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# Identification of human DNA topoisomerase I as a cofactor for activator-dependent transcription by RNA polymerase II

(transcriptional activation/repression/coactivator/acidic activator GAL4-AH)

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ABSTRACT **The transcriptional activation of eukaryotic class II genes by sequence-specific regulatory proteins requires cofactors in addition to the general transcription factors. One cofactor (termed PC3) was purified from HeLa cells and identified by sequence analysis and functional assays as human DNA topoisomerase I (EC 5.99.1.2). Under identical conditions PC3 mediates both a net activation of transcription by the acidic activator GAL4-AH and repression of basal transcription, thereby leading to a large induction of transcription by the activator. PC3-mediated activation of transcription is dependent on the presence of both the GAL4-AH activation domain and the TATA-binding protein (TBP)-associated-factors (TAFs) in natural transcription factor TFIID, while repression of basal transcription is observed with either TFIID or the derived TBP alone. These results suggest novel functions, apparently through distinct mechanisms, for human DNA topoisomerase I in the regulation of transcription initiation by RNA polymerase II.**

At least three different functional classes of proteins are involved in the process of transcription initiation by RNA polymerase II in mammalian cells: general transcription factors (GTFs), which include TFIIA, -B, -D, -E, -F, -H, and -G/J and RNA polymerase II itself (1); gene-specific transcriptional activators (2); and another group of factors which mediate the effects of activators on the general transcription machinery. This last group of factors includes both the TATA-binding protein (TBP)-associated factors (TAFs), which are tightly bound to TBP in the large TFIID complex (3–5), and other cofactors (COFs) which can be separated chromatographically from TBP and the other GTFs (6). The molecular mechanisms by which TAFs and COFs facilitate communication between activators and GTFs, and thereby enhance transcription, are still not understood, but identification of the various polypeptides involved will be crucial for elucidation of this important process. Progress in understanding the structure and function of the GTFs during formation of a preinitiation complex has been facilitated greatly by their purification and by the cloning of their respective cDNAs (1). However, while GTFs alone can lead to high basal transcription *in vitro*, activators cannot stimulate transcription efficiently in systems containing highly purified GTFs (including holo-TFIID) but lacking COFs (7–9). Whereas TAFs have been identified recently by affinity methods targeting the TBP molecule, and several of their cDNAs subsequently have been cloned (5), there have been no reports of COFs which have been characterized with respect to polypeptide composition.

In this study we have subjected HeLa cell nuclear extracts to biochemical fractionation in an attempt to isolate, from the COF fraction USA (7), components which can mediate transactivation by the acidic activator GAL4-AH. GAL4-AH contains amino acids 1–147 of the yeast activator GAL4 (including its DNA-binding domain) fused to a short acidic peptide that serves as the activation domain (10–12). We have purified to near homogeneity and functionally characterized one COF (PC3) which can both repress basal and enhance activator-dependent transcription. We have identified this factor as DNA topoisomerase I.

## MATERIALS AND METHODS

**Purification of PC3.** As described previously (7) a HeLa cell nuclear extract-derived phosphocellulose 0.85 M KCl fraction was further fractionated on two successive DEAE-cellulose columns (DE52, Whatman), and the 0.17 M KCl pool of the second DE52 step was adjusted to 0.04 M KCl and applied to a Mono Q column (FPLC, Pharmacia). The flowthrough was loaded without dialysis onto a heparin-Sepharose column (Pharmacia) and washed with buffer A (identical to buffer C in ref. 8, but without Nonidet P-40) containing 40 mM KCl. PC3 was eluted with buffer A containing 500 mM KCl and dialyzed against buffer A containing 100 mM KCl. For Superose-12 chromatography (Smart system, Pharmacia), an aliquot (250 μg of protein) of the heparin-Sepharose fraction was concentrated to 40 μl by centrifugation in Centricon-30 (Amicon) and loaded onto the column. Fractions (50 μl) were collected and dialyzed against buffer A containing 100 mM KCl. For phenyl-Superose chromatography (Smart system, Pharmacia), an aliquot (500 μg of protein) of the heparin-Sepharose fraction was adjusted to buffer B [20 mM Tris·HCl, pH 7.9 at 4°C/10% (vol/vol) glycerol/0.25 mM EDTA/1.5 M ammonium sulfate] and loaded onto the column, which was then developed with a 1.5 M to 0.0 M ammonium sulfate gradient in buffer B. Fractions (100 μl) were collected and dialyzed against buffer A containing 100 mM KCl.

**Natural and Recombinant Transcription Factors.** Recombinant TFIIB, TBP, and GAL4(Δ94) were expressed in bacteria as fusion proteins with hexahistidine N-terminal tags and purified by modifications of previously described methods (8, 13). GAL4-AH was isolated as described (14). Other natural GTFs (TFIIA, TFIID, TFIIE/F/H, and RNA polymerase II) were purified as described (7).

***In Vitro* Transcription.** The GAL4-AH-activated pMRG5 template ("GAL" in the figures) contains five GAL4 recognition sites (14) upstream of the TATA element of human immunodeficiency virus type 1 (7), and the control pMLΔ53 template ("MLΔ" in the figures) contains the adenovirus major late core promoter (8). Transcription assays were conducted as described (7). If not indicated otherwise the reaction mixtures included 10 ng of each template, 0.25–0.5

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Abbreviations: TBP, TATA-binding protein; TAF, TBP-associated factor; COF, cofactor; GTF, general transcription factor.

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μl of TFIIA (Mono Q fraction, 2.70 mg/ml), 5 ng of recombinant TFIIB (heparin-Sepharose fraction), 0.3–0.6 μl of TFIID (DE52 fraction, 0.35 mg/ml), 1 μl of TFIIE/F/H (Sephadex A-25 fraction, 0.5 mg/ml) and 0.02–0.2 μl of RNA polymerase II (DE52 fraction, 0.50 mg/ml). PC3 fractions (concentrations indicated in the figure legends) were added shortly (<1 min) after GAL4-AH and just before the GTFs.

**Preparation of Proteins for Sequence Analysis.** Purified PC3 (phenyl-Superose fraction) was subjected to SDS/PAGE. Resolved proteins were transferred to membranes, eluted, and digested with endoproteinase Lys-C, and the resulting peptides were separated and subjected to microsequence analysis as described (15).

## RESULTS

**Identification of a Positive COF (PC3) as DNA Topoisomerase I.** During purification, COF activity was assayed in a reconstituted transcription system containing both purified natural factors (TFIIA, -D, -E, -F, and -H and RNA polymerase II) and purified recombinant factors (TFIIB and GAL4-AH). Initially, a previously described COF activity, USA (7, 8), was obtained by fractionation of HeLa nuclear extracts on phosphocellulose and DEAE-cellulose columns, and further dissection of this USA fraction led to the identification of several distinct COF activities. One was separated from PC1 (positive COF 1) on the second DE52 gradient and subsequently termed PC2 (M.K., Gertraud Stelzer, R.G.R., and M.M., unpublished work), whereas the others were isolated from the crude PC1 fraction (therefore termed PC1 pool) following the heparin-Sepharose column (see *Materials and Methods*). One of these coactivators has been enriched and highly purified on both hydrophobic (phenyl-Superose) and gel filtration (Superose 12) columns. The fractions from phenyl-Superose chromatography were analyzed by SDS/PAGE, and one (no. 16), which was eluted at 1.3 M ammonium sulfate and contained a nearly homogeneous 100-kDa polypeptide (Fig. 1*A*), was found initially to have a positive COF activity (selectively enhancing GAL4-AH-dependent transcription) that was termed PC3. On Superose 12, PC3 was eluted with a native molecular size of about 300 kDa (data not shown).

More detailed analyses of fractions 14–18 by SDS/PAGE (Fig. 1*A*) and by functional assays (Fig. 1*B*) revealed clearly that the 100-kDa polypeptide and the positive COF activity were coeluted, with a peak in fraction 16. A minor band of about 80 kDa also peaked in fraction 16. Because of its perfect coelution with COF activity, the 100-kDa polypeptide was subjected to sequence analysis. Two internal peptide sequences were obtained (PVFAPPYEPLPEN and LQLFMENK) and a homology search revealed complete identity with sequences (amino acids 225–237 and 551–558, respectively) in human DNA topoisomerase I, an enzyme known to relax negatively supercoiled DNA through single-strand nicking, DNA unwinding, and subsequent religation. Consistent with this observation an assay for topoisomerase I

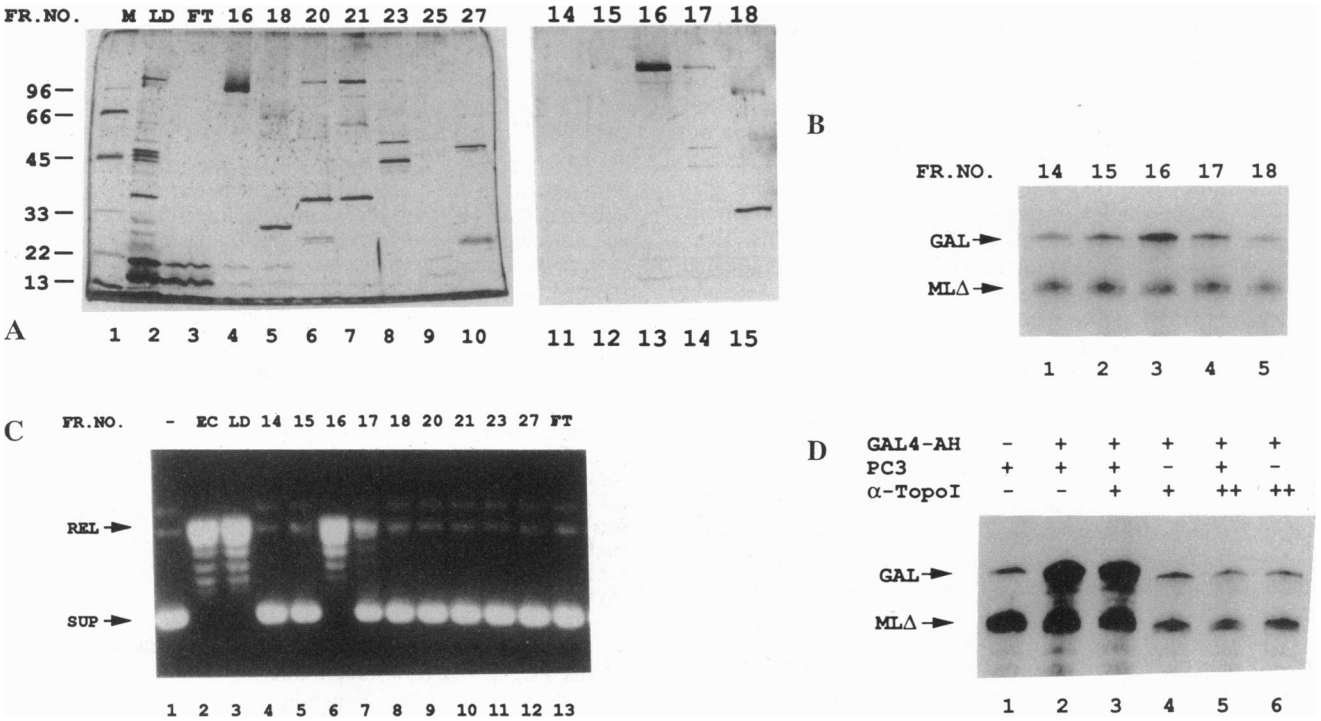


FIG. 1. Identification of topoisomerase I as the active component of PC3. (*A*) SDS/PAGE analysis of phenyl-Superose fractions. Gels were stained with silver, and molecular size markers are indicated at left in kilodaltons. Lane 1, marker proteins (M); lane 2, 5 μl of the sample loaded on the column (LD); lane 3, 5 μl of the flowthrough (FT); lanes 4–15, 2 μl each of the fractions indicated. (*B*) Transcription assays of phenyl-Superose fractions 14–18 (1 μl each) for COF activity. All reaction mixtures contained GTFs and GAL4-AH, and specific transcripts from the reporter (GAL4-AH-activated) and control templates are indicated by GAL and MLΔ, respectively. (*C*) Analysis of phenyl-Superose fractions for topoisomerase I enzymatic activity. Fractions were preincubated with 2 μg of supercoiled plasmid DNA (the transcription template) for 1 hr at 30°C in buffer conditions similar to those used in the transcription assays. The reaction mixtures were then subjected to electrophoresis in a 0.8% agarose gel and bands were visualized by UV irradiation after the gel was soaked in an ethidium bromide solution. Lane 1, no fraction; lane 2, 2 units of *Escherichia coli* topoisomerase I; lane 3, 1 μl of the load; lanes 4–12, 0.02 μl each of the fractions indicated; lane 13, 0.2 μl of the flowthrough. REL, relaxed; SUP, supercoiled. (*D*) Effect of topoisomerase I antiserum (α-TopoI) on PC3-dependent activation by GAL4-AH. A PC3 fraction (Superose-12, 1 μl) was preincubated for 1 hr at 4°C with a high-titer human autoimmune antiserum against human topoisomerase I before addition to the transcription mixtures (lanes 3 and 5). In control mixtures PC3 was treated identically, with buffer replacing the antiserum (lanes 1 and 2). Two different dilutions of the antiserum were tested (lanes 3 and 4, 1:500 dilution; lanes 5 and 6, 1:5 dilution). Reaction mixtures contained 5 ng of each template DNA and were otherwise treated as described (*Materials and Methods*).

enzymatic activity (relaxation of supercoiled plasmid DNA) demonstrated a perfect coelution (peak in fraction 16) with the COF activity and the 100-kDa polypeptide (Fig. 1*C*). The identity of the COF activity with topoisomerase I was further verified by testing the effect of an anti-topoisomerase I antiserum on transcription (Fig. 1*D*). Whereas lower amounts of the antiserum only slightly reduced transcription from the reporter template in the presence of GAL4-AH and PC3 (lane 3 versus lane 2), higher amounts completely and specifically inhibited the PC3-dependent stimulation of transcription by GAL4-AH (lane 5 versus lane 2). Basal transcription from either the GAL4 site-containing template or the control template was barely affected even at the higher serum concentration (lane 6 versus lanes 4 and 1). The specific inhibition of transcriptional activation through PC3 by the topoisomerase I antiserum provided further proof for the COF function of topoisomerase I. In immunoblots the same antiserum recognized specifically not only the 100-kDa polypeptide but also the minor 80-kDa polypeptide, indicating that the latter is a degradation product of native topoisomerase I (data not shown).

Interestingly, topoisomerase I does not seem to stimulate activator-dependent transcription by simply relaxing the supercoiled template DNA. Comparison of the quantities of topoisomerase I used in the enzymatic assay (Fig. 1*C*) and the *in vitro* transcription assays (e.g., Fig. 2*A*) revealed that optimal transcriptional activation through topoisomerase I requires a several thousandfold excess of the enzyme over the amount sufficient to completely relax the transcription template.

**PC3 Mediates Both Basal Repression and Transcriptional Activation and Is Dependent upon an Acidic Activation Domain for Activation.** Although PC3 was discovered and characterized initially as a positive COF, titration of this fraction in the absence of an activator revealed both a weak stimulation of basal transcription at lower concentrations and a repression of basal transcription at higher concentrations (Fig. 2*A*, lanes 1–5). GAL4-AH had no significant effect on transcription from the reporter template at low PC3 concentrations (Fig. 2*A*, lanes 6 and 7 versus lanes 1 and 2), whereas a significant stimulation by GAL4-AH (especially relative to basal activity) was observed at intermediate PC3 levels (Fig. 2*A*, lane 4 versus lane 9; see also Fig. 3*A*, lane 6 versus lane 3). At very high concentrations of PC3, transcription from the GAL and MLΔ templates was completely repressed in the absence of activator (Fig. 2*A*, lane 5 versus lane 1), whereas in the presence of GAL4-AH transcription from the GAL template was maintained at a level which was close to that of basal (unrepressed) transcription (lane 10 versus lane 6). It is likely that the level of transcription seen in the presence of GAL4-AH and high concentrations of PC3 (lane 10) reflects superimposed repression and activation effects that are mediated by PC3.

To test whether the COF activity of PC3 was dependent on the activation domain of GAL4-AH, the abilities of GAL4-AH and GAL4(Δ94) to activate transcription were compared under different PC3 concentrations. GAL4(Δ94) consists of the amino-terminal 94 residues of the GAL4 protein, which includes the DNA-binding and dimerization domains but no defined activation domain. Activation was specific for GAL4-AH versus GAL4(Δ94) both under conditions where PC3 completely repressed basal transcription (Fig. 2*B*) and under conditions where there was only slight repression by PC3 (Fig. 2*C*), demonstrating that the effects mediated by PC3 are not caused solely by DNA binding of GAL4 but require an activation domain as well.

Surprisingly, a 5-fold increase of the amount of template DNA (with a simultaneous 2.5-fold increase of TFIID) in otherwise identical reactions completely eliminated repression by PC3 (Fig. 2*A*, compare lanes 7 and 8 with lanes 1 and 2), indicating a strong dependency of PC3 regulatory effects on the PC3/DNA ratio (see below).

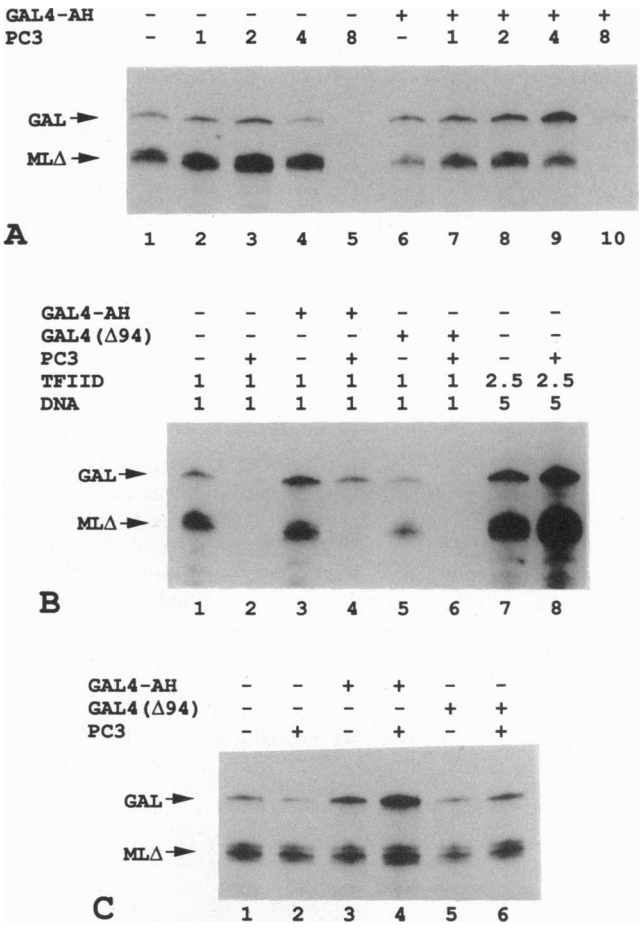


FIG. 2. PC3 is a repressor of basal transcription and a positive COF for activated transcription dependent upon the acidic activation domain of GAL4-AH. (*A*) Effects of various amounts of PC3 on basal and activator-dependent transcription. One unit of PC3 corresponds to 0.25 μl of fraction 16 (phenyl-Superose). Assays with variable levels of PC3 (units indicated at top) were conducted in the absence (lanes 1–5) or presence (lanes 6–10) of the activator GAL4-AH. (*B*) Activation at PC3 levels that strongly repress basal transcription requires the activation domain of GAL4-AH. GAL4-AH, GAL4(Δ94), and PC3 (2 μl of phenyl-Superose fraction 16) were tested in the combinations indicated, with each reaction mixture containing 10 ng of each template DNA and 0.4 μl of TFIID (lanes 1–6). Reaction mixtures for lanes 7 and 8 contained equivalent amounts of GTFs and PC3 but 5 times as much template DNA and 2.5 times as much TFIID. (*C*) Activation at PC3 levels that only weakly repress basal transcription requires the activation domain of GAL4-AH. The reaction mixtures were identical to those in *B* (lanes 1–6) except that less PC3 was used (1 μl of phenyl-Superose fraction 16).

**Factors Affecting PC3 Function.** A transcription assay with varying amounts of both PC3 and DNA confirmed the observation that the ratio of PC3 to DNA determines the effects of PC3 on transcription (Fig. 3*A*). At the standard (low) DNA concentration, increased levels of PC3 showed the typical repression of (basal) transcription in the absence (lanes 1–3) and activation of transcription in the presence (lanes 4–6) of the activator. The level of GAL4-AH-dependent transcription is always maximal at concentrations of PC3 which otherwise repress basal transcription and, significantly, reflects an overall net increase in transcription compared with unrepressed basal transcription. Thus, superposition of the basal repression and the activator-dependent transcription activities mediated by PC3 leads, at increasing

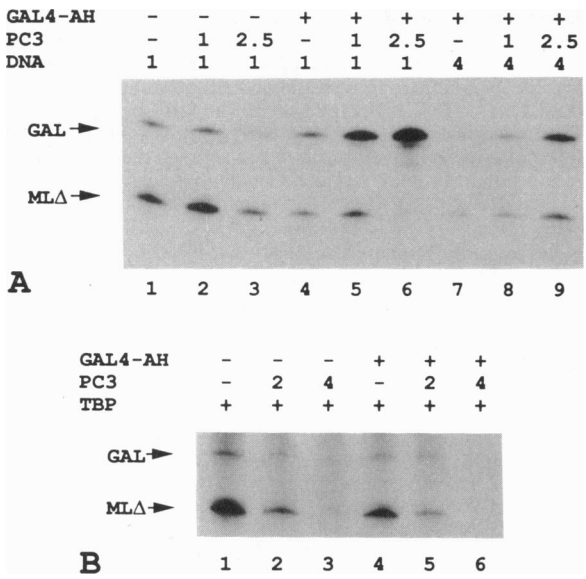


FIG. 3. Transcriptional regulation by PC3 (topoisomerase I) is influenced by DNA concentration and by TAFs. (*A*) Effects of variable DNA concentration on PC3-mediated repression and activation. Either 1 unit or 2.5 units of PC3 (Superose-12 fraction) was added to reaction mixtures containing 10 ng of each template DNA in the absence (lanes 1–3) or presence (lanes 4–6) of GAL4-AH and to reaction mixtures containing 40 ng of each template DNA in the presence of GAL4-AH (lanes 7–9). (*B*) PC3 represses basal transcription but does not mediate GAL4-AH-dependent transcription in the presence of recombinant TBP. Either 2 or 4 units of PC3 (Superose-12 fraction) was added to reactions containing recombinant TBP (5 ng of nickel-NTA-agarose fraction) in place of natural TFIID in the absence (lanes 1–3) or presence (lanes 4–6) of GAL4-AH.

PC3 concentrations, to higher ratios of transcription induction by the activator. Under otherwise identical conditions, a 4-fold higher concentration of template DNA eliminated repression of transcription and significantly reduced activation of transcription at the higher PC3 concentration (Fig. 3*A*, compare lanes 4–6 with lanes 7–9). Quantitation of these effects demonstrated that, indeed, the amount of PC3 needed for transcriptional regulation was directly proportional to the amount of DNA present in the assay. An equivalent experiment conducted with DNA lacking a functional promoter region gave a virtually identical outcome, indicating that the amount of DNA, rather than the number of functional promoters, is important for PC3 activity (data not shown). In contrast, at the same DNA concentrations higher amounts of TFIID (4-fold excess) did not significantly influence the repression and activation potentials of PC3 (data not shown).

The functional role of PC3 in transcription was investigated further by asking whether repression and/or activation by PC3 depend on the presence of TAFs in the TFIID complex. Repression by increasing concentrations of PC3 was effective with recombinant TBP (Fig. 3*B*, lanes 1–3), whereas the PC3-mediated activation by GAL4-AH was clearly dependent on the presence of TAFs in TFIID (compare lanes 4–6 in Fig. 3*B* with lanes 4–6 in Fig. 3*A*). Because of the use of a homogeneous cofactor this analysis both confirms and strengthens earlier observations that both TAFs and COFs are required for optimal function of transcriptional activators (refs. 7–9; M.K., Gertraud Stelzer, R.G.R., and M.M., unpublished work). Furthermore, it has allowed an uncoupling of the repression activity of PC3 from its positive COF activity, indicating that these are two separable properties of the same molecule. Maximal transcriptional activation (induction) in the presence of native TFIID is therefore achieved by superimposing two distinct molecular mechanisms that are both mediated by PC3.

## DISCUSSION

We have isolated and characterized a cofactor (PC3) for class II gene transcription which can effect both repression of basal transcription and a net increase in activator dependent transcription, together leading to a high transcription induction ratio in the presence of the activator. The transcriptional stimulation mediated by PC3 was shown to be dependent on the presence of both the activation domain of the acidic activator GAL4-AH and the TAFs in the TFIID complex. By sequence analysis and various functional assays the cofactor was identified as human DNA topoisomerase I.

Topoisomerase I has been suggested for some time to be involved in eukaryotic transcription (16). However, studies to date have proposed a role in elongation rather than initiation, since most of the topoisomerase I binding and cleavage sites are scattered along regions of DNA which are actively transcribed (both RNA polymerase I and II transcription units) and are not evident on the same genes when silent (17–20); moreover, the enzymatic activities of RNA polymerase II and topoisomerase I are tightly linked both temporally and physically during transcription of at least one gene (21). From a mechanistic point, topoisomerase I might serve as a "swivel" to relieve torsional stress (in the template) caused by the generation of positive supercoils downstream and negative supercoils upstream of the moving RNA polymerase (22). However, no direct evidence for a role of topoisomerase I in elongation has been presented.

As a possible mechanism for the stimulatory role of topoisomerase I in transcription initiation, as suggested in the present study, we propose that the enzyme may cause conformational changes of the DNA at the promoter region, either by DNA binding itself or by enzymatic release of conformational stress, which then may either directly facilitate binding of other transcription factors or energetically favor open-complex formation. Changes of DNA conformation in positions relevant to their function (e.g., TATA box, initiator element) have been observed upon variations of the superhelical density of the template DNA or upon interaction of the promoter with components of the transcription machinery (e.g., refs. 23–25, 29). One likely target for topoisomerase I in the preinitiation complex would be RNA polymerase itself, if topoisomerase I and RNA polymerase II might travel together along the DNA subsequent to initiation (i.e., during elongation). However, given the correlation between the amount of DNA in the transcription assay and the amount of topoisomerase I needed for cofactor function (Fig. 3*A*), such a specific interaction of topoisomerase I would appear to have a binding affinity lower than that for nonspecific DNA and would therefore occur only when the DNA templates are already mostly covered by topoisomerase I itself or by other DNA-binding proteins. In the physiological situation, most of the DNA is covered with nucleosomes and other chromosomal proteins, which could largely exclude topoisomerase I from DNA regions other than those opened up by binding of sequence-specific regulatory proteins and the general transcription machinery. The amounts of topoisomerase I in normal somatic cells ($10^5$–$10^6$ molecules per cell) should therefore be sufficient to allow binding of topoisomerase I to the promoters of transcribed genes (26, 27).

PC3 (topoisomerase I) did not require the addition of any transcriptional repressor to the highly purified reconstituted system in order to elicit a stimulatory effect of GAL4-AH, indicating that it does not function by a mere antirepression mechanism. Moreover, the net increase of transcriptional activity in the presence of the activator and PC3 (as compared

to the unrepressed basal transcription) also argues for a more direct positive role of PC3 in stimulating function of the other transcription factors. However, we cannot exclude the possibility of inhibitory factors (repressors) contaminating the transcription system or being integral components of the general transcription machinery. Recent studies have shown, for example, that *Drosophila* TFIID contains a TAF (TAF230) which can negatively regulate the DNA-binding capacity of the TATA-box-binding subunit (28).

The ability of PC3 to function as a transcriptional coactivator was dependent on the presence of the activation domain of GAL4-AH, thus providing an example of a COF of defined polypeptide composition that works in conjunction with the acidic activation domain of GAL4-AH. This specificity may reflect either a direct interaction between the COF and the activation domain or the preferential enhancement of an independent process mediated specifically through this activation domain. Preliminary studies of other activators on respective target promoters have indicated a PC3 COF specificity for certain types of activation domains (M.K., unpublished observation).

The positive COF function of PC3 was dependent on the natural TFIID complex with TAFs. This observation demonstrates with a structurally defined positive COF that both TAFs and COFs are required for transcriptional activation. In contrast to the TAF requirement for activator function, the inhibitory effect of PC3 on basal transcription was evident with either TFIID or TBP. Thus, the basal repression by PC3 must reflect a distinct (and separable) function, although the exact mechanism of the inhibition remains to be determined.

Our fractionation of HeLa nuclear extracts has led to the identification and purification of several distinct COF activities (PC1, PC2, PC3, and NC1) and a preliminary study of possible additive or synergistic effects between them has shown that PC3 (topoisomerase I) can indeed enhance the transcription induction ratio by an activator in the presence of saturating amounts of a distinct COF (M.K., unpublished observation). This indicates that different COFs utilize distinct activation mechanisms which are not mutually exclusive but can function additively and, potentially, synergistically. Thus the maximal levels of transcriptional activation as seen in less purified cell-free systems and in living cells most likely reflect the combined effects of several distinct COF activities, to which PC3 (topoisomerase I) may contribute significantly.

**Note Added in Proof.** A recent report by Merino *et al.* (30) demonstrates identity between topoisomerase I and a cellular factor (Dr2) that was initially identified as an inhibitor of basal transcription and isolated from a chromatographic fraction equivalent to that reported by us (7) to contain USA and the derived positive-cofactor activities. Their demonstration of both repression and activation functions for Dr2/topoisomerase I is in complete accord with the results presented here.

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