Sequence-specific transactivators counteract topoisomerase II-mediated inhibition of *in vitro* transcription by RNA polymerases I and II

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ABSTRACT

An inhibitor of RNA polymerase II transcription *in vitro* has been purified from HeLa cell nuclear extracts. Partial amino acid sequences derived from the purified protein revealed that the inhibitor of transcription corresponded to human topoisomerase II. Order of addition experiments provided evidence indicating that topoisomerase II inhibited transcription by binding over the core promoter and blocking preinitiation complex formation. Topoisomerase II-mediated repression could be relieved by sequence-specific transcriptional activators, having different activating and/or DNA binding domains, but antirepression required a transcriptional activation function in addition to a DNA binding domain. Moreover, transcription by RNA polymerase I was also inhibited by topoisomerase II and this inhibition could be relieved by the RNA polymerase I transactivator UBF. These observations suggest that topoisomerase II may participate in a general repression of transcription which can be counteracted by transcriptional activators.

INTRODUCTION

Initiation of transcription by RNA polymerase II is an important target step in the regulation of gene expression. Elements within the core promoter are recognized by a set of general transcription factors which are necessary and sufficient for initiation of transcription *in vitro*. The assembly of the preinitiation complex by the general transcription factors is an ordered process the first step of which is the binding of the TATA binding protein (TBP), present in the multi-protein TFIID complex, to its cognate element in the basal promoter. The binding of TFIID, together with TFIIA, promotes the stepwise assembly of the other general

initiation factors [TFIIB, -IIE, -IIF, -IIH (BTF2) and -IIJ] and RNA polymerase II in a stable, template-committed preinitiation complex (for review see 1, 2, 3). Additional factors and alternative pathways may be involved in the formation of preinitiation complexes on TATA-less promoters (see reviews above). Human TBP is a 38 kd polypeptide which in higher eukaryotes is complexed with multiple TBP-associated factors (TAFs) to form a heterogeneous set of high molecular weight complexes (reviewed in 4, 5, 6; and see 7). TBP is required not only for transcription by RNA polymerase II, but also for transcription by RNA polymerases I and III. Each of these different functional forms of TBP is associated with a distinct set of TAFs. (reviewed in 5, 8, 9, 10).

The basal activity of promoters can be modulated either by sequence-specific transcriptional activators or by transcriptional repressors (11, 12, 13; and references therein). The molecular mechanisms by which activators or repressors regulate the activity of the basic transcriptional machinery have yet to be clarified. In the absence of site-specific transcriptional activators transcription has been found to be significantly lower *in vivo* than *in vitro* suggesting that in the cell basal promoter activity is severely restricted. One mechanism for restricting basal promoter activity is the packaging of genes into chromatin. Numerous studies have shown that proteins which are involved in chromatin structure such as nucleosomes, histone H1, high mobility group (HMG) proteins and others can inhibit transcription (for reviews see 14, 15, 16, 17). *In vitro* experiments with nucleosome assembled templates suggest that transcriptional inhibition results from the positioning of nucleosomes over the promoter sequences, blocking preinitiation complex formation. This inhibition appears to be specific to nucleosomes located on promoters as downstream nucleosomes do not prevent elongation (18). The *in vivo* phenotypes of yeast strains harboring mutations in genes encoding

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histones also point to a regulatory role for nucleosomes in transcriptional repression. When histone synthesis is halted in genetically altered yeast cells many inducible genes are preferentially switched on in response to nucleosome depletion (19).

In vivo experiments indicated that promoters of transcribed genes are depleted in histone H1, relative to non-transcribed genes (20, 21, 22), suggesting a role for H1 in transcriptional inhibition. Several reports have shown that association of histone H1 with transcription templates *in vitro*, in the presence or absence of nucleosome cores, inhibits preinitiation complex formation (23, 24; and refs. therein). The transcriptional repression due to nucleosomes or histone H1 can be overcome (antirepression) *in vitro*, in crude transcription extracts, by sequence-specific transactivators (23, 25, 26). However, the nucleosome- or histone H1-mediated inhibition was not relieved by GAL-VP16 in transcription systems reconstituted from purified basal transcription factors indicating the requirement of additional factors for this antirepression effect (24, 27).

Another group of proteins, which negatively regulate basal transcription, have been described. These proteins, designated as NC1, NC2, Dr1 and Dr2, interact directly with TBP to inhibit its association with TFIIA and/or TFIIB (11, 12, 28, 29). This interaction with TBP may account for the ability of these proteins to inhibit basal transcription. At present the existence of other proteins which would interact with basal transcription factors other than TBP to prevent preinitiation complex formation cannot be excluded.

In this study we describe the purification of a HeLa cell protein which acts as a repressor of RNA polymerase II transcription *in vitro*. Peptide sequences obtained from the purified protein indicated that it was human topoisomerase II, an enzyme which modulates higher order chromatin structure and plays a critical role in regulating many aspects of DNA metabolism. Sequence-specific transcriptional activators, having different activating and/or DNA binding domains, were shown to counteract the topoisomerase II-mediated repression. For antirepression a transcriptional activation function was required in addition to a DNA binding domain. Moreover, transcription by RNA polymerase I was also inhibited by topoisomerase II and the inhibition was relieved by the transactivator UBF. Order of addition experiments indicated that transcription was not inhibited when topoisomerase II was added after preinitiation complex formation. These results provide evidence indicating that, as for histone H1, topoisomerase II inhibits transcription *in vitro* by binding over the promoter region and blocking the formation of preinitiation complexes.

MATERIALS AND METHODS

Purification of negative regulatory factor 1 (NERF-1, topoisomerase II)

NERF-1 was purified from HeLa cell nuclear extracts on the basis of its ability to repress *in vitro* transcription (see Fig. 1A). All procedures were carried out at 4°C. Nuclear extract preparation, heparin-Ultrogel and phosphocellulose (PC) chromatography has been described in (7) (see also Fig. 1A). The 0.6 M KCl-derived PC1.0 M KCl fraction (~90 mg protein), which contained NERF-1 activity, was adjusted to 0.9 M ammonium sulfate (AS) and loaded on a preparative HPLC TSK-phenyl-5PW column [50 ml (2.15 cm ID × 15 cm)]. Proteins were eluted with a linear 5 column vol 0.9–0 M AS gradient in buffer D [50 mM

Tris-HCl pH 7.9, 0.5 mM DTT, 0.1 mM EDTA, 10% glycerol (v/v)]. The fractions containing NERF-1 activity (between 0.8–0.6 M AS) were pooled and dialysed against buffer D containing 0.2 M KCl, and loaded on a heparin-5PW column [3.3 ml (0.75 cm ID × 7.5 cm)]. Bound protein was eluted with a linear 5 column vol 0.2–0.5 M KCl gradient in buffer D. NERF-1 activity eluted between 0.40–0.45 M KCl. Active fractions were pooled, diluted to 0.25 M KCl with buffer D and further chromatographed on a single stranded (ss) DNA cellulose column (2 ml). The flow through, containing NERF-1, was diluted to 0.1 M KCl with buffer D and loaded on a DEAE-5PW column [3.3 ml (0.75 cm ID × 7.5 cm)]. Bound protein was then eluted with a linear 5 column vol 0.1–0.25 M KCl gradient in buffer D. The active fractions (between 0.15–0.17 M KCl) were pooled and dialysed against 10 mM potassium phosphate buffer [containing 0.01 mM CaCl₂ and 10% glycerol (v/v)] and loaded on HPLC hydroxyapatite column HA-1000 (3.3 ml). Bound protein was eluted with a linear 5 column vol 0.28–0.42 M potassium phosphate gradient. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue and/or silver staining.

Yeast topoisomerase II was overexpressed and purified as described in (30, 31).

1 unit of topoisomerase II activity (see Results) contained approximately 200 ng of purified topoisomerase II.

Amino acid sequencing of NERF-1

NERF-1 containing fractions from three separate purifications (see above) were pooled, dialysed against buffer S (5 mM NaCl, 0.5 mM Tris-HCl pH 7.9, 0.005 mM DTT, 0.01% SDS) and lyophilized. Proteins (40 µg) were separated by preparative 7% SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted on a PVDF membrane (Millipore, Immobilon P 0.45 µm) overnight in 50 mM Tris-borate pH 8.3 buffer. The PVDF membrane was briefly stained with Coomassie blue and the two protein species which closely corresponded to the NERF-1 activity, were excised and subjected to digestion with trypsin (Promega, sequencing grade). The resulting peptide mixtures from the two species were separated by micro bore HPLC using a Vydac C18 250 × 1.6 mm reverse-phase column on a Applied Biosystems 1000S HPLC with a diode array detector. Optimum fractions were chosen and subjected to automated Edman degradation on a Applied Biosystems 477A protein sequencer.

Reporter plasmids

The reporter plasmid pAL7 contains the Adenovirus major late promoter (AdMLP -33 to +33) fused to the rabbit β-globin gene at -9 as previously described (32). (17M)5-pAL7 was derived from pAL7 by the insertion of five GAL4 binding sites upstream of the AdMLP (32). pG1 contains the rabbit β-globin promoter as previously described (33). (ERE)4-pAL10 was constructed by inserting the following sequence 5'-CTAGGG-TCACAGTGACCATGAGTCAGGTCACAGTGACC-3' between the Xho I and Xba I sites of pERE121 (34).

In vitro transcription assays

For pol II transcriptions all protein fractions were dialysed against buffer B [25 mM Tris-HCl pH 7.9, 50 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 20% glycerol (v/v)] prior to *in vitro* transcription assays. Reactions (20–25 µl) contained 25 ng of each supercoiled (in all figures) DNA template (as indicated in the legends of the

figures) along with 5 μ g of PC0.5 fraction, 0.5 μ g of partially purified TFIIA [PC0.5+TFIIA system (7)], and aliquots of the chromatography fractions (as indicated). Activated transcription was achieved by the addition of 20–80 ng of purified recombinant activator proteins (as indicated) along with the PC1.0 M-KCl derived phenyl-5PW fractions containing TFIID (7). The order of assembly of the transcription reactions is indicated in the legends to the figures. After 30 min preincubation at 25°C transcription was initiated by addition of nucleoside triphosphates (to 0.5 mM) and MgCl_2 (to 5 mM) and incubated at 25°C for 45 min. Reactions were stopped by adding 300 μ l of 0.1 % SDS and 5 μ g tRNA, extracted with phenol and then with chloroform and ethanol precipitated. Correctly initiated transcripts were analysed by quantitative S1 nuclease analysis as described in (35).

The purification of murine pol I transcription factors and the reconstituted transcription system was as described in (36). 25 μ l assays contained 5–10 ng of template DNA (pMrWT linearized with Nde I or Nar I yielding transcripts of 371 nt or 319 nt, respectively), partially purified pol I, 2 μ l of TIF-IB, 3 μ l of TIF-IA/TIF-IC and 5 ng of UBF. To assay transcription repression, the first template (pMrWT/Nde I) was preincubated for 15 min at 30°C with the topoisomerase II containing fraction before the second template (pMrWT/Nar I), pol I, the transcription factors and nucleotides were added (37). Transcription was stopped after 60 min and transcripts were analysed by gel electrophoresis and autoradiography.

Overexpression and purification of the chimeric activator proteins

GAL(1-147) comprises amino acids 1–147 of the yeast activator GAL4 containing the DNA binding and dimerisation domains. GAL-VP16 and GAL-TEF1 are chimeric activators comprising the GAL(1-147) fused to the acidic activation domain of the Herpes simplex virus-1 virion protein (VP)16, or the activation function of the human transcription enhancer factor 1 (TEF-1). Each protein was overexpressed in *E. coli* and purified as described previously (7, 38, 39) using ion exchange and DNA affinity chromatography. The human estrogen receptor (HEG0) and GAL-ER(EF), containing activation function 2 (AF-2) of the estrogen receptor, were overexpressed in *Spodoptera frugiperda* (Sf 9) cells using the baculovirus overexpression system and GAL-ER(EF) was purified as described in (38). The estrogen receptor purification was similar to that of GAL-ER(EF) except that for the DNA affinity purification an affinity column containing estrogen responsive elements (EREs) was used.

RESULTS

Purification of an inhibitor of RNA polymerase II basal transcription

During the purification of transcriptional intermediary factors from HeLa cell nuclear extracts several fractions were isolated which repressed basal RNA polymerase II (pol II) transcription. To characterize further the inhibitory fractions, a previously described, partially purified HeLa cell basal transcription system was used. This system comprises a heparin-Ultrogel 0.6 M KCl-derived phosphocellulose 0.5 M KCl fraction (PC0.5) supplemented with partially purified TFIIA (STF) (see 7; and Fig. 1A). This system (PC0.5+TFIIA) contains all the basal transcription factors necessary for initiation of transcription at the Adenovirus major late promoter (AdMLP), and the rabbit β -globin promoter (7). To test the ability of the different

Figure 1. (A) The chromatography protocol used to purify negative regulatory factor 1 (NERF-1) from HeLa cell nuclear extract (N.E.). The concentration of KCl and ammonium sulfate (AS) used in column elutions and precipitation are indicated. Arrows indicate the use of a linear gradient between the concentrations indicated at each end of the arrow. (B) The purified NERF-1 containing fractions inhibit basal transcription. The indicated fractions (5 μ l from each), eluting from the DEAE-5PW HPLC column, were preincubated with 25 ng of each DNA template (pAL7 and pG1 supercoiled plasmids) for 5 min at 25°C, prior to the addition of the general transcription factors [PC0.5+TFIIA system (7)]. After a further 30 min incubation the nucleoside triphosphates (NTPs) were added. Quantitative S1 nuclease analysis was used to determine the amount of correctly initiated transcripts from the AdMLP (AdMLP+1) and the rabbit β -globin promoter (Glob+1). (C) Analysis of 10 μ l of the indicated DEAE-5PW fractions by electrophoresis on a 7.5% polyacrylamide-SDS gel. Proteins were visualized by Coomassie blue staining. The arrows indicate the positions of the two protein species (2 and 4) whose elutions followed closely that of the NERF-1 activity. These two species were subjected to microsequencing following an additional hydroxyapatite (HAP) chromatography step (see panel A).

chromatographic fractions to repress transcription they were incubated with two template DNAs, containing either the AdMLP or the rabbit β -globin promoter, prior to addition of the PC0.5+TFIIA system and nucleoside triphosphates. When the proteins from the heparin-Ultrogel 0.6 M KCl-derived PC 1.0 M KCl fraction were eluted from the phenyl-5PW HPLC column with a linear ammonium sulfate (AS) gradient (from 0.9 M AS to 0 M AS, see Fig.1A), an inhibitor of basal transcription eluted around 0.7 M AS. Transcriptional inhibition was observed when these fractions were incubated with the DNA template, prior to the addition of the transcription factors and ribonucleoside triphosphates (NTPs), but not when they were added 15–30 minutes after the the transcription factors and the NTPs (data not shown and see below). This result indicates that inhibition was not due to ribonucleases, DNAases or proteases, but rather to the selective inhibition of preinitiation complex formation. Furthermore, this repressor was not histone H1, another inhibitor of pol II transcription *in vitro*, since the starting protein fraction eluting from the heparin-Ultrogel column (between 0.24 and 0.6 M KCl, see Fig.1A) was precipitated with 2.26 M AS which does not precipitate histone H1 (23). In addition, histone H1 elutes between 1.44 and 1.05 M AS from phenyl-Sepharose columns (40). Note also that these phenyl-5PW fractions and the subsequent chromatographic fractions did not contain factors which interact with the recombinant human TBP in gel mobility shift experiments (data not shown and see Introduction). Thus,

these observations suggested that the present phenyl-5PW fractions contained a novel repressor of pol II transcription *in vitro*, designated negative regulatory factor 1 (NERF-1).

NERF-1 was then purified to near homogeneity as outlined in Fig. 1A (see also Materials and Methods). Preincubation of the highly purified NERF-1 containing fractions with the two DNA templates resulted in a repression of transcription in each case (compare lane 1 with lanes 5–9 in Fig. 1B). Following purification on seven chromatographic columns (see Fig. 1A) the NERF-1 activity copurified with four polypeptides with relative molecular masses (MMs) between 150 and 180 kDa. The elutions of two of these proteins correlated particularly closely with that of the repressor activity (see species 2 and 4 in Fig. 1C).

NERF-1 is human topoisomerase II

To determine the identity of this repressor the purest NERF-1 preparations were pooled and separated by preparative SDS polyacrylamide gel electrophoresis. The proteins were electrotransferred to a PVDF membrane and stained briefly with Coomassie blue. Species 2 and 4 (see Fig. 1C) were excised and digested with trypsin. The tryptic peptides were separated by reverse phase chromatography and microsequenced (see Materials and Methods). The sequences of six peptides from species 4 and five from species 2 were determined (see Table 1). Comparison of the amino acid sequences of these peptides with a protein data base revealed that they all, originating from either species 2 or 4, corresponded exactly to the amino acid sequence of human topoisomerase II (41). Species 2 has an approximate MM of 170 kDa which is in good correlation with the calculated MM of topoisomerase II (174334 Da). Species 4 may be a proteolytic degradation product of species 2. These findings strongly suggested that NERF-1 was in fact topoisomerase II. To further test the ability of topoisomerase II to inhibit transcription, recombinant yeast topoisomerase II, purified by a different protocol (31), was included in the transcription reactions. As expected both the human (NERF-1) and the purified yeast topoisomerase II repressed basal (and activated; see below) transcription in a similar manner (Fig. 2A compare lane 1 with

lanes 3 and 5). Consistent with the presence of topoisomerase II in the purified protein fractions containing the inhibitory activity they also relaxed supercoiled DNA (data not shown). Linear DNA templates were prepared to eliminate the possibility that inhibition was due to the ability of topoisomerase II to relax, nick and/or introduce double stranded breaks in the circular plasmids used in the transcription reactions. Using linear templates, both the human and the yeast topoisomerase II inhibited *in vitro* transcription in a manner indistinguishable from that observed with circular templates (data not shown and see Fig. 2A). These data indicate that the enzymatic activities of topoisomerase II are not involved in the inhibition of transcription *in vitro*.

Topoisomerase II inhibits the formation of stable preinitiation complexes

It has been shown that incubation of purified topoisomerase II with DNA fragments can lead to the formation of insoluble protein–DNA aggregates (42). These aggregates are also formed under the present *in vitro* transcription conditions, since following preincubation with topoisomerase II, for 10 min at 25°C, the DNA templates can be pelleted (data not shown). The possible reversibility of the formation of insoluble topoisomerase II/DNA template aggregates was then examined to determine whether inhibition of transcription was due to the formation of these aggregates. Increasing amounts of nonspecific Bluescribe plasmid

Table 1. The amino acid sequences of the tryptic peptides obtained from either species 2 (2) or 4 (4) of NERF-1 (see also Fig. 1C).

276	286	
DKLDETGNSLK		(4)
324	335	
HVDYVADQIVTK		(2,4)
450	465	
NSTECTILILTEGDSAK		(2)
512	518	
IVGLQYK		(4)
625	631	
EYFADMK		(4)
827	834	
FLYDDNQR		(2,4)
949	957	
TPPLITDYR		(2)
958	966	
EYHTDTTVK		(2)
1075	1079	
VLIQR		(4)

The numbers indicate the amino acids of the peptides in the human topoisomerase II amino acid sequence (41).

Figure 2. (A) The purified NERF-1 inhibits transcription in a similar manner as purified recombinant yeast topoisomerase II (Topo. II). NERF-1 containing fractions or topoisomerase II purified from yeast were incubated with the two DNA templates [(17)5-pAL7 and pG1] in the presence (+) or the absence (–) of purified GAL-ER(EF) activator protein and the transcription reactions were carried out as described in legend to Fig. 1B. (B) Reversibility of the topoisomerase II-mediated transcriptional repression. 50 ng of AdMLP containing plasmid (pAL7) was preincubated either with buffer or topoisomerase II (Topo. II, 1 unit). After 10 min at 25°C Bluescribe plasmid DNA (BSM) was added in increasing concentrations to the reactions (as indicated), prior to the general transcription factors. The reactions were further incubated for 30 min at 25°C and then the nucleoside triphosphates were added. The position of the correctly initiated transcript (AdMLP+1) is shown. (C) Preformed preinitiation complexes are resistant to topoisomerase II-mediated transcriptional inhibition. Template DNAs (pAL7 and pG1) were mixed with general transcription factors (TFs) and to this mixture topoisomerase II (Topo. II, 0.4 or 1 unit) was added at the different times as indicated. The schematic assembly of the components of the transcription reactions is depicted under the figure.

DNA (BSM) were added to the template DNA, which was either preincubated with buffer or with topoisomerase II for 10 min at 25°C, before the addition of the general transcription factors (PC0.5+TFIIA system). Increasing the amount of BSM in the reactions (where transcription was inhibited by preincubation with topoisomerase II) resulted in a progressive recovery of transcription (compare lanes 1–3 and 4–6 in Fig.2B). These results suggest that topoisomerase II bound to the template DNA could be competed by a 10-fold excess of BSM rendering the AdMLP template accessible to the general transcription factors. Thus, the interaction of topoisomerase II with the DNA is dynamic and the formation of topoisomerase II/DNA aggregates is reversible. Note that by using the aggregation assay described by (42), no preferential topoisomerase II binding sites could be detected within the two template plasmids (data not shown).

Next it was determined whether, under the present *in vitro* transcription conditions, the assembly of preinitiation complexes on promoter DNA could be inhibited by topoisomerase II. In the presence of template DNA the general pol II transcription factors assemble into a preinitiation complex in 15–30 minutes at 25°C (43). 1 unit of topoisomerase II (1 unit being defined as the amount of topoisomerase II required to inhibit the transcription from 50 ng of template DNA by more than 90%) efficiently inhibited transcription when added before, or at the same time as the general transcription factors (Fig.2C lane 3 and Fig. 1B), but had much less effect on transcription when added 15 or 30 min after the transcription factors, when stable preinitiation complexes were already formed (compare lane 3 with 6 or 9 in Fig.2C). These results provide strong evidence indicating that topoisomerase II inhibits transcription by binding over the promoter region and preventing the assembly of preinitiation complexes, but that it has no inhibitory effect on the activity of preformed complexes.

Sequence-specific transcriptional activators counteract topoisomerase II-mediated repression *in vitro*

The ability of topoisomerase II to repress transcription activated by several recombinant sequence specific activators was next examined. The purified activators were either chimeras in which different, well characterized, activation functions were fused to the DNA binding domain of GAL4 [(GAL-ER(EF), GAL-VP16, GAL-TEF-1; see 7, 32, 38, 39], or the intact human estrogen receptor (ER) (see HEG0 in 44). Reporter templates, containing either five GAL4 binding sites [(17M)5-pAL7] or four ER binding sites [(ERE)4-pAL10], respectively, upstream of the AdMLP TATA box were then used (32 and see Materials and Methods). The rabbit β -globin promoter, which has no binding sites for either the GAL-chimeras or ER, serves as an internal control. The efficient function of these activators in the PC0.5+TFIIA system was assured by the addition of 5 μ l of the phosphocellulose 1.0 M KCl-derived phenyl-5PW fractions (eluting between 0.4 and 0 M AS) containing TFIID. The TBP-associated factors in this fraction have been previously shown to mediate stimulation *in vitro* by these activator proteins (7, 38). In the absence of topoisomerase II, and at close to saturating TFIID concentrations, GAL-ER(EF), GAL-VP16, GAL-TEF-1 or hER stimulated transcription 2.5–4 fold (Fig.3). At low topoisomerase II concentrations (0.2 or 0.4 units), which inhibited basal transcription by 40–60% (compare AdMLP+1 in lane 9 with lane 13, and lane 17 with lane 21 in Fig.3), activated transcription was not inhibited (compare AdMLP+1 in lane 10

with lanes 12 and 14, and in lane 18 with lanes 20 and 22 in Fig.3). At higher topoisomerase II concentrations, where basal transcription was almost completely repressed, the level of activated transcription in the presence of the four different activators remained readily detectable (compare lanes 15–16, 23–24, 27–28, and 31–32 in Fig.3). Thus, depending on the concentration of topoisomerase II, a transcriptional stimulation of up to 50-fold was obtained in the presence of the transactivators, indicating that they efficiently counteract topoisomerase II-mediated inhibition. Since the four activators behaved as transcriptional antirepressors, irrespective of the nature of their activating or DNA binding domains and/or their cognate binding sites, it is probable that antirepression by sequence-specific activators is a general effect on topoisomerase II-mediated transcriptional inhibition *in vitro*.

To investigate the role of the activation functions in antirepression, the GAL4 DNA binding domain [GAL(1-147)] was tested for its ability to relieve the topoisomerase II-mediated inhibition. In contrast to GAL-ER(EF), GAL-TEF-1 or GAL-VP16, all of which contain an activation function, GAL(1-147) was much less efficient in counteracting the topoisomerase II-mediated inhibition (compare AdMLP+1 for example in lane 6 with 14, or with 22 in Fig.3). These data indicate that, by itself,

Figure 3. Sequence-specific transactivators counteract topoisomerase II-mediated inhibition. Reactions in lanes 1–28 contained 25 ng of (17M)5-pAL7 reporter plasmid containing five GAL 4 binding sites, together with 25 ng of the reference plasmid pG1, in the presence (+) or (–) or absence of purified GAL(1-147) (lanes 1–8), GAL-ER(EF) (lanes 9–16), GAL-TEF1 (lanes 17–24) and GAL-VP16 (lanes 25–28) proteins as indicated. In lanes 29–32, 25ng of (ERE)4-pAL10 reporter, containing four estrogen responsive elements (ERE), was used in the presence or absence of purified ER (HEG0). Increasing concentrations of purified topoisomerase II (Topo. II) were added to the reactions (as indicated), in addition to the general transcription factors and a partially purified, endogenous TFIID containing fraction (see Results and 7). After a further 30 min incubation nucleoside triphosphates were added. The positions of the correctly initiated transcripts from the two test promoters are indicated by arrows to the left of each panel. In several independent experiments the addition of 0.4 to 1 unit of topoisomerase II to the transcription reactions increased the fold stimulation by GAL(1-147) from 1.5 fold in the absence of topoisomerase II to 3–4 fold, by GAL-ER(EF) from 2 fold to 15–18 fold, by GAL-TEF1 from 2.5 fold to 12–14 fold, by GAL-VP16 from 4–5 fold to almost 50 fold and by HEG0 from 2 fold to 8–10 fold.

the GAL4 DNA binding domain alone is not sufficient for antirepression and that the presence of an activation function is necessary for this effect.

In the above experiments the sequence-specific activators were added to the DNA templates concomitantly with the topoisomerase II. Thus, these results suggest that, by binding to their cognate binding sites upstream of -65 on the AdMLP, the transactivators counteract topoisomerase II-mediated inhibition by preventing topoisomerase II binding, and/or facilitating a limiting step in preinitiation complex formation. GAL-VP16 was next added to the template DNA 15 min after the topoisomerase II to determine whether it could reverse the repression from templates which already had topoisomerase II bound over the promoter region. While transcription was repressed in the presence of only the basal transcription factors (Fig.4 lanes 3 and 7, see also Fig. 2C), in the presence of GAL-VP16 transcription could still be readily detected (lanes 4 and 8 in Fig.4). Thus, GAL-VP16 counteracted topoisomerase II-mediated inhibition irrespective of whether it was added to the template DNA either 5 min before or 15 min after topoisomerase II. Hence, the presence of topoisomerase II on the template does not prevent the interaction of the activator with its binding site, even when up to 1 unit of topoisomerase II was added. Note that in these experiments saturating concentrations of TFIID were used. Under these conditions a high level of basal transcription is obtained in the absence of topoisomerase II (lanes 1 and 5) and consequently the stimulation of transcription by GAL-VP16 is relatively low (1.5 fold; compare lanes 1 and 2, or lanes 5 and 6). However, in the presence of 1 unit of topoisomerase II such high TFIID concentrations are required to overcome the inhibitory effect of topoisomerase II and to detect transcriptional stimulation by GAL-VP16 (compare lanes 3 and 4, or 7 and 8).

Figure 4. GAL-VP16 is able to counteract topoisomerase II-mediated transcriptional inhibition even after the formation of topoisomerase II/template DNA complexes. In lanes 1–4 either buffer or GAL-VP16 was incubated with the reporter [(17)5-pAL7] and the reference (pG1) plasmids for 5 min at 25°C and then purified topoisomerase II (Topo. II) was added to the reactions (as indicated) together with fractions containing the general transcription factors and TFIID (TFs) (see Results and 7). In contrast in lanes 5–8 either buffer or topoisomerase II was first incubated with the two test plasmids together with the general transcription factors (TFs) for 15 min at 25°C and then either buffer or GAL-VP16 was added to the reactions (as indicated). The schematic representation of the order of addition of the transcription reaction components is depicted under each panel. The positions of the correctly initiated transcripts from the AdMLP and β -Globin promoters are shown.

Topoisomerase II inhibits transcription by RNA polymerase I

To investigate whether topoisomerase II-mediated transcriptional repression was restricted to polymerase II promoters or whether transcription by other classes of RNA polymerases would be similarly affected, a polymerase I system was used. Recently, using a template commitment assay and partially purified transcription factors, repression of RNA polymerase I transcription by a negative-acting Ku related factor has been demonstrated. The transcriptional repression was counteracted by the nucleolar transcription factor UBF (37). This assay system was used to test the effect of topoisomerase II on ribosomal gene transcription. Two identical rDNA promoter templates which yield run off transcripts of 371 and 319 nt were employed. In the absence of preincubation with topoisomerase II or UBF, both of these templates were transcribed with the same efficiency (Fig.5 lane 1). Transcription from the first (generating the longer transcript), but not the second of the rDNA templates, was inhibited following preincubation with topoisomerase II before addition of the second template, the pol I transcription factors, and the nucleotides (Fig. 5 lane 2). If, however, UBF or UBF and TIF-IB were present during the preincubation period, together with topoisomerase II, transcription from the first template was not repressed, but rather showed a preferential transcriptional commitment (Fig.5 lane 3 and 4). These results indicate that topoisomerase II can also prevent the formation of RNA polymerase I preinitiation complexes and that repression can be overcome by the RNA polymerase I-specific transactivator UBF.

DISCUSSION

The mechanism of topoisomerase II-mediated transcriptional repression and antirepression by sequence-specific activators

We have identified, purified and characterized a general inhibitor of *in vitro* transcription from HeLa cell nuclear extracts. This inhibitor was identified as human topoisomerase II. Topoisomerases change the degree of DNA supercoiling by

Figure 5. Repression of RNA polymerase I gene transcription by topoisomerase II. Transcription reactions contained 5 ng of each of pMrWT/Nde I (template 1) and pMrWT/Nar I (template 2). Lane 1 shows a control reaction without preincubation. The first template was preincubated for 15 min at 30°C with 1 unit of topoisomerase II in the absence (lane 2) or presence of pol I- specific factors UBF (lane 3) or TIF-IB and UBF (lane 4) respectively. Then the second template, the NTPs, RNA polymerase I and missing factors were added and the incubation was continued for another 60 min.

transiently nicking either one (type I) or both strands (type II) of the DNA helix (reviewed in 45, 46). A link between supercoiling and transcriptional activity has been an attractive idea. However, the ability of topoisomerase II to change the degree of supercoiling does not seem to be the mechanism by which it inhibits transcription *in vitro*, since the inhibition was obtained using either supercoiled or linear DNA templates. In addition, topoisomerase II does not relax supercoiled DNA during the preincubation period of the transcription reactions, in the absence of ATP and magnesium ions (our unpublished data).

The exact distribution of topoisomerase II along naked DNA is not known. However, it has previously been suggested that one topoisomerase II dimer interacts with 200 bp of DNA (42). In the experiments described in this study, the amounts of topoisomerase II required to inhibit basal transcription by more than 90% (1 unit) represented approximately a one to fifty ratio between the DNA template and topoisomerase II, resulting in approximately one molecule of topoisomerase II per 80–100 bp of DNA. This concentration should be sufficient to generate a continuous topoisomerase II distribution along the naked DNA templates used in the *in vitro* transcription assay. Under these conditions, essentially no basal transcription could be observed showing that preinitiation complexes cannot form on promoters which are covered with topoisomerase II. The DNA template/topoisomerase II complexes can be pelleted by centrifugation indicating that they form insoluble protein/DNA aggregates (our unpublished data). However, the formation of these aggregates is not irreversible since the addition of a 10 fold excess of non-specific plasmid DNA to the promoter containing DNA templates can restore transcription. Hence, there must exist a dynamic exchange of the topoisomerase II molecules when free DNA templates are available. The exchange of topoisomerase II from the promoter containing plasmids to the BSM allows access of the transcription factors to the promoter regions and preinitiation complex formation, and transcription can subsequently take place.

Topoisomerase II inhibits basal transcription when added to the DNA templates either prior to or at the same time as the basal transcription factors. In contrast, a much diminished transcriptional inhibition was observed when preinitiation complexes were preformed on the promoters of the DNA templates, prior to the addition of topoisomerase II. These results indicate that the binding of topoisomerase II to the promoter regions prevents the formation of preinitiation complexes, but that once formed the preinitiation complexes cannot be displaced by topoisomerase II. These results also indicate that topoisomerase II does not prevent the transition from the preinitiation to elongation phase. Nevertheless, irrespective of the order of addition, at high topoisomerase II concentrations, the DNA templates can be pelleted by centrifugation indicating that they are in the form of insoluble aggregates. RNA transcripts of the expected length were detected, even in the presence of 1 unit of topoisomerase II which is sufficient to cover the entire DNA template (with the exception of the promoter regions containing preinitiation complexes). These results indicate that the transcribing RNA polymerase II can displace topoisomerase II from the DNA, and that transcription can proceed on DNA templates which are aggregated by topoisomerase II. Thus, topoisomerase II-mediated transcriptional inhibition is not due to the formation of insoluble protein/DNA aggregates *per se*, but is the consequence of topoisomerase II binding to the promoter region preventing preinitiation complex formation.

Several transcriptional activators are able to counteract repression mediated by purified human or yeast topoisomerase II. All of the activator proteins tested counteracted the topoisomerase II-mediated inhibition (both human and yeast, Fig.3 and data not shown), irrespective of their activation and/or DNA binding domains, suggesting that antirepression by sequence specific activators *in vitro* is a general phenomenon. Furthermore, this transcriptional antirepression requires the presence of an activation function since GAL(1-147) alone could not efficiently counteract the topoisomerase II-mediated inhibition (Fig.3). GAL(1-147) has been reported to contain a weak activation function (47; and refs. therein), but this domain has no detectable activity in HeLa cell transfection experiments (51), and had only a weak effect on transcription *in vitro* (38; and see Fig.4). GAL-VP16 was able to counteract topoisomerase II-mediated inhibition when the plasmid templates were preincubated with 1 unit of topoisomerase II indicating that GAL-VP16 binding takes place even when its binding sites are covered with topoisomerase II. Thus, the activator has a stronger affinity for its binding sites than topoisomerase II for the DNA in general, and it can displace topoisomerase II from the promoter region where its binding sites are located. In contrast, under the same conditions, the basal transcription factors alone were not able to displace topoisomerase II from the AdMLP or β -globin promoters, as no basal transcription is observed when the transcription factors are added after topoisomerase II. Note that in the presence of the transactivator, insoluble template DNA/topoisomerase II aggregates are still formed (our unpublished data). Why does the binding of GAL-VP16 lead to antirepression? It is possible that, by binding to its cognate site and displacing topoisomerase II, the activator also displaces topoisomerase II from the adjacent TATA box and thus, preinitiation complexes can form. However, as antirepression requires the presence of an activation function the activator may also induce or stabilize a step in preinitiation complex formation which cannot otherwise occur on promoters covered with topoisomerase II. These two possibilities, however, are not mutually exclusive.

The mechanism by which topoisomerase II inhibits transcription initiation by RNA polymerase I from rDNA templates appears to be similar to that described above for RNA polymerase II. Moreover, UBF a transactivator of RNA polymerase I transcription was able to counteract the topoisomerase II-mediated inhibition suggesting that its mode of action *in vitro* is analogous to that of the transactivators of polymerase II transcription.

It is important to note that the present results demonstrating topoisomerase II-mediated transcriptional inhibition and antirepression by sequence-specific activators are analogous to those obtained with histone H1 (23, 24). Recent *in vitro* results indicated that nucleosomes could be destabilized by the binding of an activator to the DNA and that this destabilization did not require an activation function (63). Antirepression, however, cannot be achieved without an activation function, and requires auxiliary factors not required for basal transcription (23, 26, 27; and this study). Nevertheless, in addition to antirepression, transactivators also interact with the basal transcription machinery to activate transcription from naked DNA templates. In these interactions intermediary factors or coactivators, some of which are associated with TBP, seem to play a critical role (7, 38; and refs. therein). Thus, one should bear in mind that transcription extracts, prepared in different ways from various cell lines or tissues, may contain not only topoisomerase II and histones, but possibly other similar inhibitory activities. Therefore

transcriptional activation in these extracts may result from both antirepression and transcriptional stimulation. Consequently, at present it seems that transcriptional activation *in vivo* can be ascribed to two different, but not mutually exclusive, molecular mechanisms.

What is the physiological significance of topoisomerase II-mediated repression of transcription observed *in vitro*?

If the topoisomerase II-mediated transcriptional inhibition *in vitro* is due to the binding of topoisomerase II directly over the promoter sequences then it is unlikely that this reflects a general function of topoisomerase II *in vivo*. However, it cannot be excluded that such a situation may be found in cells at a limited set of promoters where topoisomerase II molecules are transiently binding and that this would be of physiological significance. It should also be noted that *in vitro* a high topoisomerase II/DNA ratio is required to efficiently inhibit transcription from naked DNA templates. *In vivo*, however, few if any regions of naked DNA exist, thus a lower topoisomerase II/DNA ratio may be sufficient to inhibit transcription, particularly if the promoter DNA regions are closely associated with the nuclear scaffold. Nevertheless, if topoisomerase II represses transcription *in vivo* through the condensation of the chromatin structure then it is possible that the displacement of topoisomerase II by a transcriptional activator, as observed *in vitro*, may lead to a local decondensation of the chromatin, thus facilitating the formation of preinitiation complexes.

It is intriguing to note that histone H1 and other non-histone components of the chromatin structure, like topoisomerase II or HMG 1, all bind DNA non-specifically and inhibit transcription *in vitro* (14, 15, 17, 23, 24) and, thus, they may contribute to some (possibly limited) extent in the general repression effect of chromatin on transcription *in vivo*. At present the only evidence linking topoisomerase II with transcriptional repression *in vivo* comes from the studies of Schultz et al. (49) who observed an increase in rRNA transcription when topoisomerase II was inactivated. Thus, topoisomerase II may also repress RNA polymerase I transcription in yeast *in vivo* as suggested by the present *in vitro* results. Nevertheless, it is possible that the repression of transcription *in vitro* by non-specific DNA binding proteins is an artefact which has no physiological significance. In the future genetic studies in yeast will be necessary to elucidate the precise function of topoisomerase II and other non-histone DNA binding proteins in regulating transcription and chromatin structure.

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REFERENCES

1. Roeder, R.G. (1991) *TIPS* **16**, 402–408.
2. Sawadogo, M., and Sentenac A. (1990) *Annu. Rev. Biochem.* **59**, 711–54.
3. Zawel, L., and Reinberg D. (1992) *Current Opinion Cell Biol.* **4**, 488–495.
4. Gill, G. and Tjian R. (1992) *Curr. Opin. Genet. Dev.* **2**, 236–242.
5. Pugh, B.F., and Tjian R. (1992) *J. Biol. Chem.* **267**, 679–682.
6. Sharp, P.A. (1992) *Cell* **68**, 819–821.
7. Brou, C., Chaudhary S., Davidson I., Lutz Y., Wu J., Egly J.M., Tora L., and Chambon P. (1993) *EMBO J.* **12**, 489–499.
8. Gill, G. (1992) *Current Biology* **2**, 565–567.
9. Rigby, P.W.J. (1993) *Cell* **72**, 7–10.
10. White, R.J., and Jackson S.P. (1992) *TIG* **8**, 284–288.
11. Hahn, S. (1992) *Current Biol.* **2**, 152–154.
12. Inostroza, J.A., Mermelstein F.H., Ha I., Lane W.S., and Reinberg D. (1992) *Cell* **70**, 477–489.
13. Mitchel, P.J., and Tjian R. (1989) *Science* **245**, 371–378.
14. Fedor, M.J. (1992) *Current Biology* **4**, 436–443.
15. Felsenfeld, G. (1992) *Nature (London)* **355**, 219–224.
16. Grunstein, M. (1992) *Scientific American*, october 40–47.
17. Wolffe, A.P. (1992) *FASEB J.* **6**, 3354–3361.
18. Lorch, Y., LaPointe J.W., and Kornberg R.D. (1987) *Cell* **49**, 208–210.
19. Han, M., and Grunstein M. (1988) *Cell* **55**, 1137–1145.
20. Bresnick, E.H., Bustin M., Marsaud V., Richard-Foy H., and Hager G.L. (1992) *Nucl. Acids Res.* **20**, 273–278.
21. Nacheva, G.A., Guschin D.Y., Preobrzhsenskaya O.V., Karpov V.L., Elbradise K.K., and Mirzabekov A.D. (1989) *Cell* **58**, 27–36.
22. Postnikov, Y.V., Shick V.V., Belyavsky A.V., Khrapko K.R., Brodolin K.L., Nikolskaya T.A., and Mirzabekov A.D. (1991) *Nucl. Acids Res.* **19**, 717–725.
23. Croston, G.E., Kerrigan L.A., Lira L.M., Marshak D.R., and Kadonaga J.T. (1991) *Science* **251**, 643–649.
24. Croston, G.E., Laybourn P.J., Paranjape S.M., and Kadonaga J.T. (1992) *Genes & Dev.* **6**, 2270–2281.
25. Laybourn, P.J., and Kadonaga J.T. (1991) *Science* **254**, 238–245.
26. Workman, J.L., Taylor I.C.A., and Kingston R.E. (1991) *Cell* **64**, 533–544.
27. Lorch, Y., LaPointe J.W., and Kornberg R.D. (1992) *Genes & Dev.* **6**, 2282–2287.
28. Meisterernst, M., and Roeder R.G. (1991) *Cell* **67**, 557–567.
29. Meisterernst, M., Roy A.L., Lieu H.M., and Roeder R.G. (1991) *Cell* **66**, 981–993.
30. Lindsley, J.E., and Wang J.C. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10485–10489.
31. Worland, S.T., and Wang J.C. (1989) *J. Biol. Chem.* **264**, 4412–4416.
32. White, J.H., Brou C., Wu J., Burton N., Egly J.M., and Chambon P. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7674–7678.
33. Wasyluk, C., and Wasyluk B. (1986) *EMBO J.* **5**, 553–560.
34. Ponglikitmongkol, M., White J.H., and Chambon P. (1990) *EMBO J.* **9**, 2221–2231.
35. Tora, L., White J.H., Brou C., Tasset D., Webster N., Scheer E., and Chambon P. (1989) *Cell* **59**, 477–487.
36. Schnapp, A., and Grummt I. (1991) *J. Biol. Chem.* **266**, 24588–24595.
37. Kuhn, A., and Grummt I. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 7340–7344.
38. Brou, C., Wu J., Ali S., Scheer E., Lang C., Davidson I., Chambon P., and Tora L. (1993) *Nucl. Acids Res.* **21**, 5–12.
39. Xiao, J. H., Davidson I., Matthes H., Garnier J.M., and Chambon P. (1991) *Cell* **65**, 551–568.
40. Croston, G.E., Lira L.M., and Kadonaga J.T. (1991) *Protein Exp. and Purif.* **2**, 162–169.
41. Tsai-Pflugfelder, M., Liu L.F., Liu, A.A., Tewey K.M., Whang-Peng J., Knutsen T., Huebner K., Croce C.M., and Wang J.C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7177–7181.
42. Adachi, Y., Käs E., and Laemmli U.K. (1989) *EMBO J.* **8**, 3997–4006.
43. White, J.H., Brou C., Wu J., Lutz Y., Moncollin V., and Chambon P. (1992) *EMBO J.* **6**, 2229–2240.
44. Tora, L., Mullick A., Metzger D., Ponglikitmongkol M., Park I., and Chambon P. (1989) *EMBO J.* **8**, 1981–1986.
45. Wang, J.C. (1985) *Annu. Rev. Biochem.* **54**, 665–697.
46. Wang, J.C. (1991) *J. Biol. Chem.* **266**, 6659–6662.
47. Marmorstein, R., Carey M., Ptashne M., Harrison S.C. (1992) *Nature* **356**, 408–414.
48. Workman, J.L., and Kingston R.E. (1992) *Science* **258**, 1780–1784.
49. Schultz, M.C., Brill S.J., Ju Q., Sternglanz R., and Reeder R.H. (1992) *Genes & Dev.* **6**, 1332–1341.