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Interaction of PC4 with melted DNA inhibits transcription

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PC4 is a nuclear DNA-binding protein that stimulates activator-dependent class II gene transcription *in vitro*. Recent biochemical and X-ray analyses have revealed a unique structure within the C-terminal domain of PC4 that binds tightly to unpaired double-stranded (ds)DNA. The cellular function of this evolutionarily conserved dimeric DNA-binding fold is unknown. Here we demonstrate that PC4 represses transcription through this motif. Interaction with melted promoters is not required for activator-dependent transcription *in vitro*. The inhibitory activity is attenuated on bona fide promoters by (i) transcription factor TFIIH and (ii) phosphorylation of PC4. PC4 remains a potent inhibitor of transcription in regions containing unpaired ds DNA, in single-stranded DNA that can fold into two antiparallel strands, and on DNA ends. Our observations are consistent with a novel inhibitory function of PC4.

Keywords: cofactors/DNA-binding proteins/general transcription factors/transcriptional regulation/transcriptional repression

Introduction

PC4 was originally isolated from a mammalian cofactor activity, termed upstream-factor stimulatory activity (USA), that stimulates activator-dependent transcription by RNA polymerase II in reconstituted class II gene transcription systems (Meisterernst *et al*., 1991; Ge and Roeder, 1994a; Kretzschmar *et al*., 1994a; Kim and Maniatis, 1997). Cloning of the corresponding cDNA and molecular characterization of PC4 revealed contacts to both general factors and activators (Ge and Roeder, 1994a). A mechanistic study supported the hypothesis that PC4 facilitates binding of the TFIID complex to promoters (Kaiser *et al*., 1995). Thus, PC4 is one member of an expanding list of eukaryotic co-activators which appear to bridge physically and functionally between activators and the class II gene machinery, and which help to recruit general factors to core promoters. Other examples include the factors associated with the TATA box-binding protein, TBP (TAFs, Dynlacht *et al*., 1991; Burley and Roeder, 1996) and RNA polymerase II mediator/SRB subunits (Kim *et al*., 1994; Koleske and Young, 1994).

Several of the factors that stimulate activator-dependent transcription *in vitro* are DNA-binding proteins. Examples are members of the positive cofactors (termed PCs, reviewed in Kaiser and Meisterernst, 1996) topoisomerase I/PC3 (Kretzschmar *et al*., 1993; Merino *et al*., 1993; Shykind *et al*., 1997), topisomerase II (Brou *et al*., 1993), poly-ADP-ribose-polymerase (PARP, PC1), PC4 (Kaiser *et al*., 1995) and HMG proteins (Ge and Roeder, 1994b; Stelzer *et al*., 1994; Shykind *et al*., 1995; Zwilling *et al*., 1995; Zappavigna *et al*., 1996; Jayaraman *et al*., 1998). Despite this functional relationship, there is no general molecular model for the role of DNA binding in co-activation. For example, it has been suggested that HMG proteins support sequence-specific DNA-binding of activators, such as the tumour suppressor p53, to their promoter-proximal target sites (Jayaraman *et al*., 1998 and references therein). In the case of PC4, a related mechanism has never been reported. PC4 binds double-stranded (ds) DNA in a sequence-independent manner. The regions required for dsDNA binding and for co-activation overlap in PC4. However, the cofactor also requires interaction with preinitiation complexes (Ge and Roeder, 1994a; Kaiser *et al*., 1995), arguing for the relevance of DNA contacts, but arguing against a model in which DNA binding alone suffices for co-activation.

Recent investigations revealed another unique DNA-binding property of PC4. The cofactor binds tightly to melted dsDNA and single-stranded (ss) DNA that can fold into two antiparallel strands, respectively (Werten *et al*., 1998). X-ray analysis of crystals identified a novel fold located within the C-terminal domain of PC4 (PC4–CTD), spanning amino acids (aa) 63–127 (Brandsen *et al*., 1997). The dimeric fold provides an intriguing binding surface for two antiparallel ssDNA strands (Brandsen *et al*., 1997). This interaction surface is also suggested by comparisons with the replication protein A ssDNA co-crystal structure (Bochkarev *et al*., 1997). The affinity for ssDNA that can fold into two antiparallel strands is indeed very high, exceeding that for dsDNA at least 100 times (Werten *et al*., 1998). High affinity for melted DNA suggests a critical role of this fold in the cellular function of PC4, the latter being at present unknown.

Here, we have studied the role of PC4–CTD in transcription. Point mutants in PC4–CTD were constructed that eliminated binding to melted DNA, thereby providing strong evidence for the predicted DNA-binding interface. Surprisingly, contacts with open promoters are not required for co-activator function *in vitro*. In fact, PC4 represses transcription via binding to melted promoters, and this is

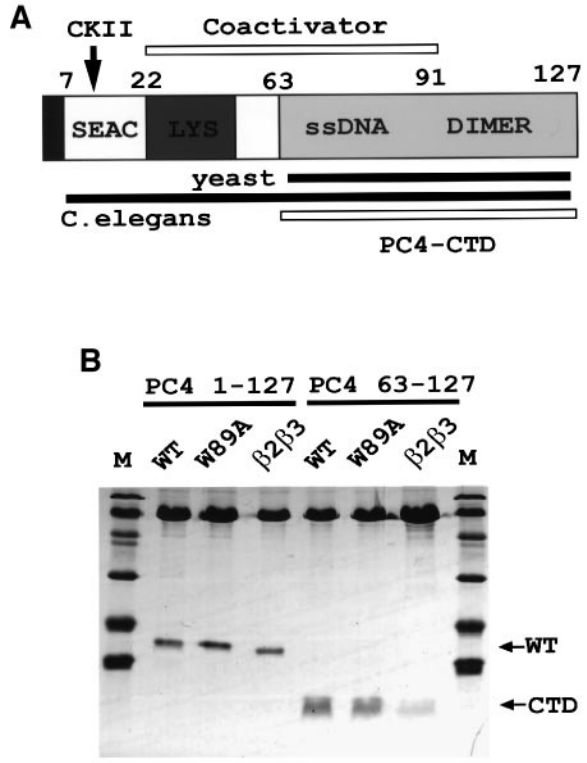

Fig. 1. (**A**) Schematic representation of PC4. Minimal co-activator region (coactivator), SEAC (serine-acidic) regions containing the major CKII phosphorylation sites (CKII), lysine-rich N-terminal (LYS), the ssDNA-binding and dimerization region in the C-terminal domain (PC4–CTD) and to yeast and *Caenorhabditis elegans* conserved regions are indicated. (**B**) Coomassie Blue-stained SDS gel of purified PC4 (WT) and mutants W89A, and β2–β3 in the full-length context, PC4 1–127 (arrow WT) and in PC4 63–127 (arrow CTD). M lanes contained the BioRad low-molecular-weight marker proteins (14–96 kDa range). The major band on top of the gel and minor bands above originate from BSA added exogenously in order to stabilize proteins.

antagonized by TFIIH. PC4 was further shown to be a very potent repressor of transcription on specific DNA structures such as ssDNA, DNA ends and heteroduplex DNA. Related structures serve as effective initiation sites for RNA polymerase II. Evolutionary conservation of PC4–CTD as well as quantitative considerations suggests that inhibition of RNA polymerase II activity in these non-promoter regions could be important.

Results

PC4 consists of an N-terminal region (aa 7–22), rich in serines and acidic residues, that is phosphorylated by Casein Kinase II (CKII) *in vitro* (Ge *et al*., 1994; Kretzschmar *et al*., 1994a). This so-called SEAC region precedes a lysine-rich motif (aa 23–41) that together with C-terminal regions (including aa 91) are sufficient for co-activator function (Figure 1A, Kaiser *et al*., 1995). Crystallographic studies of PC4–CTD (aa 63–127, Brandsen *et al*., 1997) demonstrated that PC4 dimerizes through PC4–CTD (Figure 2A and B). This was also suggested by yeast two-hybrid screens using PC4 as a bait, which yielded many independent PC4 clones, all of which contained PC4–CTD (data not shown). The PC4–CTD dimer contains a β-ridge region flanked by two channels, reminiscent of quarter pipes, that provide a putative surface for two antiparallel ssDNA strands (Brandsen *et al*., 1997). The global structure of the PC4–CTD dimer is unique in that it resembles a plough (Figure 2A). In agreement with this picture, PC4 binds to melted DNA and is able to unwind DNA at high concentrations in an ATP-independent process (Werten *et al*., 1998).

Construction of PC4 mutants affected in ssDNA binding

In order to prove the DNA-binding hypothesis, we designed point mutants that would specifically affect binding to ssDNA. From superposition of the PC4 anti-parallel channels onto the ssDNA-binding channels found in the RPA–ssDNA co-crystal structure, it was expected that both Trp89 and the β2–β3-loop (connecting β-strands 2 and 3) would be particularly important for the interaction of PC4–CTD with ssDNA (see Figure 2). Trp89, which is located in the β-ridge separating the two antiparallel channels in PC4, according to the superposition by Brandsen *et al*. (1997) corresponds to Phe238 in the A and Trp361 in the B subregion of RPA, both of which residues interact with ssDNA in the co-crystals. Loop β2–β3 of PC4 is a typical ssDNA-binding loop found in several other SSBs, reminiscent of the L45-loop of OB-fold proteins (Murzin, 1993). It contains two positive charges (Lys78 and Lys80) and an aromatic residue (Phe77). In the two RPA subunits, the corresponding strands and the connecting loop β4′–β5′ are seen to bind ssDNA through stacking of the aromatic residue Phe269 (A) or Phe386 (B) onto a DNA base, as well as through interactions of the positively charged residue Lys263 (A) or Arg382 (B) with the phosphate backbone. The importance of both Trp89 and the β2–β3 loop of PC4 for ssDNA binding was further supported by NMR experiments, as all residues concerned showed large amide resonance changes in the HSQC spectrum upon addition of a single-stranded oligonucleotide (S.Werten, manuscript in preparation). Hence, we constructed a PC4 mutant in which Trp89 was replaced by Ala (W89A) and another (triple) mutant, in which both of the Lys residues and the Phe residue of the β2–β3-loop were replaced by Gly and Ala, respectively (F77A/K78G/K80G, henceforth referred to as β2–β3). W89A and β2–β3 alterations were introduced into the isolated PC4–CTD as well as the full-length PC4. These mutants were expressed in and purified from *Escherichia coli* to apparent homogeneity (Figure 1B) and subsequently analysed in DNA-binding and in transcription.

Electrophoretic mobility shift assay (EMSA) with an oligo-dT20 probe (Figure 3A) showed that both PC4–CTD(W89A) and PC4–CTD (β2–β3) are severely affected in binding to ssDNA. No significant binding to oligo-dT20 is observed at any of the protein concentrations tested (up to 500 ng per 20 μl reaction), whereas as little as 0.5 ng of the wild-type PC4–CTD shifts more than 50% of the probe in this experiment. Thus, the equilibrium dissociation constant (K_d) is increased by more than two orders of magnitude in the case of the dT20 oligonucleotide, from 0.07 nM (Werten *et al*., 1998) to at least 50 nM in the mutants. It has been shown in earlier work (Werten *et al*., 1998) that optimal binding of PC4–CTD to ssDNA requires a 16–20-nucleotide-binding site, that presumably bends by 180° in the middle so as to form

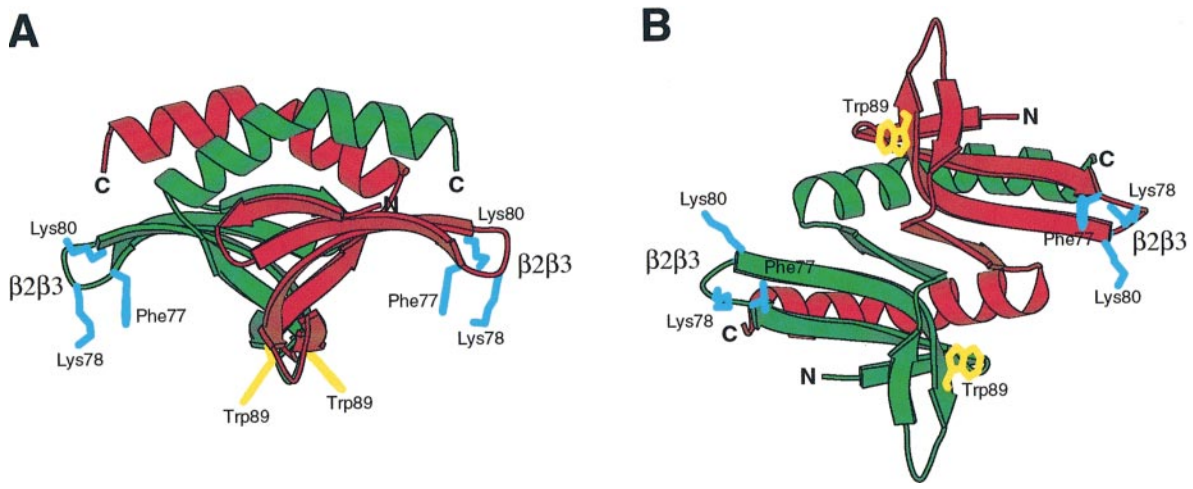

Fig. 2. Position of mutations in the dimeric PC4-fold including aa 63–127 (PC4–CTD region) in two different perspectives. (**A**) and (**B**) The two chains of PC4 dimers are shown in green and red, respectively. Trp89 in the β-ridge region was changed to Ala (W89A), shown in yellow, and Phe77, Lys78 and Lys80 in the loop between the corresponding β-strands to Ala, Gly and Gly, respectively (β2–β3), displayed in blue.

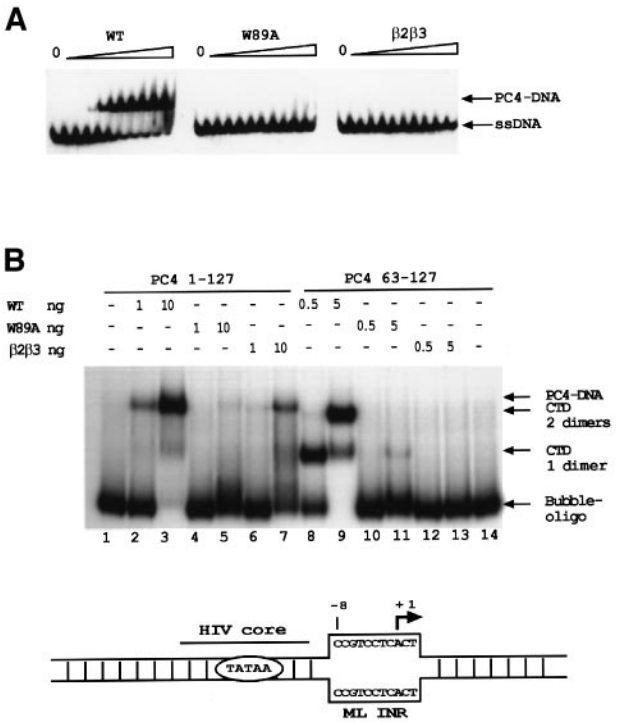

Fig. 3. Analysis of PC4 mutants during DNA-binding. (**A**) Binding of PC4–CTD and mutants in PC4–CTD, as indicated, to labeled oligo-dT 20-mer (ssDNA). Each panel contains, starting from the left corner, 0, 0.002, 0.008, 0.03, 0.12, 0.49, 2.0, 7.8, 31, 125 and 500 ng of expressed PC4–CTD-derivatives. (**B**) Binding of full-length and CTD derivatives in the indicated amounts to a 51-bp promoter fragment containing an unpaired region of 11 bp (bubble oligonucleotide), as indicated below and described in detail in Materials and methods. The positions of the dimeric PC4–DNA and a complex containing two copies of PC4 dimers bound to the oligonucleotide are indicated.

two antiparallel regions that can simultaneously occupy both of the ssDNA-binding channels of the protein.

Hence, we tested double-stranded core promoter oligonucleotides that contained unpaired bases surrounding the start site of transcription (positions –8 to +2) flanked by double-stranded regions. The bubble oligonucleotide was efficiently bound by PC4 and PC4–CTD (Figure 3B) with PC4–CTD displaying moderately higher affinity and forming a double instead of a single complex with DNA (lanes 8 and 9). Mutant W89A interacted far less efficiently with the bubble oligonucleotide, both in PC4–CTD (lanes 11 and 12) and in the full-length context (lanes 4 and 5). β2–β3 could not recognize bubble oligonucleotide in the PC4–CTD context, while DNA-binding was impaired but not fully eliminated in the presence of the N-terminal region of PC4 (lanes 6 and 7, and 12 and 13). In summary, both mutants are severely impaired when bound to melted dsDNA.

Binding of PC4 to open promoters is dispensable for transcriptional activation

Given that PC4 has been characterized as a transcription cofactor both in man (Ge and Roeder, 1994a; Kretzschmar *et al*., 1994a; Orphanides *et al*., 1998) and in the yeast *Saccharomyces cerevisiae* (Henry *et al*., 1996; Knaus *et al*., 1996), the question arises as to which role the evolutionarily highly conserved PC4–CTD plays in transcription. PC4–CTD, lacking the N-terminal lysine-rich region, is insufficient for co-activation (Kaiser *et al*., 1995, cf. Figure 1A). Hence, full-length PC4 derivatives were tested for activation of an HIV promoter by GAL4-Sp1 fusion proteins in a purified class II gene transcription system (Figure 4). Both mutants mediated activation of Gal4–Sp1 moderately better than did wild-type PC4. Thus, interaction with unpaired DNA during opening of the promoter is not required for co-activator function *in vitro*.

PC4 represses transcription through CTD, which is alleviated by TFIIH

PC4 derivatives were also tested in minimal transcription systems containing supercoiled templates, recombinant TBP, TFIIB, TFIIEα, TFIIEβ, RAP30 and RAP74, as well as purified RNA polymerase II, but lacking TFIIH. In these minimal systems PC4 repressed transcription at concentrations comparable to or below those required for trans-activation (Figure 5A, lanes 1–4). PC4–CTD also inhibited transcription, although less efficiently, requiring ~4-fold higher concentrations. Importantly, W89A (Figure 5A, lanes 9–12 and Figure 5B, lanes 4 and 5) as well as β2–β3 (Figure 5B, lanes 6 and 7) lost the ability to repress

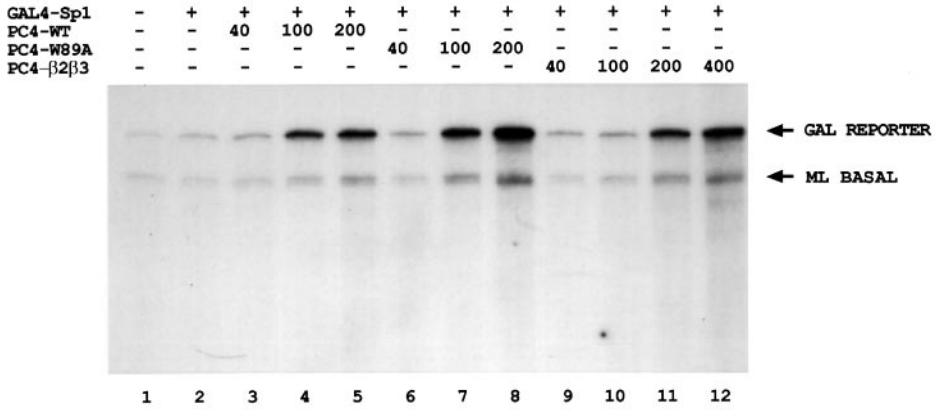

Fig. 4. Analysis of PC4 mutants in transcriptional activation. PC4 wt and mutants in the full-length context and in the indicated amounts (ng) were added to a complete purified transcription system, also containing TFIID and TFIIH complexes, in the presence of recombinant purified GAL4-Sp1 (if indicated). GAL4-Sp1 does not activate in the absence of PC4 (lane 1 versus lane 2). Approximately 100 ng of wild-type PC4 saturate transcriptional activation (lane 4), whereas levels of activated transcription [GAL-reporter corresponds to the vector pMRG5 (Kaiser *et al*., 1995)] are further stimulated at higher concentrations of mutants (lanes 8 and 12), probably owing to reduced repression activity through binding to open promoters.

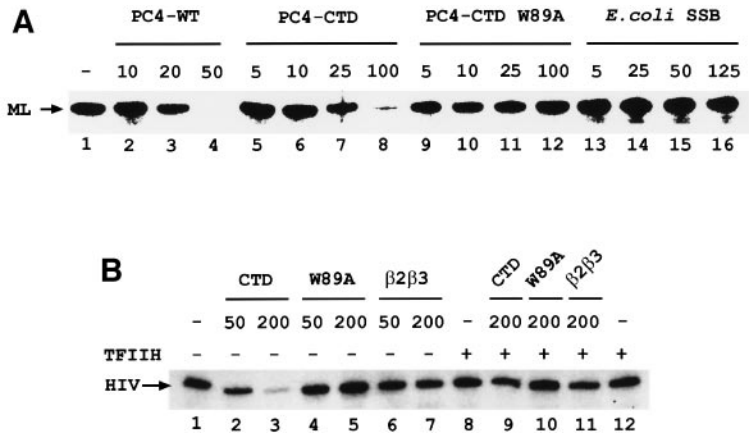

Fig. 5. PC4 represses transcription in the absence of TFIIH. (**A**) Effects on transcription from the adenovirus major late promoter (ML) by the indicated amounts of full-length PC4 (PC4-wt), PC4 63–127 (PC4–CTD), W89A mutant in PC4–CTD and recombinant *E.coli* SSB in minimal systems, lacking TFIIH and containing TBP instead of TFIID and supercoiled template. (**B**) Repression by PC4–CTD and mutants in PC4–CTD on the HIV promoter (pMRG5) and antirepression by TFIIH. Numbers refer to ng of PC4-derivatives in 20 μl transcription reactions.

transcription (lanes 9–12). *Escherichia coli* SSB had no effect on transcription at comparable concentrations, indicating that these effects are PC4 specific (Figure 5A, lanes 13–16). Mutants in full-length PC4 behaved similarly, provided that DNA was supplied in excess (data not shown).

Repression by PC4–CTD is alleviated if TFIIH is included in otherwise identical (TBP-containing) transcription reactions (Figure 5B, lane 3 versus lanes 8 and 9). This phenomenon explains the lack of repression in systems containing TFIIH (Figure 4) and has been one reason for the addition of TFIIH to transcription reactions in former analysis of PC4 activity. Taken together, repression of transcription and binding to melted DNA appear to be correlated.

A second mode of repression through interactions with dsDNA

High concentrations of PC4 inhibit transcription even in the presence of TFIIH in TBP- as well as in TFIID-containing systems (Figure 6A, lanes 2–4 versus 9–11). This second mode of repression could result from non-specific interactions with dsDNA leading to competition with General Transcription Factors (GTFs), based on the following arguments: repression is not seen with PC4–CTD, but requires the lysine-rich N-terminal regions of PC4 that were shown earlier to enhance interaction with dsDNA. PC4 binds dsDNA, although with much lower affinity than bubble DNA, as has been shown in earlier work (Kaiser *et al*., 1995; Werten *et al*., 1998) and is again demonstrated in a competition experiment in Figure 6B. Efficient competition of PC4–bubble-DNA complexes required a more than 100-fold excess of dsDNA. Finally, raising template concentrations eliminates the second but not the first pathway (data not shown). At low DNA concentrations both modes will operate in parallel.

Phosphorylated PC4 inhibits solely via binding to melted DNA

PC4 is mostly phosphorylated in logarithmically growing mammalian cells (Figure 6C). CKII introduces up to seven phosphate groups into the N-terminal SEAC motif, which leads to marked mobility changes in SDS gels (Ge *et al*., 1994; Kretzschmar *et al*., 1994a; see also Figure 6C). We

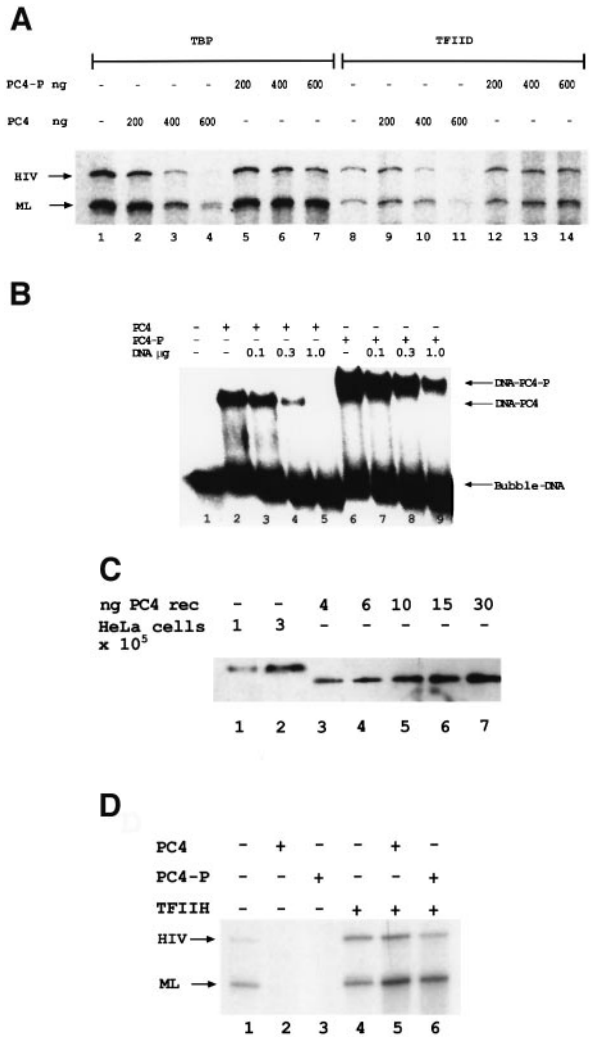

Fig. 6. (**A**) Repression in the presence of TFIIH. Inhibition is attenuated by phosphorylation of PC4 with CKII (PC4-P). Transcription reactions contained either TBP or TFIID, demonstrating that TAFs do not significantly influence repression by PC4 under our conditions (cf. Malik *et al*., 1998). (**B**) Comparison of PC4 and PC4-P in DNA-binding. Conditions and bubble oligonucleotide are comparable to the experiment shown in Figure 3B. Plasmid pMRG5 was added as a competitor in the indicated amounts to reactions containing 10 ng PC4-derivatives and 50 fmol (1.7 ng) of labelled promoter bubble-oligonucleotide. (**C**) Quantitation of PC4 in HeLa nuclei. Recombinant PC4 the amounts indicated was analysed together with SDS lysates of HeLa nuclei (isolated in a standard NP-40 protocol) in Western blots with polyclonal antibodies against PC4. Note the reduced mobility of cellular PC4 which results from phosphorylation, as has been shown previously (Kretzschmar *et al*., 1994a). (**D**) Effects of phosphorylation by CKII on repression by PC4 and antirepression by TFIIH. Reactions were conducted with 50 ng of pMRG5 and pMLΔ53 templates, recombinant TBP, TFIIB, TFIIE, RAP30, RAP74, purified RNA polymerase II and 400 ng of PC4 and PC4-P (cf. Figure 5).

have tested the effects of phosphorylation by CKII on transcriptional repression. In the absence of TFIIH, phosphorylated PC4 (termed PC4-P) represses transcription equally well as non-phosphorylated PC4 (Figure 6D). Again, PC4-P effects on transcription are fully reversed by TFIIH. In contrast, CKII relieves repression at high PC4 concentrations and in the presence of TFIIH (Figure 6A). PC4-P binds the bubble oligonucleotide with high affinity (Figure 6B). PC4-P/bubble-oligonucleotide complexes are less well competed by dsDNA (Figure 6B) and PC4-P shifts circular plasmids less efficiently in agarose gels (data not shown), arguing for reduced affinity and/or stability of dsDNA–PC4-P complexes.

The concentration of PC4 in HeLa cell nuclei is estimated from Western blots to be ~1 μM (Figure 6C). These concentrations would suffice for co-activation (Figure 4) as well as repression in both the absence and presence of TFIIH (Figures 5 and 6). However, phosphorylated PC4, PC4-P, barely represses transcription, even at 2 μM concentrations in the presence of TFIIH (Figure 6A). Thus, PC4 will probably generally not repress transcription from bona fide promoters via non-specific dsDNA-binding in mammalian cells, provided that we do not underestimate PC4 concentrations and do not fail to consider possible local fluctuations that may have an affect in specific situations.

Mechanistic characterization of relief of repression by TFIIH

The general transcription factor TFIIH supports unwinding of class II gene promoters via its intrinsic helicase subunit ERRC3 (Schaeffer *et al*., 1993; Stelzer *et al*., 1994; Timmers, 1994). We reasoned that PC4 effects functionally relate to competition with TFIIH on open promoters (Wang *et al*., 1992 and references therein; Tantin and Carey, 1994). Experiments were designed to identify the molecular event during initiation complex formation that is subject to repression. We made use of templates that can be elongated to either of positions +2 or +5 in the absence of UTP and GTP because they contain the sequence ACT (ML) and ACCCAT (MLIn4) downstream of position –1 of the adenovirus major late promoter and upstream of G-free cassettes of 300 and 380 bp length, respectively. When we added PC4 together with GTFs, both promoters were repressed (Figure 7A, lane 1 versus lane 3). However, when PC4 was included together with UTP after formation of initiation complexes, MLIn4 was more resistant to PC4 (lane 5) and to PC4–CTD (lane 9) than was ML. Thus, RNA polymerase II that has transcribed through the first nucleotides becomes resistant to repression. Transcriptional repression by PC4 was not alleviated by TFIIH when ATP in the reaction was replaced by ATPγS (Figure 7B), the latter blocking preferentially ATP-dependent helicases but not protein kinases (Eckstein, 1985; Serizawa *et al*., 1993). In contrast, H8, an inhibitor of protein kinases that target the largest subunit of RNA polymerase II (references in Stelzer *et al*., 1994), does not interfere with TFIIH function (Figure 7C). Hence, relief of repression requires the helicase activity of TFIIH.

Heteroduplex DNA is targeted by RNA polymerase II, and this is efficiently antagonized by PC4

To analyse further the effects of PC4 on melted DNA, we used templates that contained unpaired heteroduplex regions between positions –4/+2 and –8/+2 of the adenovirus major late promoter. We had previously shown that PC4 binds with high affinity to melted regions larger than five bases (Werten *et al*., 1998). Non-template sequences were introduced into both strands, generating an unpaired bubble surrounding the initiation site of transcription, as described previously (Holstege *et al*.,

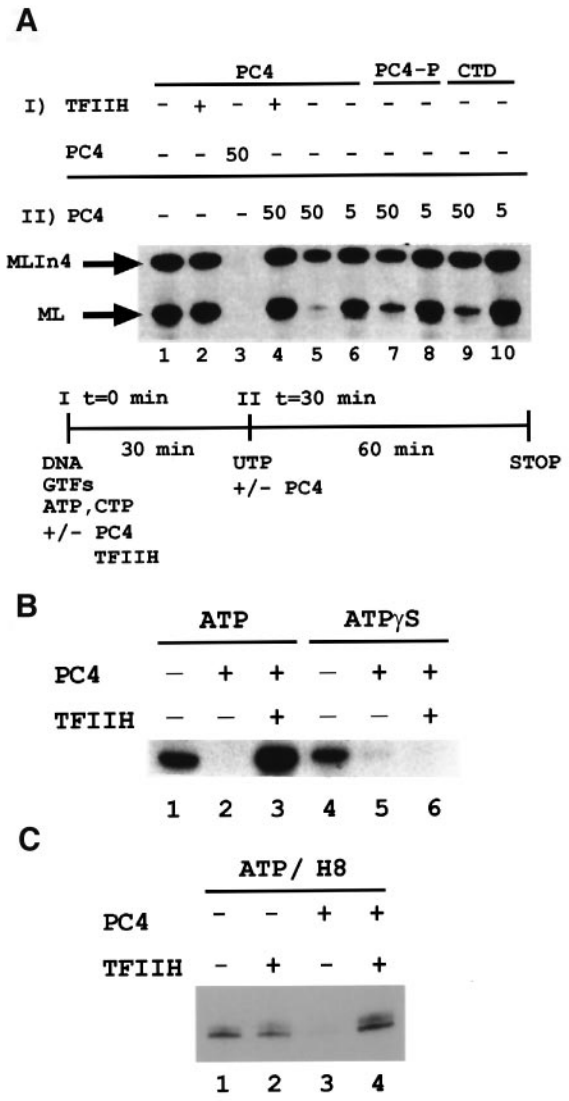

Fig. 7. PC4 represses early on in elongation. (**A**) Transcription templates MLIn4 or ML contain the sequences ACT and ACCCAT, with the first A being the initiation site. Reactions were conducted according to the scheme (below) with the indicated amounts of PC4, PC4–CTD (CTD) and phosphorylated PC4 (PC4-P), (I) before and (II) after a preincubation period (30 min) with GTFs, ATP and CTP. (**B**) Relief of repression by TFIIH is lost if ATP-γS is used instead of ATP in transcription reactions. (**C**) Relief of PC4-repression (200 ng) by TFIIH is maintained in the presence of H8 (1 μM), an inhibitor of the kinase of the largest subunit of RNA polymerase II, under standard conditions. H8 does not inhibit transcription in purified systems (our data not shown; Serizawa *et al*., 1993).

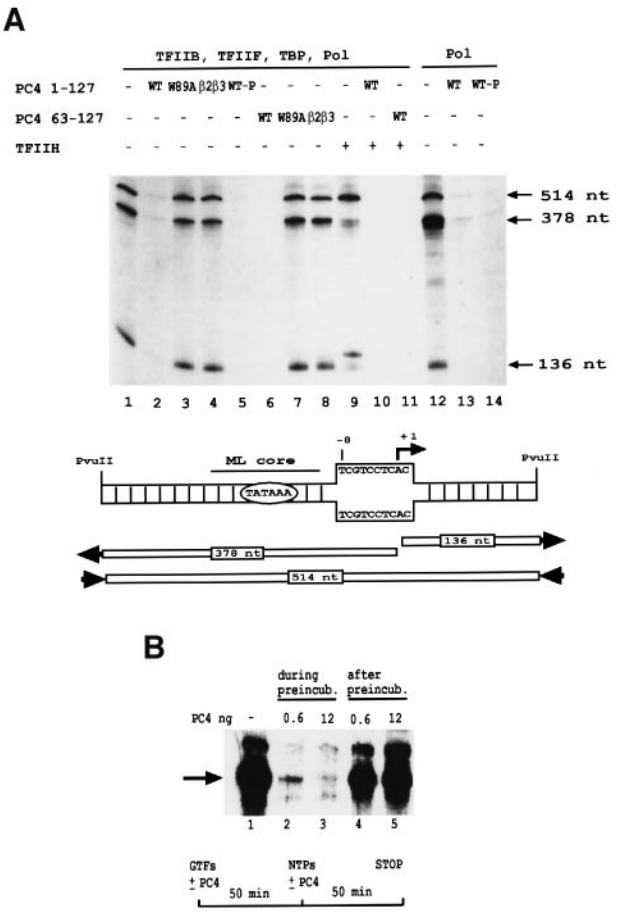

Fig. 8. Effects of PC4 on transcription from heteroduplex templates. (**A**) 514 bp DNA fragments contained a unpaired region between positions –8 to +2, which lead to a correct 136-nucleotide transcript and an antisense 387-nucleotide transcript, as well as a 514-nucleotide end-to-end transcript, as schematically indicated below. Reactions were conducted with minimal systems in the absence of TFIIE and TFIIH, or with RNA polymerase II alone (A, lanes 12–14). Note that GTFs were not required on the (–8 to +2) template, but also did not change PC4 effects if they were present. TFIIH has negative effects on transcription and generates multiple start sites for as yet unknown reasons (lane 9), but it cannot relieve PC4 effects (lanes 10 and 11). Mutations in PC4 (10 ng) abolish repression capacity, whereas CKII-phosphorylated-PC4 (wt-P) represses better than non-phosphorylated full-length PC4 (wt). (**B**) Repression of a minimal system on a (–4 to +2) bubble template. On this template, the GTFs TFIIB, TFIIF and TFIID, but not TFIIE and TFIIH, were used and were required for transcription. Preincubation of templates with GTFs and RNA polymerase II alleviates represson by PC4 (lanes 4 and 5).

1996). These templates can be targeted by GTFs and transcribed in the absence of TFIIE and TFIIH (–4/+2), or even by RNA polymerase II alone on the larger bubble (Figure 8A). RNA polymerase II transcribes the –8/+2 template in both directions. Both PC4 and PC4-P, but not the mutants, repressed transcription from bubble templates. We also noted that PC4 repressed end-to-end transcription on these linear promoter fragments (Figure 8A), probably because PC4 unwinds DNA ends, generating bubble-related binding sites (Werten *et al*., 1998). Repression of transcription is very efficient: >90% repression was seen at ~1 nM concentrations of dimeric PC4 (Figure 8B), at least 100 times lower than the levels that are necessary for co-activation and repression of homoduplex promoters, and ~1000 times lower than cellular concentrations. We noted that PC4–CTD binds to and represses more efficiently through premelted templates than does full-length PC4 (Figures 3 and 8), whereas bona fide promoters are less efficiently repressed by PC4–CTD (compare Figure 5A). This is consistent with differences between bubble structures generated during initiation of transcription and those introduced in the premelted templates. In contrast to the situation on bona fide promoters, TFIIH could not relieve repression either on –4/+2 or on –8/+2 heteroduplex templates, whereas preincubation of the template with TBP, TFIIB, TFIIF and RNA polymerase II blocked repression by PC4 (Figure 8B). These data suggest that PC4 is a very potent inhibitor of RNA polymerase II transcription in unpaired DNA regions.

Discussion

We have investigated the role of a unique DNA-binding fold contained in the C-terminal domain of PC4 that tightly interacts with melted DNA. The X-ray and biochemical characterization of the C-terminal 65 aa (PC4–CTD) of PC4 led to the hypothesis that PC4 binds unpaired melted DNA via the surface provided by a cluster of antiparallel β-strands that form two quarter-pipe-like structures (Brandsen *et al*., 1997; Werten *et al*., 1998). Although a detailed picture must await protein–DNA co-crystals, the analyses of point mutations in the putative DNA-binding surface both in DNA-binding and in transcription provide strong evidence that this model is generally correct. Surprisingly, this novel fold is a potent repressor of RNA polymerase II, whereas it is not important for transcriptional activation *in vitro*. Our study describes mechanisms by which the inhibitory activity of PC4 might be controlled on promoters. It also predicts novel functions of PC4 on defined DNA structures.

Relationship to other DNA-binding co-activators/ repressors

PC4 shares the ability to repress transcription at high concentrations with other DNA-binding co-activators such as HMG proteins and DNA topoisomerase I (Kretzschmar *et al*., 1993; Merino *et al*., 1993; Stelzer *et al*., 1994; Shykind *et al*., 1997). In some cases it has been suggested that repression is directly correlated with the ability to bind dsDNA, leading to competition with general transcription factors for core promoter regions (Kretzschmar *et al*., 1993; Meisterernst *et al*., 1997). PC4–dsDNA interactions could play a role during co-activation (Kaiser *et al*., 1995). It has also previously been noted that high concentrations of PC4 repress transcription (Malik *et al*., 1998 and references therein). PC4 is a very abundant protein that might well repress transcription via binding to dsDNA through direct competition with general factors in the presence of TFIIH, unless DNA is present in sufficient excess. However, at least 10-fold higher concentrations are needed for TFIIH-independent repression, compared with co-activation, while 1000-fold higher concentrations of PC4 are required to see repression on dsDNA as compared with heteroduplex DNA templates. Ubiquitous protein kinases such as CKII alleviate the dsDNA-repression mode. In sharp contrast, phosphorylated PC4 maintains its inhibitory potential on premelted templates. Other protein kinases may also modify PC4 and thereby differentially influence DNA interactions (Malik *et al*., 1998). We cannot fully exclude the possibility of high local concentrations of PC4, allowing the different modes to operate in specific situations. However, if concentrations do not greatly exceed the calculated values, the nuclear factor PC4 might not generally repress transcription from promoters in living cells. In any event, PC4 is predicted preferentially to inhibit transcription from unpaired pyrimidine-rich DNA regions.

Co-activation and repression are separate properties

PC4 has previously been characterized as a positive cofactor that stimulates activator-dependent transcription but has little effect on basal promoter activity (Ge and Roeder, 1994a; Kretzschmar *et al*., 1994a; Orphanides *et al*., 1998; reviewed in Kaiser and Meisterernst, 1996). The cofactor facilitates binding of TFIID early on in preinitiation complex formation, but it also stimulates activator function in an as yet unidentified later step (Kaiser *et al*., 1995). Lack of effects of mutants in the bubble-DNA-binding surface on co-activation argued clearly against the possibility that the affect after TFIIA–TFIID-complex formation is related to binding to open promoters and/or unwinding of template DNA. Instead, PC4–CTD inhibits transcription. This finding generates a paradox, given that PC4 can strongly enhance transcription in the presence of activators. The general initiation factor TFIIH solves this problem by means of antagonizing PC4 inhibitory effects early on in elongation. Antagonism of TFIIH is consistent with investigations by Roeder and colleagues that were performed when this work was in progress (Malik *et al*., 1998). Our data further show that PC4 represses and TFIIH antagonizes PC4 effects through open promoters before formation of the fourth phosphodiester bond by RNA polymerase II. Assuming that TFIIH is constitutively expressed and distributed, this reinforces the earlier observation that TFIIH can act as a general antirepressor (Stelzer *et al*., 1994).

It remains to be analysed whether the various mechanisms are relevant in yeast. Yeast contains a protein termed Sub1 or Tsp1 that is highly related to the human factor within PC4–CTD. Kornberg and colleagues suggested that yeast PC4 exerts positive effects on basal transcription in the presence of Mediator and TFIIH in yeast (Henry *et al*., 1996). In an other study, the yeast homologue was shown to facilitate activator function through release of TFIIB from preinitiation complexes (Knaus *et al*., 1996). Notably, the yeast homologue of PC4 is much larger than human PC4, which may result in functional differences. To our knowledge, the yeast factor has not been analysed on premelted templates. However, both yeast and human PC4 bind ss- and dsDNA (Henry *et al*., 1996), which, together with the strict conservation of PC4–CTD, indicates that Sub1/Tsp1 might perform as human PC4 in unpaired DNA regions. The extent to which the chromatin evironment and the presence of other accessory factors, as present for example in holo RNA polymerase (Kim *et al*., 1994; Koleske and Young, 1994), affect the various modes of PC4, both in yeast and mammals, is presently unknown and must await further investigations.

A novel role of PC4 as a transcriptional inhibitor on ssDNA

From the biological point of view a constitutive counterplay of TFIIH and PC4 does not satisfactorily explain the need for PC4–CTD. More puzzling is the previous finding that only part of PC4–CTD (including aa 91, Kretzschmar *et al*., 1994a) is necessary for co-activation, whereas the evolutionarily conserved region extends to the C-terminus of PC4. It is presently unknown whether the counterplay of TFIIH and PC4 is subject to regulation. In any event, one would predict a second evolutionarily conserved function of PC4 mediated by the conserved structure in PC4–CTD (the PC4-fold). Here we propose that this second function might be repression of transcription in non-promoter regions, although we cannot formally

exclude the possibility of other transcription-coupled or alternative functions of the PC4-fold. Our estimation of PC4 levels in cell nuclei strongly suggests that pyrimidine-rich ssDNA structures will be targeted in mammalian cells. This idea is further supported by the observation that the physiologically phosphorylated form of PC4 maintains the ssDNA-binding and transcriptional properties of PC4–CTD. Suitable DNA structures, unpaired DNA and DNA ends, may well occur during DNA replication or as a result of DNA damage (compare with Pan *et al*., 1996). Moreover, both PC4 and RNA polymerase II preferentially target pyrimidine-rich sequences, whereas both show significantly reduced binding and transcriptional activity on purine-rich bubble templates (unpublished observations), which further correlates the function of the PC4-fold to inhibition of transcription from non-promoter regions.

Materials and methods

Generation of mutant PC4 constructs

For the introduction of mutations in our PC4 and PC4–CTD expression vectors we used a modification of the recombinant PCR strategy described by Higuchi (1990), allowing us to generate two mutants in a single procedure. An antisense oligonucleotide that contained the desired base substitutions in the W89 codon (5′-TTCAGGATCCATCGCATATTCT-CTAAT-3′) was used in combination with a sense promoter primer to create an N-terminal W89A PCR product. A sense oligonucleotide containing the β2–β3-loop mutations (5′-TTAGTGTTCGCGATG-CTGGAGGCGGAGTGCTAATTGATA-3′) was used in combination with an antisense terminator primer to create a C-terminal PCR product containing β2–β3-loop mutations. A mixture of both overlapping PCR products was subsequently used as the template in an additional PCR reaction with promoter and terminater primers, leading to a mixture of full-length products containing either the W89A or the β2–β3-loop mutations. Resulting DNA fragments were cloned back into pET-11a via the *Nde*I and *Eco*RI sites. Mutant constructs were selected on the basis of restriction digests and verified by dideoxy-sequencing.

Protein purification

PC4–CTD and mutants were expressed in *E.coli* and purified as described previously (Werten *et al*., 1998), using heparin–Sepharose and S-Sepharose columns. The PC4–CTD β2–β3 protein, owing to the loss of two positive charges, did not bind to S-Sepharose and was present in the flow-through. However, since in the original purification scheme the majority of *E.coli* proteins that remained after passage through the heparin column remained bound to S–Sepharose at the salt concentration at which PC4–CTD eluted, this flow-through contained protein that like the wild-type PC4–CTD and PC4–CTD W89A proteins was essentially pure. Full-length PC4 and mutants were expressed in *E.coli* and purified using a heparin–Sepharose column followed by Superdex 200 gel filtration.

In vitro transcription reactions

Transcription templates comprising HIV and adenovirus major late core promoter regions (pMRG5 and pMLΔ53) have been described previously (Kretzschmar *et al*., 1994a; Goppelt *et al*., 1996 and references therein). MLIn4 and ML contained ML promoter sequences from positions –38 to +2 linked to CCA (positions + 3 to +5) in MLIn4, upstream of a 380 bp G-less cassette. Bubble templates carrying five GAL4 sites cloned into the *Eco*RI site upstream of the major late promoter were prepared as described previously (Holstege *et al*., 1996). In short, two templates that differ in the (–4 to +2) or (–8 to +2) regions, in that one of them carries template sequences instead of non-template sequences, were digested with *Pvu*II, releasing a 514 bp promoter fragment, were hybridized against each other, and the resulting isoforms were separated on and eluted from MDE gels (Serva), and purified by standard procedures. Transcription reactions were performed with 20 ng of each of the supercoiled transcription templates and 20 ng of bubble fragments, respectively. If not indicated as otherwise in the figure legends, standard reactions included 20 ng of recombinant purified TFIIB, 8 ng of TBP or 2 µl of a partially purified TFIID fraction (DE-52 fraction, 0.6 mg/ml), 10 ng of recombinant TFIIEα, 5 ng of recombinant TFIIEβ, 10 ng of baculovirus-expressed and -purified RAP30/RAP74 (cf. Stelzer *et al*., 1994), 1.0 µl of RNA polymerase II purified from HeLa nuclear extracts by phosphocellulose, DE-52 and Superose 6 chromatography, 1 µl TFIIH DE-52 fraction (0.2 mg/ml) and purified PC4-derivatives in concentrations indicated in the figures. GAL4-Sp1 consisted of the N-terminal 94 aa of yeast GAL4-protein and the complete activation region of human Sp1. Approximately 50 ng were used in the transcription reaction. In addition to the buffer introduced by general factors (adding up to <10 mM Tris–HCl pH 7.3 at RT), transcription reactions included 25 mM HEPES KOH pH 8.2, 4 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 8–12% (v/v) glycerol, 60–70 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mg bovine serum albumin (BSA) per ml, 20 U RNase inhibitor, 0.1 mM ATP (if not indicated otherwise in the figure legends), 0.1 mM UTP, 0.1 mM 3′-*O*-methyl-GTP, 5 mM CTP and 10 µCi [α-^{32}P]CTP. Ultrapure nucleotides and deoxynucleotides were purchased from Pharmacia LKB Biotechnology Inc., ATP-γS (lithium salt) and BSA (grade: for molecular biology) from Boehringer Mannheim and [α-^{32}P]CTP (3000 Ci/mmol) from Amersham Inc. Transcription reactions were incubated for 1 h at 28°C and processed as described (Meisterernst *et al*., 1991).

Phosphorylation of PC4

PC4 was modified with CKII (Promega) as described previously (Kretzschmar *et al*., 1994a). In brief, reactions containing 12.5 mM HEPES pH 8.2, 100 mM NaCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM DTT, 1 mM PMSF, 0.05 mM ATP, 250 ng/µl PC4 and 0.05 U/µl CKII were performed for 40 min at 30°C. For comparison, non-phosphorylated PC4 was treated identically, leaving out solely CKII, and aliquots were taken and frozen at –80°C. Phosphorylated proteins tested for binding or repression were shown to be as inactive in activator-dependent transcription as a standard (data not shown).

Electrophoretic mobility-shift assay (EMSA)

The oligonucleotide dT20 used in mobility-shift assays was obtained from Pharmacia, endlabelled, and subsequently gel-purified according to standard protocols. HIV/ML promoter oligonucleotides (Kaiser *et al*., 1995) were gel-purified and labelled by T4-polynucleotide-kinase. Heteroduplex oligonucleotides (52-mers spanning positions –43 to +9 of plasmid pMRG5, Kaiser *et al*., 1995) contained an unpaired region with the sequence CCGTCCTCACT on both strands, or, where indicated, the corresponding template strand sequences, with A being the initiation site of transcription. EMSA reactions were performed in a buffer containing 10 mM Tris–HCl pH 7.3, 5% glycerol, 1 mM DTT, 1 mM EDTA, 10 ng/µl BSA and 200 mM KCl if not detailed otherwise in the figure legends. After incubation of binding reactions (20 µl) at 4°C for 2 h, samples were loaded onto a 10% polyacrylamide (PAA) gel (0.5×TBE, 0.1% Nonidet P-40) at 4°C, while an electric field of 15 V/cm was applied to the gel to ensure rapid entering of the samples into the matrix. After 15 min, electrophoresis was continued at 5 V/cm for several hours. Gels were then transferred to Whatmann filter paper, dried and autoradiographed.

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