formation. However, like PC4, PC5 failed to activate transcription when added after binding of TFIID and TFIIA to the promoter. As discussed previously, the activator itself binds qualitatively to its recognition sites after formation of DA complexes (33). Thus, one likely explanation for these observations might be that PC5 fails to enter into the transcription complex if added after binding of TFIID, whereas it does stimulate an event during assembly of the other general transcription factors (such as TFIIB or RNA polymerase II). It could equally well enhance transcription after initiation of transcription (e.g. during promoter clearance or elongation by RNA polymerase II). Both topological promoter mutants and mutants of general transcription factors will be required to further elucidate the mechanism by which positive cofactors stimulate transcription.

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